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Enhanced hydrogen production from glucose using *ldh*- and *frd*-inactivated *Escherichia coli* strains

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Abstract We improved the hydrogen yield from glucose using a genetically modified *Escherichia coli*. *E. coli* strain SR15 (Δ*ldhA*, Δ*frdBC*), in which glucose metabolism was directed to pyruvate formate lyase (PFL), was constructed. The hydrogen yield of wild-type strain of 1.08 mol/mol glucose, was enhanced to 1.82 mol/mol glucose in strain SR15. This figure is greater than 90 % of the theoretical hydrogen yield of facultative anaerobes (2.0 mol/mol glucose). Moreover, the specific hydrogen production rate of strain SR15 (13.4 mmol h^{-1} g^{-1} dry cell) was 1.4-fold higher than that of wild-type strain. In addition, the volumetric hydrogen production rate increased using the process where cells behaved as an effective catalyst. At 94.3 g dry cell/l, a productivity of 793 mmol h^{-1} l^{-1} (20.2 l h^{-1} l^{-1} at 37 °C) was achieved using SR15. The reported productivity substantially surpasses that of conventional biological hydrogen production processes and can be a trigger for practical applications.

Introduction

Hydrogen is a most promising alternative energy source given the projected depletion of fossil fuels and the development of fuel cells (Benemann 1996). It is currently produced chemically by refining oil or natural gas at high temperatures and pressures. In contrast, biological hydrogen production occurs at ambient temperatures and pressures. Furthermore, it has the striking merit of obviating production of carbon monoxide, which is harmful to the electrodes of fuel cells.

Biological hydrogen production is classified into two main categories: photosynthesis and dark fermentation. In general, the specific hydrogen production rate of dark fermentation is much higher than that of photosynthesis (Nandi and Sengupta 1998; Nath and Das 2004). Although biological hydrogen has been hampered by its low volumetric production rate when compared to chemical processes, improvements using various approaches have been reported recently (Chin et al. 2003; Kim et al. 2004; Kumar and Das 2001; Lee et al. 2003; Oh et al. 2002; Rachman et al. 1998; Wu et al. 2005). By using formic acid and recombinant *E. coli* as catalyst, we previously reported enhanced volumetric production rate to 11.8 mol h^{-1} l^{-1} (300 l h^{-1} l^{-1} at 37 °C) (Yoshida et al. 2005).

As a renewable energy source from biomass, glucose is one of the most important substrates for hydrogen production because of its high hydrogen content and its abundant availability in the form of biomass. In theory, facultative anaerobes like *E. coli* or *Enterobacter* species evolve 2 mol of hydrogen from each mole of glucose consumed, whereas, strict anaerobes like *Clostridium* species, which grow much more slowly, evolve 4 mol of hydrogen from each mole of glucose consumed. However, adequate hydrogen yields from glucose have not been achieved so far (Nath and Das 2004).

The glucose metabolic pathway of facultative anaerobes, especially *E. coli*, is understood in detail (Clark 1989). There are two possible ways through which improved hydrogen yields from glucose can be achieved. The first involves directing glucose metabolism toward pyruvate formate lyase (PFL) by disrupting the lactate-producing and succinate-producing pathways. The second encompasses enhanced downstream pathways of PFL through overexpression of the formate hydrogen lyase (FHL) complex.

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In this study, we demonstrate the improvement of hydrogen yield from glucose by genetically modifying the hydrogen-producing pathways of *E. coli*. We consequently use the resultant strain exhibiting enhanced volumetric hydrogen production rate in a manipulated process where cells behave as effective catalysts.

Materials and methods

Construction of recombinant strains

The strains, plasmids, and primers used in this study are shown in Table 1. SR14 and SR15 were constructed by using a modification of the method described previously (Sauter et al. 1992). pSR203 was obtained by amplifying the *ldhA* gene of *E. coli* K-12 strain W3110 using primers ldhA-fw and ldhA-rv, digesting the product with *Bam*HI and *Pst*I, and inserting the resulting digest within the *Bam*HI and *Pst*I sites of pHSG398. pSR205 was constructed from pSR203 by inverse amplification using primers ldhAinv-fw and ldhAinv-rv, digesting the product with *Spe*I and *Xba*I, followed by re-ligating with *Spe*I- and *Xba*I-digested chloramphenicol cassette, which was obtained by amplifying the Cm^r region of pHSG398 with primers cat-fw and cat-rv. pSTK102 was constructed by inserting the *Bam*HI- and *Pst*I-digested *ldhA*::Cm^r region of pSR205 within the *Bam*HI and *Pst*I sites of pSTK1. pSTK102 was electroporated into strains SR13 and W3110, and the resulting transformant strains were cultivated in LB medium containing chloramphenicol (50 mg/l) at 43 °C. The recombinant strains were cultivated in minimal medium containing sucrose (Yoshida et al. 2005) at 30 °C for 24 h, and the *sacB* region was excised to obtain *ldhA*-disrupted strains. In the same manner, *frdBC*-disrupted strains SR14 and SR15 were obtained each from *ldhA*-disrupted SR13 and W3110 by using plasmids and primers listed in Table 1. All genetic manipulations were performed using *E. coli* JM109.

Cultivation and reaction conditions

E. coli K-12 strain W3110 and the recombinant strains (Table 1) were initially cultivated aerobically in test tubes at 37 °C in 10 ml BC medium (Yoshida et al. 2005)

Table 1 Strains, plasmids, and primers used in this study

	Genotype	Reference
Strain		
W3110	$F^- \lambda^-$ IN(*rrnD-rrnE*)1 *rph-1*	ATCC 27325
SR13	W3110 Δ*hycA*, overexpression of *fhlA*	Yoshida et al. 2005
SR14	SR13 *ldhA*::Cm^r, *frdBC*::Km^r	This work
SR15	W3110 *ldhA*::Cm^r, *frdBC*::Km^r	This work
JM109	*endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ(*lac-proAB*), *recA*1, F′[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	ATCC 53323
Plasmid		
pHSG398	Cm^r	TaKaRa
pUC4K	Km^r	Pharmacia
pSTK1	rep-ts, Km^r, *sacB*	Yoshida et al. 2005
pSR203	pHSG398, with a 4.2-kb *Bam*HI–*Pst*I fragment containing the *E. coli* W3110 *ldhA* gene	This work
pSR204	pHSG398, with a 3.3-kb *Bam*HI–*Sac*I fragment containing the *E. coli* W3110 *frdABCD* gene	This work
pSR205	pHSG398, a 0.9-kb Cm^r cartridge inserted into pSR203 *Spe*I–*Xba*I site	This work
pSR206	pHSG398, a 1.0-kb Km^r cartridge inserted into pSR204 *Xho*I site	This work
pSTK102	pSTK1 with a 5.1-kb fragment of *ldhA*::Cm^r from pSR205	This work
pSTK103	pSTK1 with a 4.3-kb fragment of *frdBC*::Km^r from pSR206	This work
Primer		
ldhA-fw	5′-CCTGTTTCGCTTCACCGGTCAG-3′	This work
ldhA-rv	5′-TCTTTGGTTCTGTCCAGTACCG-3′	This work
frdBC-fw	5′-GCGAGCTCGTGCAAACCTTTCAAGCCGA-3′	This work
frdBC-rv	5′-CGGGATCCGACACCAATCAGCGTGACAA-3′	This work
ldhAinv-fw	5′-GGACTAGTCTGGCGTTCGATCCGTATCC-3′	This work
ldhAinv-rv	5′-GCTCTAGACCAAAACCTTTCAGAATGCGCA-3′	This work
frdBCinv-fw	5′-CCGCTCGAGCTGAACCCAGAGTTCATCGG-3′	This work
frdBCinv-rv	5′-CCGCTCGAGAACGTACGCTTTCGCCAGTT-3′	This work
cat-fw	5′-GCTCTAGAACGGAAGATCACTTCGCAGAAT-3′	This work
cat-rv	5′-GGACTAGTTTAAGGGCACCAATAACTGCCT-3′	This work
kan-fw	5′-CCGCTCGAGGAAGATGCGTGATCTGATCCT-3′	This work
kan-rv	5′-CCGCTCGAGGCCACGTTGTGTCTCAAAATC-3′	This work

supplemented with 30 mM glucose and 50 mg/l ampicillin, kanamycin, or chloramphenicol where necessary. The 24-h cell suspension was centrifuged at 6,500×*g* for 20 min and washed appropriately with BC medium in an anaerobic chamber (model A; Coy). As the FHL induction phase, anaerobic cultures were carried out in an atmosphere comprising 95 % nitrogen and 5 % hydrogen. Cells were inoculated to an initial OD_{610} of 0.2 in an appropriate volume of BC medium supplemented with 150 mM glucose and 50 mg/l ampicillin, kanamycin, or chloramphenicol where necessary, maintaining the pH at 6.0 using 5 M NaOH. After 12 h of incubation under anaerobic conditions, a sample of the cell suspension was harvested by centrifugation, washed appropriately with BC medium, and resuspended to an appropriate cell density in 100 ml reaction buffer [yeast extract 5 g/l, tryptone peptone 10 g/l, 100 mM phosphate buffer pH 6.2, 100 µl antifoam (Antifoam SI; Wako Chemicals)].

Measurement of hydrogen production ability

Cell density during cultivation was measured spectrophotometrically (Novaspec II; Amersham Bioscience). An OD_{610} of 1.0 was equivalent to 0.41 g (dry cell weight) cells/l. The specific and volumetric hydrogen production rate was measured as the rate of hydrogen produced from a stirred cell suspension at an appropriate OD_{610} in 100 ml reaction buffer in a reactor equipped with pH sensor and ports for NaOH-feed, gas exhaustion, substrate-feed and sampling, in the presence of 60 mM glucose. The atmosphere in the reactor was completely anaerobic. The concentration of hydrogen and carbon dioxide in the evolved gas phase was analyzed by gas chromatography (GC-14B; Shimadzu) using a thermal conductivity detector (Kawaguchi et al. 2001), and the hydrogen production rate was calculated from the value detected by gas chromatography and collected gas volume. The concentration of glucose was detected by glucose analyzer (BF-4; Oji Scientific Instruments), the concentration of metabolites from glucose (lactate, succinate, acetate, formate, malate) was detected by high-performance liquid chromatography (CCP & 8020 series, Tosoh) equipped with an electric conductivity detector, and the concentration of ethanol was detected by gas chromatography (Inui et al. 2004).

Results

Improvement of hydrogen yield by genetic modification

To demonstrate enhanced hydrogen yield from glucose, the effects of genetic modification (overexpression of the FHL complex, which degrades formate to hydrogen, and disruption of *ldh*A and *frd*BC encoding lactate dehydrogenase and fumarate reductase, respectively, which produce undesired products) were confirmed. The modified pathway is shown in Fig. 1, and the genotypes of the constructed strains are shown in Table 1. Hydrogen yield was measured upon adding 60 mM glucose to cells suspended in phosphate buffer to OD_{610} of 20.0 and maintaining the pH at 6.0 with 5 M NaOH in a reactor at 37 °C. The yield of the FHL-overexpressing strain SR13 of 0.95 mol/mol glucose when added glucose was completely consumed was almost the same as that of wild-type strain W3110. On the other hand, the yield of the lactate dehydrogenase- and fumarate-reductase-inactivated strain SR15 of 1.82 mol/mol glucose was about 1.7-fold that of W3110. There was no striking difference in hydrogen yield between SR15 and SR14, which differs from SR15 in that its FHL complex is overexpressed. The concentration of hydrogen in the evolved gas phase was around 24 mM for all four strains.

The specific hydrogen production rate of SR13 of 9.1 mmol h^{-1} g^{-1} dry cell was the same as that of W3110. On the other hand, the rate of SR15 of 13.4 mmol h^{-1} g^{-1} dry cell was about 1.4-fold that of W3110. Moreover, there was no striking difference in specific hydrogen production rate between SR15 and SR14. The rate hardly changed in the course of the reaction. A summary of these results is shown in Table 2.

To analyze the effects of directing the carbon flux toward PFL, the concentrations of metabolites from glucose by each strain were analyzed (Table 3). With all strains, added glucose was completely consumed and more than 90 % of the carbon was recovered as metabolites. There were no differences in the concentration of metabolites upon FHL overexpression, as hydrogen productivity did not change with FHL overexpression. One-third of the added carbon was converted to lactate and succinate in W3110 and SR13. In contrast, SR14 and SR15 produced almost no lactate and succinate. SR14 and SR15 produced 1.5-fold acetate and ethanol and 1.8-fold carbon dioxide about of W3110 and SR13. Most of the carbon was confirmed to pass through

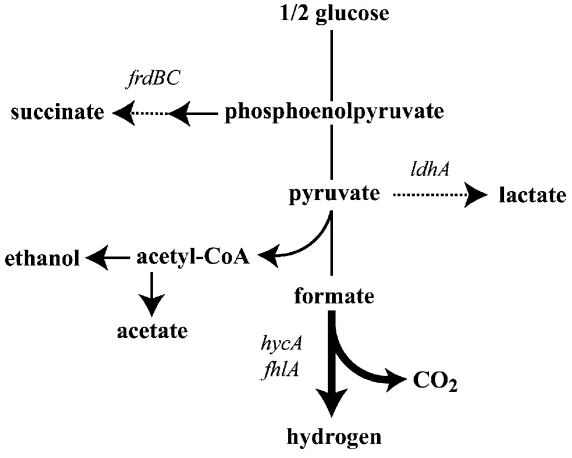

Fig. 1 Hydrogen production pathway from glucose and the strategy to improve the productivity of hydrogen. Some of the intermediates are not shown here. The *dashed lines* are the pathways inactivated by disrupting *ldhA* and *frdBC*, and the *bold lines* are the pathways enhanced by disrupting *hycA* and overexpressing *fhlA* in this study

Table 2 Hydrogen concentration, hydrogen production rate, and hydrogen yield from glucose[a]

Strain	W3110	SR13	SR14	SR15
Hydrogen concentration in evolved gas phase (mM)	24.0±0.5	24.8±0.2	24.3±0.8	24.5±0.4
Specific H_2 production rate from glucose (mmol h^{-1} g^{-1} dry cell)	9.5±0.8	9.1±0.8	13.0±1.1	13.4±1.2
Hydrogen yield from glucose (mol/mol glucose)	1.08±0.13	0.95±0.11	1.87±0.06	1.82±0.11

[a]All the values are averages of triplicate experiments

PFL in SR14 and SR15. In addition, on the basis of hydrogen balance about one-third of hydrogen in glucose was converted to lactate and succinate in W3110 and SR13, and more than 90 % of hydrogen passed through PFL in SR14 and SR15 (data not shown). These results corroborated high hydrogen yield in SR14 and SR15.

Characteristics of hydrogen production from glucose

Apart from the improved hydrogen yield shown above, the general features of hydrogen production from glucose determined using wild-type strain W3110. First, the effect of glucose concentration in the reaction buffer on the hydrogen production rate was measured. The specific hydrogen production rate was 8.0±0.7 and 8.7±0.7 mmol h^{-1} g^{-1} dry cell at 5 and 180 mM of glucose, and the hydrogen production rate was independent of the glucose concentration in the range from 5 to 180 mM.

In addition to the concentration of substrate, changes in the specific hydrogen production rate with temperature and pH were determined by comparing the initial rates of hydrogen produced in the presence of 60 mM glucose at different temperature and pH values (Fig. 2). Maximum production rate was observed at around 47 °C. Below this temperature a gradual decrease in productivity was observed, whereas, above this temperature a drastic decrease in productivity was detected (Fig. 2a). Likewise, peak productivity was observed around pH 6.0, with a drastic decrease in productivity observed below pH 5.5 (Fig. 2b). Hydrogen production was detected even at alkaline pH, although the productivity was half that at pH 6.

Construction of high-cell-density biohydrogen reactor

To improve the volumetric hydrogen production rate, the cell density in the reactor was increased. The effect of increased reactor cell density was analyzed by using strains W3110 and SR15 (Fig. 3). Considering the durability of cells, the reaction was performed at 37 °C. In W3110 at the cell density of 92.3 g dry cell/l, the reaction started immediately after addition of glucose. In 9 min, 60 mM glucose was completely degraded. The volumetric hydrogen production rate was 481 mmol h^{-1} l^{-1} (12.2 l h^{-1} l^{-1} at 37 °C). With SR15 at the cell density of 94.3 g dry cell/l, the reaction commenced immediately after the addition of glucose, and 60 mM glucose was completely used up in 14 min. The volumetric hydrogen production rate was 793 mmol h^{-1} l^{-1} (20.2 l h^{-1} l^{-1} at 37 °C). In SR15, therefore, hydrogen productivity was 1.4-fold higher than that in W3110, even at high cell density (above 90 g dry cell/l). The volumetric hydrogen productivity of both W3110 and SR15 strains improved with increasing cell density, but the specific productivity decreased gradually as cell density increased. With SR15, the specific hydrogen production rate at high cell density of (94.3 g dry cell/l) decreased 59 % compared to that achieved at low cell density (5.1 g dry cell/l).

Discussion

In this study, hydrogen yield was drastically improved by using newly constructed strains. The yield from SR14 and SR15 was substantially improved to 1.87 and 1.82 mol/mol glucose, respectively, compared to the yield of 1.08 mol/mol glucose by wild-type strain W3110. Lactate and succinate were the main metabolites required to exhaust

Table 3 The amount of the metabolites in hydrogen production reaction from glucose[a]

Strain	W3110	SR13	SR14	SR15
Lactate	0.98±0.12	1.41±0.25	0.04±0.01	0.06±0.01
Succinate	1.01±0.06	0.98±0.02	0.10±0.01	0.13±0.02
Ethanol	1.74±0.19	1.82±0.20	2.68±0.29	2.76±0.25
Acetate	0.68±0.01	0.58±0.08	0.83±0.05	0.91±0.07
Carbon dioxide	0.80±0.10	0.64±0.08	1.33±0.04	1.27±0.04
Formate	0.05±0.03	0.05±0.02	0.06±0.01	0.06±0.01
Malate	0.19±0.05	0.19±0.03	0.24±0.02	0.27±0.24
Total	5.45±0.26	5.68±0.28	5.28±0.35	5.45±0.16

[a]Each value is the ratio of carbon recovery when the carbon amount of the added glucose was defined as 6.0 (carbon number per molecule of glucose). All the values are averages of triplicate experiments

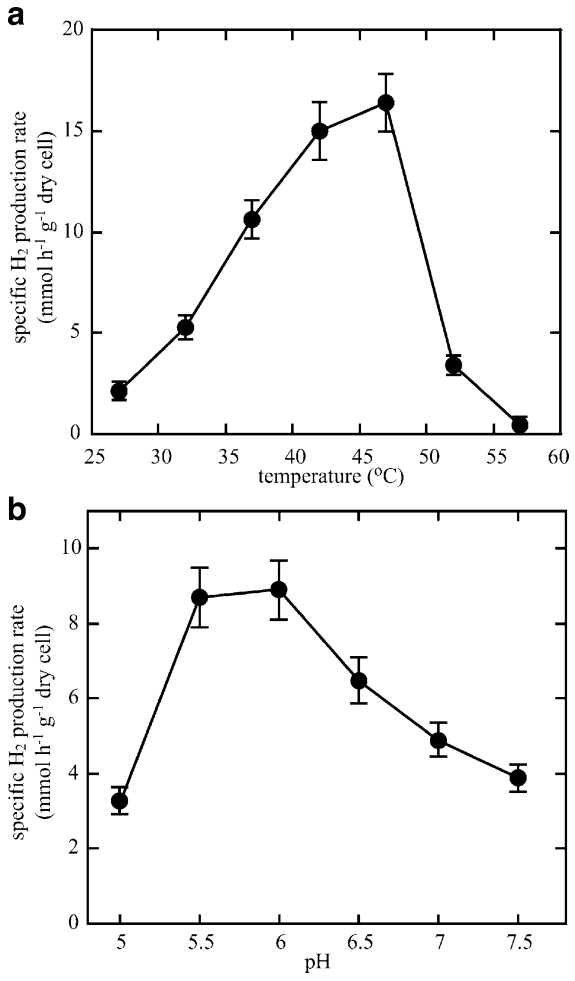

Fig. 2 Temperature and pH dependence of hydrogen production from glucose using W3110. A 100-ml cell suspension (8.2 g dry cell/l) was mixed with a stirrer, and hydrogen production was initiated by adding 60 mM glucose to the reactor. The hydrogen production rate was plotted from the results of the initial hydrogen production rate. The *error bars* indicate standard deviations. **a** Temperature dependence between 27 and 57 °C. **b** pH dependence between pH 5.0 and 7.5

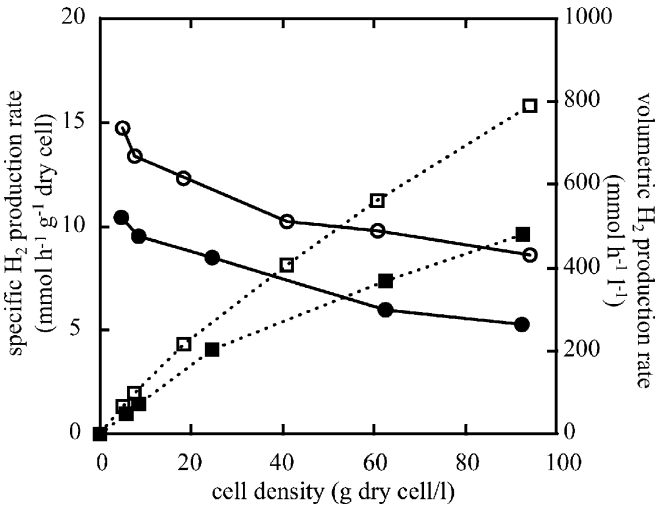

Fig 3 Effects of cell density on the volumetric hydrogen production rate from glucose of SR15 (*open squares*) and W3110 (*closed squares*) and the specific hydrogen production rates of SR15 (*open circles*) and W3110 (*closed circles*). Each value represents the initial hydrogen production rate. All the reactions were conducted using 100 ml cell suspension

excess electrons as reduced equivalents under anaerobic conditions (Alam and Clark 1989). The hydrogen yield above 90 % of the theoretical yield of 2.0 mol/mol glucose was obtained by disrupting the pathways which are used in absorbing the excess reductants.

From the viewpoint of carbon flux, one-third of the carbon of added glucose was converted to lactate and succinate in W3110 and SR13 (Table 3). In SR14 and SR15, about 1.5-fold amount of the carbon was recovered through PFL, compared to the amount in W3110 (Table 3). Moreover, the ethanol-to-acetate ratio via PFL increased to a large extent in SR14 and SR15, compared to W3110. The likely explanation of the increase of the ethanol to acetate ratio is that a reoxidation pathway of NADH exists only in the ethanol-producing pathway in SR14 and SR15, although the reoxidation pathway of NADH exists in the lactate and succinate pathways in addition to the ethanol pathway in the wild-type strain (Clark 1989).

In terms of specific hydrogen production rate, SR13 did not show improved hydrogen production rate. This indicates that the FHL complex is not a limiting factor for hydrogen production from glucose, as discussed in a different study (Sode et al. 1998). Although the FHL-overexpressing strain constructed by *hycA* disruption showed enhanced hydrogen production rate from glucose (Penfold and Macaskie 2004), the FHL overexpressing strains in this study did not exhibit improved specific production rate (Table 2). This is presumably due to the fact that in this study the FHL complex was already expressed in adequate amounts for hydrogen production because the cells were cultivated anaerobically before the reaction. The specific hydrogen production rate was 1.4-fold greater in SR14 and SR15, compared to W3110, in spite of the fact that the single *ldhA*-disrupted strain did not show any improvement in production rate (Sode et al. 1999). Among the metabolites from glucose by W3110, about 15 mM of succinate was produced from 60 mM glucose (Table 3). By disrupting the succinate and lactate pathways concurrently, the flux from glucose was directed to PFL in the resultant strain and the specific hydrogen production rate was clearly increased.

The effects of the change of glucose concentration on hydrogen production were hardly detectable, although a striking dependence on the concentration of formate was detected (Yoshida et al. 2005). The specific hydrogen production rate from glucose remained constant to the end of the reaction. This zero dimensional reaction implies the presence of multiplex enzyme reactions from glucose to hydrogen in the cell. The maximum productivity appeared at slightly acidic conditions (pH 5.5–6.0) in the case of glucose as the substrate (Fig. 2). A likely explanation for this observation is that the FHL complex is a means of maintaining pH homeostasis by degrading the accumulated

formate and the maximal activity of FHL exists at a slightly acidic condition (Sawers 1994).

When cell density in the reactor got higher, the linearity of the volumetric hydrogen production rate collapsed (Fig. 3). This phenomenon could be explained by limited substrate dispersion in the reactor due to the high cell density, or the accessibility for the produced gas in the liquid phase to be excreted out of the gas phase (Yoshida et al. 2005). The engineering improvement of the reactor is supposed to overcome these difficulties.

It has been estimated that 23.9 mol/h hydrogen is necessary to activate a 1-kW proton exchange membrane fuel cell (Levin et al. 2004). Based on the hydrogen production rate of 793 mmol h^{-1} l^{-1} (20.2 l h^{-1} l^{-1} at 37 °C) attained in this study, about 30 l of cell suspension would be sufficient to provide 1 kW of electric power. The volumetric hydrogen production rate in this study was vastly improved, compared to the productivity of conventional biological hydrogen production, and the results in this study promote biohydrogen to practical applications.

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