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Disruption of lactate dehydrogenase and alcohol dehydrogenase for increased hydrogen production and its effect on metabolic flux in *Enterobacter aerogenes*

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HIGHLIGHTS

- The genes *ldhA* and *adh* of *E. aerogenes* IAM1183 were disrupted by a conjugation.
- The enzyme activities of mutants LDH and ADH declined to 10% and 37%.
- The IAM1183-ΔldhA and IAM1183-Δadh exhibited significant changes in metabolic fluxes.
- The hydrogen production of IAM1183-ΔldhA was 2.3 times higher than the wild-type in a 5-L fermentor.

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ABSTRACT

Hydrogen production by *Enterobacter aerogenes* from glucose was enhanced by deleting the targeted *ldhA* and *adh* genes responsible for two NADH-consuming pathways which consume most NADH generated from glycolysis. Compared with the wild-type, the hydrogen yield of IAM1183-ΔldhA increased 1.5 fold. Metabolic flux analysis showed both IAM1183-ΔldhA and IAM1183-Δadh exhibited significant changes in flux, including enhanced flux towards the hydrogen generation. The lactate production of IAM1183-ΔldhA significantly decreased by 91.42%, while the alcohol yield of IAM1183-Δadh decreased to 30%. The mutant IAM1183-ΔldhA with better hydrogen-producing performance was selected for further investigation in a 5-L fermentor. The hydrogen production of IAM1183-ΔldhA was 2.3 times higher than the wild-type. Further results from the fermentation process showed that the pH decreased to 5.39 levels, then gradually increased to 5.96, indicating that some acidic metabolites might be degraded or uptaken by cells.

1. Introduction

With growing concerns about global warming and environmental problems caused by burning fossil fuels, extensive research has been conducted on renewable energy. Molecular hydrogen (H_2), due to its high conversion efficiency, recyclability and environment-friendly nature, is considered as a clean and ideal alternative energy (Fabiana et al., 2014).

Microbial hydrogen production is an ideal approach towards environment-friendly and less energy-intensive production of hydrogen compared with electrolysis and thermo-chemical processes (Lee et al., 2011; Shireen and Debabrata, 2008). Two categories of hydrogen-producing microbes have been found in nature (Hallenbeck et al., 2012; Patel and Kalia, 2013). One is photosynthetic microorganisms producing hydrogen under light, such as the bacteria *Rhodobacter sphaeroides* (Krujatz et al., 2014) and the algae *Chlamydomonas reinhardtii* (Esquivel et al., 2011; Oncel et al., 2014). The others are fermentative hydrogen-producing microorganisms such as the facultative anaerobes *Escherichia coli* (Jenni et al., 2013) and *Enterobacter* spp (Zhang et al., 2011) and the obligate anaerobe *Clostridia* spp (Calusinska et al., 2011). Fermentative hydrogen-producing microbes are preferred over photosynthetic microbes, because hydrogen can be produced in a reactor continuously without the necessity to supply light. Compared with obligate anaerobic species such as *Clostridium*

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spp, facultative anaerobes such as *Enterobacter* spp are more easily handled in the presence of oxygen (Jayasinghearachchi et al., 2009; Zhang et al., 2011).

Enterobacter aerogenes as a facultative anaerobic bacterium performs fermentative hydrogen production similar to *E. coli*. It grows fast and produces hydrogen at a high rate (Kurokawa and Tanisho, 2005). Therefore, *E. aerogenes* could be one of the most suitable and promising organisms for converting biomass to hydrogen. Two hydrogen-producing pathways are well known to be involved in the hydrogen production by *E. aerogenes* (Steuber et al., 1999; Tanisho et al., 1989). One is the formate pathway, which is based on the decomposition of formate mediated by the formate hydrogen lyase (FHL) system (McDowall et al., 2014). Another pathway is called the nicotinamide-adenine dinucleotide (NADH) pathway, in which electrons from NADH are transferred to H^+ to produce molecular hydrogen (Tanisho et al., 1989; Wang et al., 2013; Zhang et al., 2011).

In the formate pathway, hydrogen and carbon dioxide are produced at a 1:1 molar ratio. Based on the stoichiometry of the intracellular reactions, the maximum yield of hydrogen by *E. aerogenes* through the formate pathway is 2 mol H_2/mol glucose. In our previous studies, it was shown that the hydrogen yields through the formate pathway can be enhanced by genetic manipulation of the FHL system, including the knockout of the *hycA* gene which encodes the FHL repressor protein, deactivation of the uptake hydrogenase, and by a combination of the two (Zhao et al., 2009). Analysis of the metabolites formed during hydrogen fermentation by *E. aerogenes* showed that the yield of H_2 generated through the formate pathway can be calculated by the following equation:

$$V_h = V_e + V_a - V_f$$

where V_h is the amount of hydrogen produced through the formate pathway, V_e is the amount of ethanol, V_a is the amount of acetate, and V_f is the amount of formate (Lu et al., 2009; Zhao et al., 2009).

The NADH pathway is another important hydrogen-producing pathway existing in *E. aerogenes*. It was first reported by Tanisho's group in the 1980s. The thermodynamic analysis implied that a putative membrane-bound hydrogenase might catalyze the hydrogen evolution from NADH (Nakashimada et al., 2002; Tanisho et al., 1989; Zhang et al., 2011). The addition of external NADH and NAD^+ into *E. aerogenes* cultures, as well as the ratio of [NADH] to [NAD^+] were proven to affect the hydrogen yield (Zhang et al., 2009). Overexpression of the *nadE* gene encoding the NAD synthetase and knockout of the *hybO* gene encoding the small subunit of the uptake hydrogenase increased the intracellular concentration of the NAD(H) pool and thereby enhanced the hydrogen yield (Wang et al., 2013). These results showed that the regulation of the NAD (H) pool could affect cellular metabolism and the hydrogen production. It also suggested that the accumulation of intracellular NADH might enhance the hydrogen production of *E. aerogenes*.

However, in anaerobic metabolism, NADH generated from glycolysis is mainly consumed by two metabolic pathways: the lactate production pathway and the alcohol production pathway (Fig. 1). Moreover, the lactate yield accounts for more than 50% of the end products (Converti and Perego, 2002; Ma et al., 2012; Zhao et al., 2009). This could be the main reason for the rapid decrease of pH in fermentation systems without pH control. Ethanol is a metabolite known for its inhibitory activity toward microorganisms. It derives from acetaldehyde catalyzed by alcohol dehydrogenase. Production of alcohol not only consumes NADH, but also inhibits cell growth because of its toxicity (Daria and Piotr, 2014).

In this study, the *ldhA* gene encoding lactate dehydrogenase (EC 1.1.1.27, LDH) was disrupted in *E. aerogenes* IAM1183 to accumulate intracellular NAD (H) concentration, enhance hydrogen production, and reduce the lactate accumulation (Fig. 1). To prevent negative alcohol influence during anaerobic fermentation and increase NADH accumulation in cells, the *adh* gene encoding alcohol dehydrogenase (EC 1.1.1.1, ADH) was also deactivated in wild-type IAM1183 (Fig. 1). The production of hydrogen and other metabolites were analyzed in mutants of Δ*ldhA* and/or Δ*adh*. The results could provide a thorough understanding of the roles played by LDH and ADH towards producing hydrogen. Furthermore, the NADH accumulation and utilization rates directly related to the hydrogen production were analyzed to further understand the effect of the NADH perturbation on hydrogen production.

2. Methods

2.1. Microorganisms, plasmids and cultivation

The strains and plasmids used in this study are listed in Table 1. *E. aerogenes* IAM1183 was purchased from the Institute of Applied Microorganisms (IAM) of the University of Tokyo, Japan. *E. aerogenes* IAM1183, its mutants, and *E. coli* as host in genetic manipulation were all cultivated aerobically in a 250 mL flask containing 100 ml Luria Broth (LB) medium (per liter: 5 g yeast extract, 10 g tryptone, and 5 g NaCl) on a shaker at 250 rpm at 37 °C. Conjugation assays were performed on LB plates (per liter: 5 g yeast extract, 10 g tryptone, 2.5 g NaCl, agar 15 g) and incubated at 30 °C for 24 h or longer. Different antibiotics were added to the growth medium according to the particular experimental needs at the following concentrations: 100 μg/mL ampicillin, 30 μg/mL chloramphenicol, or 15 μg/mL tetracycline. Glucose medium containing (per liter) 15 g glucose, 5 g trypone, 14 g K_2HPO_4, 6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$ and 0.2 g $MgSO_4{\cdot}7H_2O$ was used for hydrogen production and metabolic flux analysis.

2.2. General genetic techniques

Standard molecular genetics techniques were employed as described in the literature (Miller, 1972). Restriction enzymes, T4 DNA ligase, DNA polymerase, and T-vector system were purchased from Takara Biotechnology Co., Ltd (Dalian, China) and New England Biolabs Inc., (NEB, USA). Genomic DNA from *E. aerogenes* IAM 1183 was prepared as described in the literature or using a Wizard Genomic DNA Purification Kit (Promega, USA). Plasmid DNA was purified using QIAprep Spin Miniprep Kit (GenScript USA Inc., USA). The detailed information of the primers used is listed in Table 2. PCR amplification was performed on a Bio-Rad PCR apparatus (Hercules, USA) with a final volume of 50 μL containing 5 μL 10× *Taq* buffer, 10 mM dNTP, 30–60 ng of DNA template, 10 pmol each of the appropriate primers and 0.5 units each of *Taq* polymerases and Pfu DNA polymerases (Takara Inc. Dalian). The thermo-cycling program used involved 35 PCR cycles of denaturation at 94 °C for 1 min, annealing at between 50 and 60 °C for 30 s and extension at 72 °C for 1 min/kb. The final extension step was carried out for 10 min at 72 °C and kept at 4 °C. PCR products were purified using Wizard PCR Preps (Promega, USA). DNA sequencing was performed by Life Technologies Corporation (Shanghai Invitrogen, China).

2.3. Assays of enzyme activities

The enzyme activities were measured under anaerobic conditions in an anaerobic chamber (Shelton Bactron II, USA) (Ma et al., 2012). The cells were harvested by centrifugation and resuspended in a buffer containing 100 mM potassium phosphate (pH 7.0). Cells were lysed by ultrasonication. Cell-free extracts were

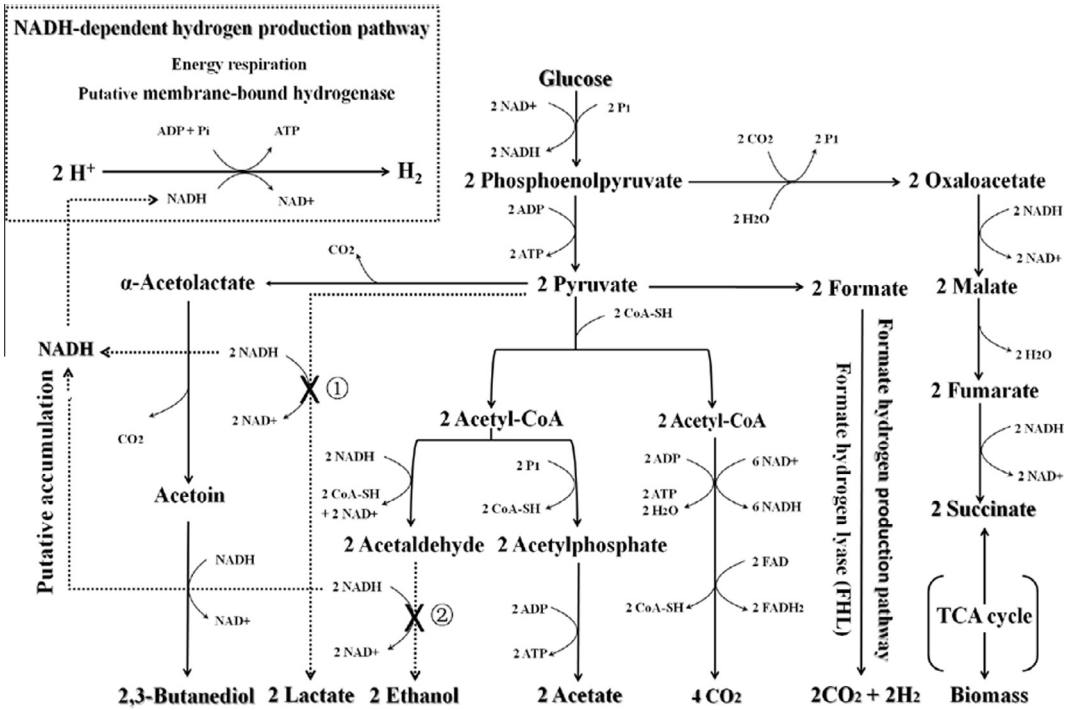

Fig. 1. NAD recycling through the salvage pathway and metabolic flux patterns of *E. aerogenes* in anaerobic fermentation, and schematic of the knockout of lactate production and alcohol production pathways in *E. aerogenes* IAM1183 (dashed lines shown on diagram) (All modified from Converti and Perego, 2002; Zhang et al., 2011; Jung et al., 2012; Wang et al., 2013). Enzymes studied in this paper: ① lactate dehydrogenase, ② alcohol dehydrogenase.

prepared and kept on ice. The activity of the lactate dehydrogenase (EC 1.1.1.27, LDH) was determined by measuring the decrease of NADH absorbance (Ma et al., 2012). The NADH absorbance was measured using an ultraviolet-visible spectrophotometer (UV757CRT, Shanghai Precision & Scientific Instrument Co., Ltd., China). The reaction system contained 1 mL imidazole-HCl (pH 6.7), 50 μL of 10 mM NADH, 1.5 mL of 0.5 mM sodium pyruvate, and 0.5 mL cell-free extracts. The measurements of alcohol dehydrogenase (EC 1.1.1.1, ADH) activity were carried out using the same procedure as that of LDH, except that 1.5 mL of 0.5 mM sodium pyruvate in the reaction mixture was replaced with 1.5 mL of 0.5 mM acetaldehyde and 1.5 mL of 10 mM acetoin.

2.4. Anaerobic serum bottle batch cultivation for hydrogen production

The anaerobic cultivations were performed using 50 mL serum bottles with top caps and PTFE/silicone rubber septa. Aseptic nitrogen was sparged for 5 min to purge the residual air in the bottle, thereby creating an anaerobic environment. The serum bottles containing 20 mL glucose medium were inoculated with the seeding cells and hermetically sealed. Serum bottles were then cultivated at 37 °C on a shaker at 170 rpm for 20 h. pH values and cell densities were measured periodically at 4 h intervals.

2.5. Fermentation for hydrogen production

Fermentation experiments were carried out at 37 °C with agitation rate at 250 rpm in a 5-L fermentor (Biof-6005A, Shanghai Biotech Co., Ltd. China) containing 3 L medium without pH control. Nitrogen sparging was performed to remove the residual air from the fermentor to create an anaerobic environment. Carbon dioxide was absorbed via 5 M sodium hydroxide and the hydrogen production was measured by Wet Gas Meter (WGM). The pH value was monitored online. Samples were taken every 2 h. The metabolite concentrations were quantified by a high performance liquid chromatography (HPLC) system (Shimadzu 10A, Japan).

2.6. Analysis of hydrogen gas and metabolites

Cell densities during cultivation at 600 nm (OD_{600}) were measured with a Shimadzu UV–Vis spectrophotometer (Shimadzu UV-1206, Japan). The dry cell weight (DCW) was subsequently calculated by the formula DCW (mg/ml) = 0.132 × OD_{600} for *E. aerogenes*. The pH values of the bacterial cultures were measured with a pH meter (Orion 828, USA). The total volume of gas produced in the serum bottle was measured by displacing pure water within an inverted cylinder. The gas production in the 5-L fermentor was measured by Wet Gas Meter (WGM) (Sinagawa Co., Japan). The composition of the gas was further measured by gas chromatography (GC) (Shimadzu GC-8A, Japan), which was equipped with a Parapak Q column (80/100 mesh) and a thermal conductivity detector. The temperatures of injector, column and detector were 120, 80 and 120 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 10 mL/min. The retention time of hydrogen was 1.815 min. The cells were centrifuged at 12,000 rpm for 10 min and the supernatant was collected for analysis of glucose and metabolites. The concentrations of glucose, organic acids, and alcohols in the culture media were analyzed by HPLC. The HPLC system was equipped with an organic acid analysis column (Shimadzu SCR-102G, Japan) and a refractive index detector (Shimadzu RID-10A, Japan). The mobile phase was 0.1% aqueous perchloric acid solution. The column temperature was maintained at 40 °C. The retention times of glucose, succinate, lactate, formate, acetate, 2,3-butanediol, and ethanol were 6.128, 6.681, 8.666, 9.404, 10.088, 11.746, and 13.670 min, respectively.

Table 1
Strains and plasmids used in the experiments.

Strain and plasmid	Relevant genotype	Reference or Source
E. aerogenes IAM1183	Wild type	IAM (Tokyo, Japan)
E. aerogenes IAM1183-ΔldhA	*Amp*^{r}, *Tet*^{r}, Δ*ldhA*	This work
E. aerogenes IAM1183-Δadh	*Amp*^{r}, *Cm*^{r}, Δ*adh*	This work
E. coli SM10	*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λpir*	Miller (1972)
E. coli SM10(pGP704-ldhA)	*Amp*^{r}, *Tet*^{r}, *thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λpir*	This work
E. coli SM10(pGP704-adh)	*Amp*^{r}, *Cm*^{r}, *thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λpir*	This work
E. coli JM109	*endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ(*lac-proAB*), *recA1*, *F'*[*traD36*, *proAB+*, *lacIq*, *lacZΔM15*]	TaRaKa
E. coli DH5α	*F-Φ80 lacZ ΔM15(lacZYA-argF) U169 endA1 recA1 hsdR17 (rk,mk-)supE44 λ-thi-1 gyrA96 relA1 phoA*	TaRaKa
E. coli DH5α(pGEM®-T Easy-ldhA)	*Amp*^{r}, Derived from *E. coli* DH5α contained pGEM®-T Easy-ldhA	This work
E. coli JM109(pGEM®-T Easy-adh)	*Amp*^{r}, Derived from *E. coli* JM109 contained pGEM®-T Easy-adh	This work
pGEM®-T Easy	*Amp*^{r}, AT clone vector	Promega
pGEM®-T Easy-ldhA	*Amp*^{r}, Derived from pGEM®-T vector contained *ldhA*	This work
pGEM®-T Easy-adh	*Amp*^{r}, Derived from pGEM®-T vector contained *adh*	This work
pGP704	*Amp*^{r}, *Tet*^{r}, Suicide vector	Miller and Mekalanos (1988)
pGP704-Cm	*Amp*^{r}, *Cm*^{r}, Suicide vector derived from pGP704	This work
pGP704-ldhA	*Amp*^{r}, *Tet*^{r}, Derived from pGP704 contained *ldhA*	This work
pGP704-adh	*Amp*^{r}, *Cm*^{r}, Derived from pGP704-Cm contained *adh*	This work

Table 2
Primers used in the experiments.

Primers	Oligonucleotide Sequence (from 5′ to 3′)	Source
ldh-forward-F (*Eco*RI)	CG*GAATTC*CCCCGCTTACCAGCGTACCC	This work
ldh-reverse-R (*Bam*HI)	CG*GGATCC*GTCAGGAATGCCTGATGCCC	This work
adh-forward-F (*Pst*I)	AG*CTGCAG* TCATAGACGG AGTCAACAAC GACC	This work
adh-reverse-R (*Sph*I)	CA*GCATGC*AGGCTGAACTGGGTATTCCTAAGT	This work
ldh-reverse-PCR-F	CCCTTTCAGA ATACGCAGC	This work
ldh-reverse-PCR-R	CGAAGATAAGTCCAATGACG	This work
ldh-orf-forward-F	ATGAAAMTCGCSGTTTATAG	This work
ldh-orf-reverse-F	CGTTCGGRCA GGTTTCGCC	This work
pGP704-ldh-F	CATGCGCTCCATCAAGAAGA	This work
pGP704-ldh-R	GTGGGTCTCG CGGTATCATT	This work
pGP704-adh-F	ATGAAACCAAGCCAACCAGG	This work
pGP704-adh-R	AAGCCCTCCCGTATCGTAGTT	This work

Note: Relevant restriction enzyme sites are underlined and in italics.

3. Results and discussion

3.1. Isolation of LDH and ADH disrupted mutants

Among all intracellular metabolic pathways in *E. aerogenes*, the lactate pathway and ethanol pathway are two main NADH-consuming pathways (Fig. 1) (Converti and Perego, 2002; Zhao et al., 2009). NADH is known to be involved in the hydrogen production by *E. aerogenes* (Zhang et al., 2009; Zhao et al., 2009). Thus, knockout of *ldhA* or *adh* genes would affect the intracellular NADH distribution, which thereby could regulate the hydrogen metabolism. Moreover, if more NADH could be regenerated, the alteration in hydrogen productivity could be more prominent (Jung et al., 2012; Lu et al., 2010).

The primer pairs added restriction enzyme sites (*ldh*-forward-F (*Eco*RI) and *ldh*-reverse-R (*Bam*HI), *adh*-forward-F (*Pst*I) and *adh*-reverse-R (*Sph*I)) were designed using the ClustalX software according to conserved regions published in GenBank (Table 2) and used to amplify partial *ldhA* and partial *adh*. The PCR fragments were purified and ligated into T-vector to create pGEM-T Easy-ldhA and pGEM-T Easy-adh. The sequence analyses revealed that the homologies of these two genes were more than 95% with those of strains from the same family (Bunch et al., 1997; Goodlove et al., 1989). A 1.5 kb PCR *ldhA* fragment obtained from one round of inverse-PCR amplification using *ldh*-reverse-PCR-F and *ldh*-reverse-PCR-R was purified and sequenced. The sequencing results showed that it was identical to that of *ldhA* published in GenBank. After splicing the twofragments, the complete *ldhA* gene of *E. aerogenes* IAM1183 was successfully obtained by reverse PCR (GenBank No: JX129363).

The pGEM-T Easy-ldhA and pGEM-T Easy-adh were cut with the restriction enzymes *Eco*RI and *Bam*HI. The 1 kb fragments of partial *ldhA* and partial *adh* were ligated into pGP704 (Miller and Mekalanos, 1988) or pGP704-Cm to create pGP704-ldhA and pGP704-adh. These plasmids were then transformed into *E. coli* SM10 and positive colonies were screened on LB agar plates supplemented with the appropriate antibiotics. LDH and ADH-disrupted mutants were generated by conjugating *E. aerogenes* IAM 1183 with *E. coli* SM10 carrying the suicide plasmid pGP704-ldhA or pGP704-adh. Conjugations were performed on LB plates and incubated at 30 °C for 24 h (Metcalf et al., 1996). The mixture of *E. aerogenes* IAM 1183 and *E. coli* SM10 was diluted from 10^{-1} to 10^{-8} to deposit on LB plates containing appropriate antibiotics. Transconjugants could be seen after 24–48 h of incubation. Colonies of transconjugants were streaked on the selective medium twice to yield single colonies. The mutants were selected and identified by colony PCR with primer pairs (pGP704-ldh-F and pGP704-ldh-R, pGP704-adh-F and pGP704-adh-R). A total of 5 colonies of Δ*ldhA* and 8 colonies of Δ*adh* from 600 transconjugants had positive signals (see Supporting Information). The isolations of the LDH-and ADH-disrupted mutants were further confirmed by sequencing. Thus, DNA gel electrophoresis images and nucleotide sequence results demonstrated that foreign plasmids (pGP704-ldhA and pGP704-adh) successfully integrated into *ldhA* and *adh* locations in the recipient chromosome by a single cross-over event (see Supporting information). These positive mutants

were used in subsequent experiments to examine the hydrogen gas yield and other variations in the metabolism.

3.2. Detection of LDH and ADH activities in the ΔldhA and Δadh mutant

To examine how the disruptions of LDH or ADH involved in the anaerobic metabolism of IAM1183-ΔldhA and IAM1183-Δadh, the activities of the two enzymes were compared between the wild-type strain and the mutant strains (Ma et al., 2012). The enzyme activities were detected in the exponential phase of cell growth ($t_{1/2}$ OD_{max}: the time for cell density to reach half of the maximum value) and in the stationary phase at the end of the batch cultivation, respectively. As shown in Fig. 2a, LDH activity of IAM1183-ΔldhA dramatically decreased, but relative to wild-type IAM1183, there was still 10% residual enzyme activity in the exponential phase and 9% in the stationary phase. This residual 10% activity is regarded as background. However, the ADH activity of IAM1183-Δadh was still 37% residual, both in the exponential phase and in the stationary phase (Fig. 2b). The ADH thus seemingly retained partial enzyme function in IAM1183-Δadh. The length of *adh* is more than 2.7 kb, as submitted to GenBank in 2012 by Shin and coauthors (Shin et al., 2012). However, only 1 kb partial *adh* was replaced in this study, which might explain why there was still 37% of activity left. Alternatively, an unknown homologous gene may exist in IAM1183. Further studies are required to elucidate the reason for the residual activity of IAM1183-Δadh.

3.3. Culture performances and hydrogen production by the mutants

The physiological characteristics pertinent to hydrogen fermentation were analyzed. The mutants of IAM1183-ΔldhA and IAM1183-Δadh were cultivated in 50 mL serum bottles with 20 mL glucose medium under anaerobic conditions (Zhao et al., 2009). The wild-type IAM1183 served as the control. As shown in Fig. 3a, the final cell density of IAM1183-ΔldhA was the highest among the three strains. The cell density (OD_{600}) of IAM1183-ΔldhA (2.5 ± 0.13) was nearly 1.72 times that of IAM1183-Δadh (1.46 ± 0.13) and IAM1183 (1.55 ± 0.18) after 20 h of incubation. The maximum specific growth rates during the exponential phase were then calculated. The maximum specific growth rates of IAM1183-ΔldhA, IAM1183-Δadh, and wild-type were 0.43 ± 0.05, 0.35 ± 0.02, and 0.36 ± 0.02 (/h), respectively. The mutant IAM1183-ΔldhA appeared to have a slightly faster growth rate than others. It is suggested that the knockout of *ldhA* could be beneficial to cell biomass synthesis. This result is also similar to that reported by Cai and coauthors (2011). As shown in Fig. 3b, the final culture pH of IAM1183-ΔldhA (5.05 ± 0.08) was higher than that of IAM1183-Δadh (4.21 ± 0.13) and IAM1183 (4.36 ± 0.09) after 20 h of incubation. These observations suggest that the knockout of *ldhA* had effects on the medium pH, but the knockout of *adh* did not affect the pH.

The hydrogen production performance in the fermentation process was further explored. The total volume of gas produced by *E. aerogenes* in the serum bottle was measured by displacing H_2O within an inverted gas cylinder. The final total gas evolved by IAM1183-ΔldhA was 87.7 ± 1.33 mL, which was up to 2.19 fold greater than that of both the wild-type and IAM1183-Δadh (Fig. 4a). The gas composition was analyzed by gas chromatography (GC). Interestingly, of the three strains examined, the hydrogen production of the IAM1183-ΔldhA was the highest, up to 1.5 times greater than both IAM1183-Δadh and wild-type IAM1183 (Fig. 4b). The molar fraction of hydrogen produced by the wild strain and IAM1183-Δadh was 58.3% and 57.97%, respectively, but the hydrogen molar fraction of IAM1183-ΔldhA was as high as 88.7% (Fig. 4b). The carbon dioxide production by these three strains was also examined. The average fractions of carbon dioxide evolved from IAM1183-ΔldhA, IAM1183-Δadh, and IAM1183 were 40.8%, 39.0%, and 40.3%, respectively (Fig. 4c).

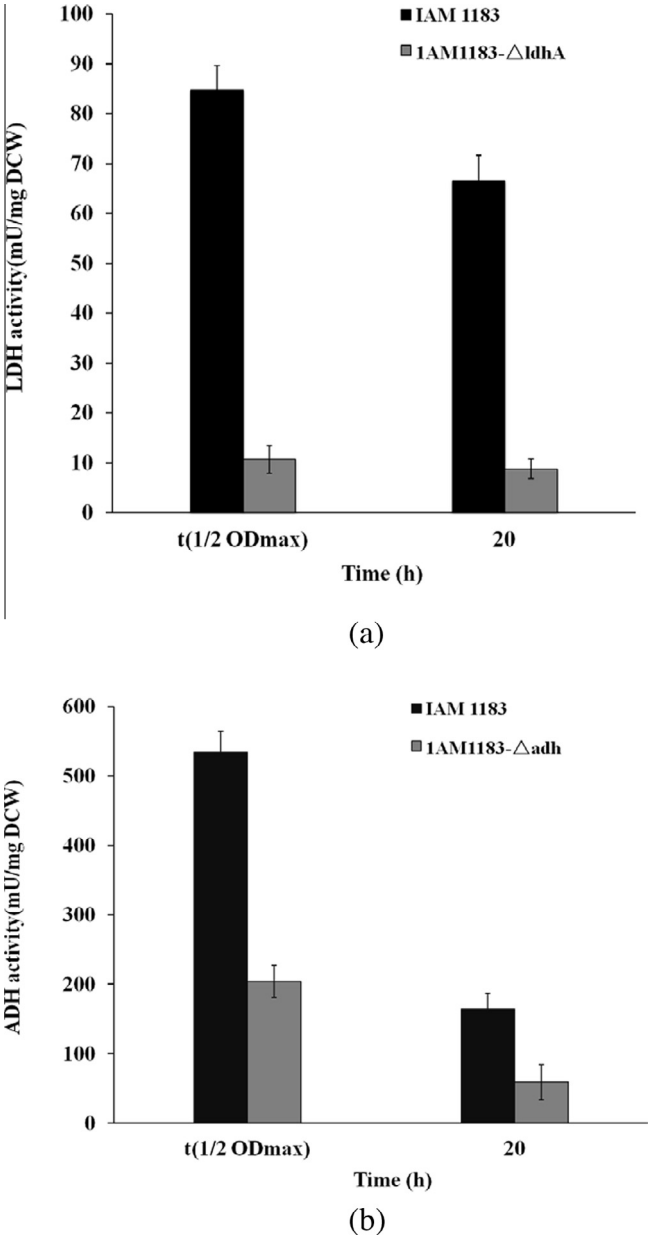

Fig. 2. Comparison of enzyme activities between wild-type strain and the mutants ($n = 3$): (a) IAM1183-ΔldhA: lactate dehydrogenase (EC 1.1.1.27, LDH); (b) IAM1183-Δadh: alcohol dehydrogenase (EC 1.1.1.1, ADH). The activities of the two enzymes were detected and compared in the middle of the exponential phase of cell growth ($t_{1/2}$ OD_{max}: the time for cell density to reach half of the maximum value) and at the end of the batch cultivation (after inoculating 20 h), respectively.

The hydrogen yield per mole glucose via the anaerobic fermentation could be analyzed in terms of the individual FHL pathway and NADH pathway, which contribute to the fermentative hydrogen production by *E. aerogenes* (Nakashimada et al., 2002; Zhang et al., 2009). Based on the central anaerobic metabolism of *E. aerogenes*, pyruvate is split into formate and acetyl-CoA via pyruvate formate lyase (Fig. 1) (Converti and Perego, 2002; Jung et al., 2012). H_2 and CO_2 are then produced from formate via formate hydrogen lyase (Fig. 1). Accordingly, the hydrogen yields through the FHL and NADH pathways were calculated by the following

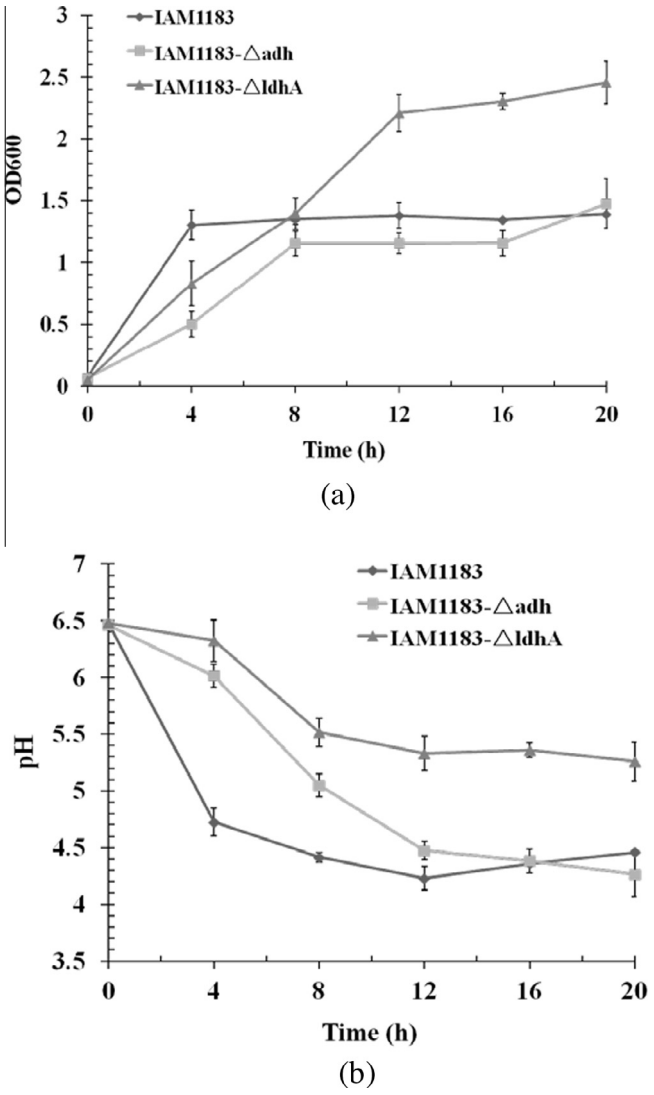

Fig. 3. Comparison of cell growth (in terms of OD_{600}) (a) and pH (b) between mutants and wild-type strain during cultivation ($n = 3$).

two equations according to the metabolites detected: (1) yield of hydrogen (mol/mol glucose FHL Pathway) = yield of ethanol + yield of acetate − yield of formate; (2) yield of hydrogen (mol H_2/mol glucose NADH pathway) = yield of total hydrogen produced − yield of hydrogen produced through formate pathway. From these equations, the hydrogen yield from the NADH pathway or formate pathway could be estimated. The H_2 yields from the FHL of IAM1183-ΔldhA, IAM1183-Δadh, and wild-type IAM1183 were 0.64, 0.00, and 0.74 mol H_2/mol glucose, respectively (Fig. 5). In comparison, the hydrogen yield of wild-type IAM1183 was slightly higher than that of IAM1183-ΔldhA. However, for the contribution of the NADH pathway to hydrogen production, the hydrogen yield of IAM1183-ΔldhA via the NADH pathway showed no obvious change from the wild-type (Fig. 5). Due to the deletion of the lactate pathway, more glucose could be consumed in the batch culture, which made the volumetric hydrogen production increase 1.5 fold greater than that of either the wild strain IAM1183 or IAM1183-Δadh after 20 h of cultivation. Interestingly, no H_2 gas was produced from the FHL pathway in IAM1183-Δadh according to the two hydrogen-producing equations (Fig. 5). These results suggest that the knockout of alcohol dehydrogenase inhibited the FHL pathway, and that the total hydrogen gas was produced through the NADH pathway. The results in Fig. 4 indicate that there might be certain relations between the NADH pathway, FHL pathway, and hydrogen uptake for hydrogen production. However, the mechanism of the NADH pathway in *E. aerogenes* is still unclear (Zhang et al., 2011). Thus, further study to elucidate the mechanism of the NADH pathway for hydrogen production in *E. aerogenes* is still required.

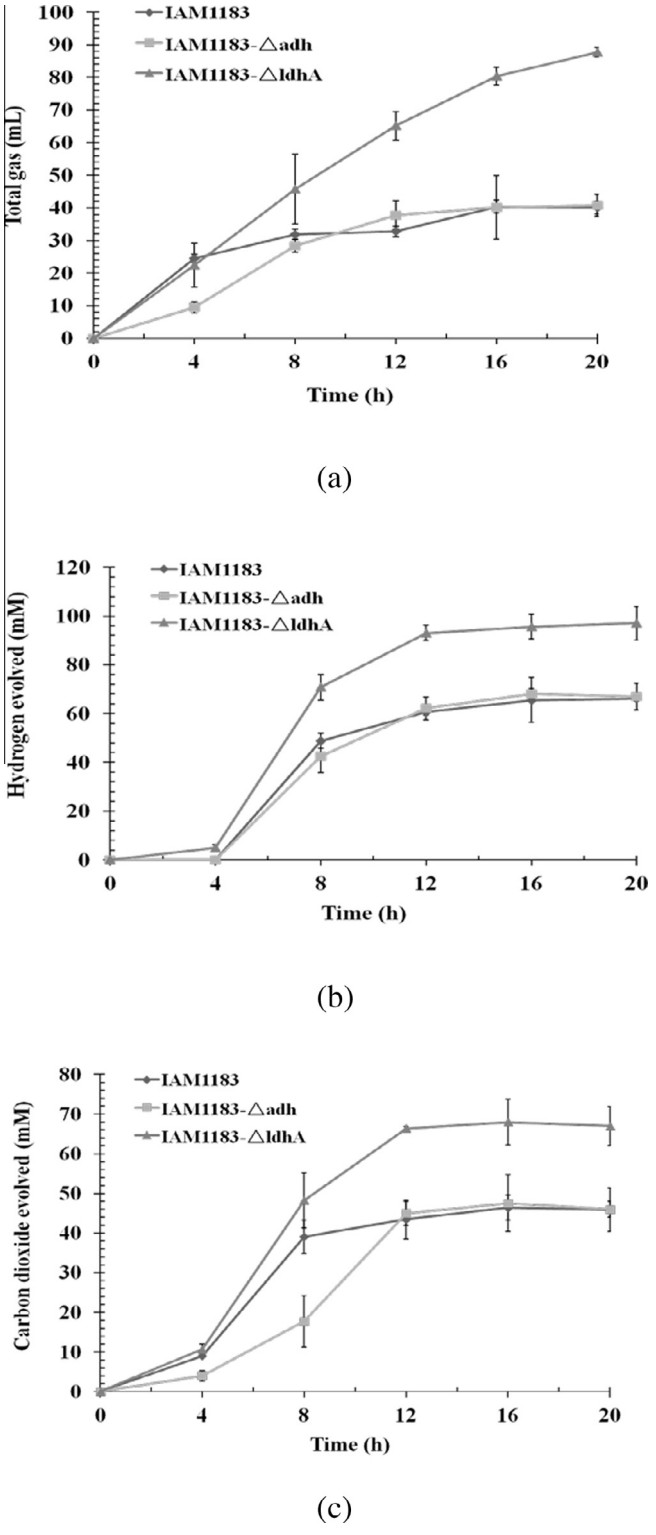

Fig. 4. Comparison of total gas (a), hydrogen gas evolved (b), and carbon dioxide evolved (c) between mutants and wild-type strain ($n = 3$).

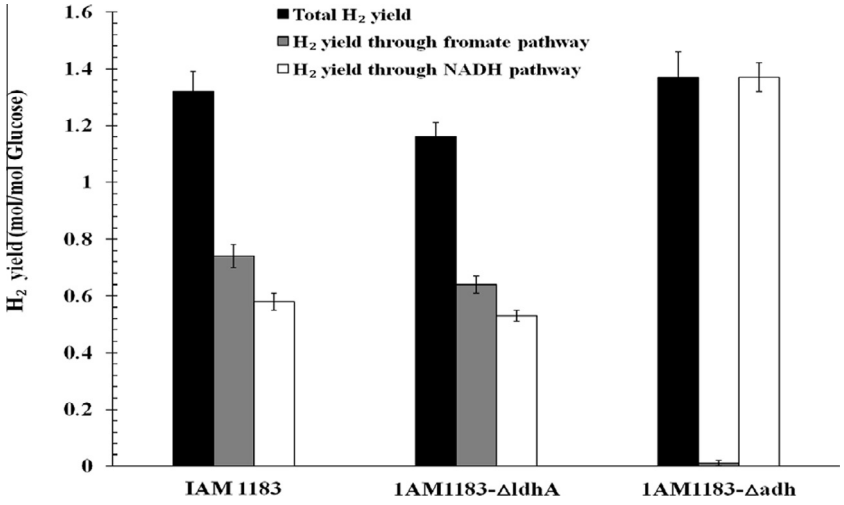

Fig. 5. Hydrogen yield from glucose via formate pathway and NADH pathway by wild-type strain, IAM1183-ΔldhA, and IAM1183-Δadh (n = 3). The data shown in Table 3 were used for the calculations.

3.4. Changes in final metabolite concentrations in the mutants

To examine how the disruption of LDH and ADH affected the cellular metabolism, the final metabolites were analyzed. The cell culture after 20 h was centrifuged at 12,000 rpm for 10 min, and the supernatant was collected and then filtered via 0.2 μm filter for HPLC analysis. The major final metabolites include residual glucose, formate, 2,3-butanediol, lactate, acetate, succinate, and ethanol (Ma et al., 2012). The changes of metabolic flux in IAM1183-ΔldhA and IAM1183-Δadh could be more clearly revealed via quantification of the metabolites. The concentrations of metabolites were accordingly measured and summarized in Table 3.

These mutants exhibited enhanced formate yields compared with the wild-type for which formate yield was zero. The formate yield of IAM1183-Δadh (16.19 mmol/mol glucose) was significantly higher than that of 1AM1183-ldhA and IAM1183, as shown in Table 3. The 2,3-butanediol, ethanol, and succinate yields of the IAM1183-ΔldhA were all higher than those of the wild-type. On the contrary, the 2,3-butanediol, ethanol, and succinate yields of the IAM1183-Δadh were all lower than that of the wild-type IAM1183. The lactate production of IAM1183-ΔldhA was significantly lower than that of the wild-type IAM1183, in which the production decreased by 91.42%. These results corresponded well with those from the reported knockout of lactate dehydrogenase in *Thermoanaerobacterium aotearoense* (Cai et al., 2011) and *E. aerogenes* KCTC 2190 (Jung et al., 2012). It is suggested that the *ldhA* was successfully knocked out and the cells accordingly lost their LDH function, which is the main reason why the culture pH of IAM1183-ΔldhA remained at 5.5 after 20 h of cultivation, while the culture pH of wild-type IAM1183 remained at 4.3. The cells stopped growing and cellular activities ceased when the culture pH reached 4.3. Therefore, when the culture pH of IAM1183-ΔldhA remained at 5.5, the cellular activities were maintained until all glucose was used up, as shown in Table 3. The ethanol production of IAM1183-Δadh was reduced 30% compared to wild-type IAM1183, suggesting that part of the ADH function was lost. The main reason for ADH function partially remaining may be due to several copy *adh* genes existing in bacteria (Shin et al., 2012).

3.5. Hydrogen production of IAM1183-ldhA in fermentor

As illustrated by the serum bottle batch results in Sections 3.3 and 3.4, IAM1183-ΔldhA has better hydrogen productivity than both wild-type and IAM1183-Δadh. Therefore, IAM1183-ΔldhA exhibited suitable performance to be a potential candidate for industrial bioproduction of hydrogen. To this end, it would be desirable to investigate its hydrogen production in a fermentor.

Table 3
Analysis of consumed substrate and anaerobic metabolites after 20 h of cultivation in serum bottle for IAM1183-ΔldhA, IAM1183-Δadh, and wild-type IAM1183.

End products	Product yield (mmol/mol glucose)		
	IAM1183-ΔldhA	IAM1183-Δadh	IAM1183
Glucose consumed percent (%)	100	57.7	60.98
Residual glucose (g/L)	0 ± 0	6.45 ± 0.1	5.85 ± 0.33
Hydrogen (H_2)	96.99 ± 4.24 ↑	66.07 ± 1.11 ↓	66.98 ± 3.38
Carbon dioxide (CO_2)	66.98 ± 4.88 ↑	46.02 ± 1.95 ↑	45.95 ± 3.51
Succinate	5.95 ± 0.29 ↑	0.45 ± 0.08 ↓	2.07 ± 0.91
Lactate	3.6 ± 0.45 ↓	36.33 ± 1.53 ↓	41.95 ± 3.66
Formate	0.54 ± 0.12 ↑	16.19 ± 0.11 ↑	0 ± 0
Acetate	13.8 ± 0.11 ↓	0.2 ± 0.2 ↓	20.85 ± 3.29
2,3-Butanediol	35.84 ± 0.36 ↑	2.3 ± 0.1 ↓	1.18 ± 0.27
Ethanol	39.97 ± 1.73 ↑	11.66 ± 0.4 ↓	16.54 ± 0.18

Note: Anaerobic culture conditions: 37 °C; initial pH 6.5; 170 rpm; 20 h of cultivation. Experimental values represent the averages of triplicate biological repeats (n = 3).

The enlarged fermentation process for hydrogen production was undertaken in a 5-L fermentor containing 3 L glucose medium. As shown in Fig. 6a, it is apparent that the mutant IAM1183-ΔldhA showed faster growth rate than wild-type IAM1183. The final OD_{600} of IAM 1183-ΔldhA was 1.7 times higher than that of the wild-type after 25 h of cultivation. The final pH values for IAM 1138-ΔldhA and wild-type were 5.96 and 4.75 (Fig. 6b), respectively. Interestingly, the lowest pH for IAM1183-ΔldhA was 5.39 at 15 h and it then gradually increased to 5.96. These observations indicated that some acidic metabolites might be degraded or uptaken by cells. The underlying cause of these pH fluctuations will be explored in future studies. The hydrogen production of IAM1183-ΔldhA was 2.3 times greater than that of the wild-type after 25 h of cultivation (Fig. 6c). Interestingly, hydrogen gas was continuously produced by IAM1183-ΔldhA during fermentation until the glucose was used up. In comparison with wild-type IAM1183, the final lactate and acetate yields of IAM1183-ΔldhA decreased (Table 4). The lactate, noticeably, deceased 94.58%. Other final metabolite levels were higher than those of the

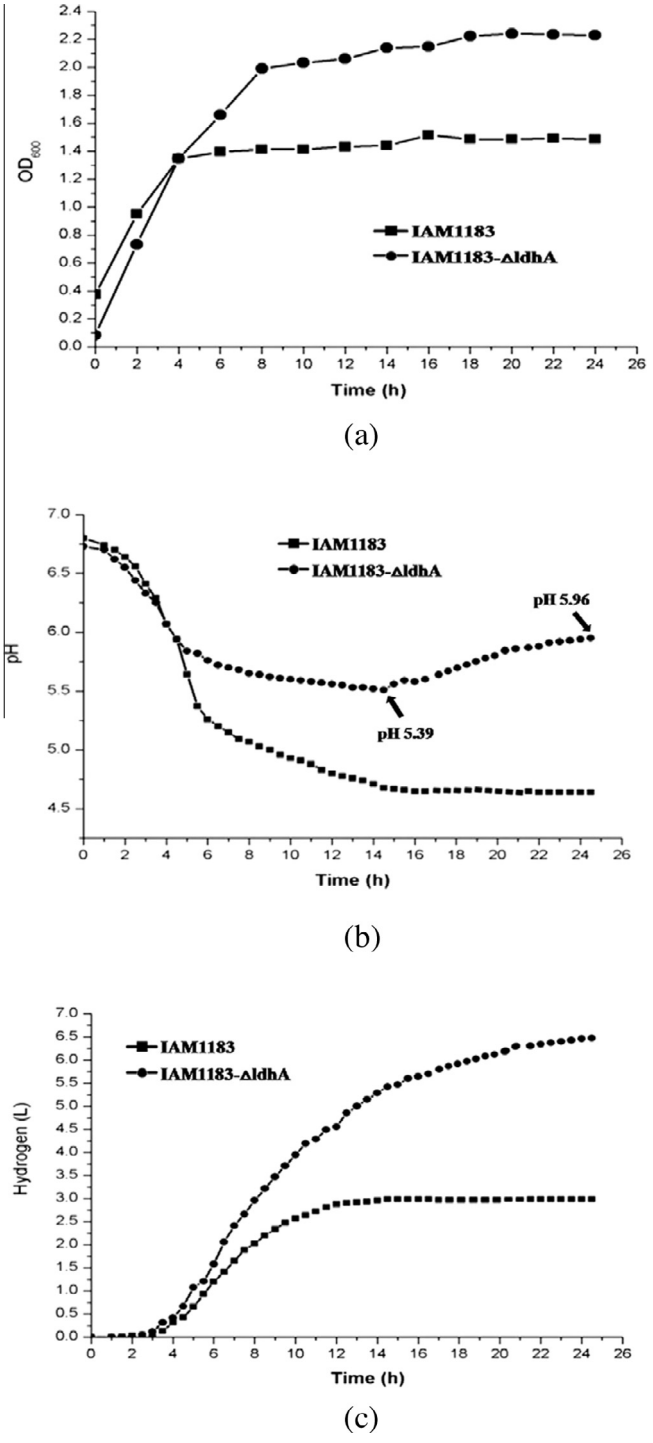

Fig. 6. Comparison of cell growth (in terms of OD_{600}) (a), pH (b) and hydrogen gas evolved (c) during the cultivation between IAM1183-ΔldhA and wild-type strain in a 5-L fermentor.

wild-type. In the fermentor system without pH control, IAM1183-ΔldhA had similar fermentative characteristics with those of the serum bottle batch system. LDH-disrupted IAM1183-ΔldhA proved to be a good candidate for hydrogen production. The main purpose of the fermentor experiment was to compare its metabolic characteristics with those in the serum bottles. The fermentative parameters need to be optimized for the bioprocess engineering of hydrogen production.

Table 4
Analysis of consumed substrate and anaerobic metabolites after 25 h of cultivation in 5-L fermenter for IAM1183-ΔldhA and wild-type IAM1183.

End products	Product yield (mmol/mol glucose)	
	IAM1183-ΔldhA	IAM1183
Glucose consumption percent (%)	98.96	85.8
Residual glucose (g/L)	0.16	2.12
Hydrogen (H_2) (L)	5.6 ↑	2.99
Succinate	2.27 ↑	0.62
Lactate	2.22 ↓	41.01
Formate	6.15 ↑	0
Acetate	11.31 ↓	14.93
2,3-Butanediol	34.4 ↑	1.14
Ethanol	29.76 ↑	4.71

4. Conclusions

Overall, LDH and ADH of IAM1183 were successfully impaired to enhance hydrogen production by regulating NADH metabolism. Metabolic flux analysis indicated that IAM1183-ΔldhA exhibited greatly enhanced fluxes for generation of hydrogen and other species while the fluxes of IAM1183-Δadh decreased except formate. The hydrogen production of IAM1183-ΔldhA was 2.3 times higher than the wild-type in the fermentor. The mutant IAM1183-ΔldhA could be a promising candidate to further improve hydrogen production using genetic technologies. The findings reported here also represent a key step towards elucidating the biochemical mechanisms of bacterial hydrogen production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.06.149.

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