Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he

Metabolic engineering of *Escherichia coli* strains for co-production of hydrogen and ethanol from glucose

*Eunhee Seol, Satish Kumar Ainala, Balaji Sundara Sekar, Sunghoon Park**

School of Chemical and Biomolecular Engineering, Pusan National University, Busan 609-735, Republic of Korea

ARTICLE INFO

Article history:
Available online 8 July 2014

Keywords:
Co-production
Hydrogen
Ethanol
EMP
PPP
EDP

ABSTRACT

The co-production of H_2 and ethanol from glucose was studied to address the low H_2 production yield in dark fermentation. Several mutant strains devoid of *ackA-pta*, *pfkA* or *pgi* were developed using *Escherichia coli* BW25113 Δ*hycA* Δ*hyaAB* Δ*hybBC* Δ*ldhA* Δ*frdAB* as base strain. Disruption of *ackA-pta* eliminated acetate production during glucose fermentation but resulted in the secretion of a significant amount of pyruvate (0.73 mol mol^{-1} glucose) without improving the co-production of H_2 and ethanol. When *pfkA* or *pgi* was further disrupted to enhance NAD(P)H supply by diverting the carbon flux from Embden-Meyerhof-Parnas (EMP) pathway to the pentose phosphate pathway (PPP), the cell growth of both strains was severely impaired under anaerobic conditions, and only the Δ*pfkA* mutant could recover its growth after adaptive evolution. The production yields of the Δ*pfkA* strain (H_2, 1.03 mol mol^{-1} glucose and ethanol, 1.04 mol mol^{-1} glucose) were higher than those of the *pfkA*$^+$ strain (H_2, 0.69 mol mol^{-1} glucose and ethanol, 0.95 mol mol^{-1} glucose), but pyruvate excretion was not reduced. The excessive excretion of pyruvate in the Δ*pfkA* mutant was attributed to an insufficient NAD(P)H supply caused by the diversion of carbon flux from the EMP pathway to the Entner-Doudoroff pathway (EDP), rather than the PPP as intended. This study suggests that co-production of H_2 and ethanol from glucose is possible, but further metabolic pathway engineering is required to fully activate PPP under anaerobic conditions.

Introduction

Hydrogen (H_2) production via dark fermentation has been considered to be the most practical biological method because of the method's high H_2 production rate, easy operation of the bioreactor, diversity of feedstock, and light independence [1,2]. Nevertheless, dark fermentation has a limited success commercially because the H_2 production yield, the amount of H_2 produced per carbon substrate consumed, is low. Strain development to improve the H_2 production yield has been extensively investigated. The most popular approach was the deletion of the competing pathways against H_2 production and/or the improvement of H_2 production activity by overexpressing (inactivating) the activator (repressor) for H_2 production enzymes [3–5]. In

* *Corresponding author*. Tel.: +82 51 510 2395; fax: +82 51 515 2716.
E-mail address: parksh@pusan.ac.kr (S. Park).
http://dx.doi.org/10.1016/j.ijhydene.2014.06.054

addition, the heterologous expression of foreign hydrogenases which are known to utilize NAD(P)H as reducing power was attempted [6,7]. Some improvement in the H_2 production yield (up to 2–4 mol H_2 mol^{-1} glucose) has been accomplished, but the yield is not still satisfactory for commercial applications.

To address the low H_2 production yield in dark fermentation, several novel processes have also been suggested. One example is the two-stage process which combines dark and photo-fermentation. In this process, the by-products of the first, dark fermentation are to be further converted into H_2 in the second, photo-fermentation. This process improved H_2 production yield substantially, but the reaction in the second stage was very slow and a very high retention time was required [8,9]. The co-production of H_2 and CH_4 (so-called 'hythane' process) using a similar two-stage system has also been studied. In this case, CH_4, instead of H_2, is produced in the second stage. Although H_2 production yield is not enhanced in this process, energy recovery from carbon feedstock is greatly enhanced. However, the value of CH_4 as biofuel is significantly lower than that of H_2 [10,11]. Also, CH_4 production proceeds more slowly than H_2 production and, thus, a longer retention time for the former reaction is required as in the photo-fermentation.

Another strategy to cope with the low H_2 production yield in the dark fermentation of glucose is the co-production of H_2 and ethanol. Ethanol is one of the attractive, alternative biofuels producible through the fermentation by various anaerobic microorganisms including the facultative anaerobe, *Escherichia coli*. Additionally, liquid ethanol can be easily separated from gaseous H_2 during co-production. According to the mixed acid fermentation of glucose by facultative anaerobes, ethanol is naturally produced along with many other acids and alcohols such as acetate, lactate, succinate, butanol etc [12]. To improve ethanol production, it is necessary to reduce the production of these metabolites, especially acetate which is produced in an equal molar quantity as ethanol. Both ethanol and acetate are produced from pyruvate and to divert acetate to ethanol, NADH should be supplied sufficiently because 2 mol of NADH per mol ethanol production from pyruvate are required. Disruption of competing pathways for the production of acetate, lactate and succinate etc. is also needed [3,4].

This study aims to co-produce H_2 and ethanol in dark fermentation of glucose by deleting the acetate production pathway and increasing the regeneration of NAD(P)H. Several mutant strains devoid of *ackA-pta*, *pfkA* or *pgi* were developed using *E. coli* BW25113 Δ*hycA* Δ*hyaAB* Δ*hybBC* Δ*ldhA* Δ*frdAB* as base strain. Although both the pentose phosphate pathway (PPP) and tricarboxylic acid (TCA) cycle regenerate NAD(P)H, the requirement of the strict anaerobic condition for H_2 production prevents the use of the TCA cycle which is not fully functional under anaerobic conditions. To divert glucose metabolism from the Embden-Meyerhof-Parnas (EMP) pathway to PPP, the reversible phosphoglucose isomerase (Pgi) or phosphofructokinase 1 (PfkA) in the EMP pathway was disrupted. This study demonstrates that co-production of H_2 and ethanol from glucose is possible, although complete activation of PPP under anaerobic conditions is challenging.

Materials and methods

Construction of recombinant E. coli strains and adaptive evolution

The bacterial strains and plasmids used in this study are presented in Table 1. The bacterial strain *E. coli* SH5 was used as parental strain for the construction of mutant strains [3]. Development of mutant strains was performed using a λ red recombinase system [13,14]. The strain SH8AE, exhibiting higher growth rates, was obtained by adaptive evolution of SH8 strain in fortified M9 media containing 5 g L^{-1} glucose and 3 g L^{-1} yeast extract. The dilution ratio of each transfer was varied from $1:10^4$ to $1:10^5$ as the cell growth rate increased. Transfer was conducted before the optical density A_{600} reached 1.5, during the mid-exponential growth phase. The adaptive evolution was conducted for 40 days for approximately 300 generations.

For the overexpression of *udhA* in SH8AE, the gene was amplified from *E. coli* BW25113. The amplified fragment was cloned into the expression vector pDK7 [15]. This recombinant plasmid was transformed into SH8AE by electroporation, yielding the strain SH8U to overexpress *udhA*.

Culture conditions

Luria Bertani medium was used for regular genetic engineering and culture maintenance work. Kanamycin at 50 μg mL^{-1}, ampicillin at 100 μg mL^{-1}, and chloramphenicol at 25 μg mL^{-1} were added to the culture media when necessary. *E. coli* strains were cultured in a modified M9 medium containing 5 g L^{-1} glucose, 0.3 or 3.0 g L^{-1} yeast extract, 3.0 g L^{-1} Na_2HPO_4, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NH_4Cl, 0.25 g L^{-1} NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 2 mL L^{-1} trace element solution. The culture medium was maintained at pH 7.0 ± 0.2 and the trace element solution contained the following components (per liter): $FeSO_4 \cdot 7H_2O$, 0.21 g; $ZnSO_4 \cdot 7H_2O$, 50 mg; $MnCl_2 \cdot 4H_2O$, 50 mg; H_3BO_3, 15 mg; $CoCl_2 \cdot 6H_2O$, 100 mg; $CuSO_4 \cdot 5H_2O$, 6.7 mg; $NiCl_2 \cdot 6H_2O$, 10 mg; Na_2SeO_3, 5.0 mg; and $Na_2MoO_4 \cdot 2H_2O$, 15 mg. To induce the

Table 1 – Strains and plasmids used in this study.

	Relevant characteristics	Description
Strains		
SH5	BW25113 Δ*hycA* Δ*hyaAB* Δ*hybBC* Δ*ldhA* Δ*frdAB*	[3]
SH6	SH5 Δ*pta-ackA*	This study
SH7	SH6 Δ*pgi*	This study
SH7U	SH7, pSTH	This study
SH8	SH6 Δ*pfkA*	This study
SH8AE	SH8, adaptive evolution	This study
SH8U	SH8AE, pSTH	This study
Plasmids		
pKD4	PCR template for Km cassette and FRT, Kan^R	CGSC
pKD46	λRed recombinase expression plasmid, Amp^R	CGSC
pCP20	FLP recombinase expression plasmid, Amp^R	CGSC
pDK7	Bacterial expression plasmid, Cm^R	ATCC
pSTH	pDK7:*udhA*, Cm^R	This study

expression of the *udhA* gene in the pDK7 plasmid, 1.0 mM of IPTG was added initially to the culture broth.

Anaerobic fermentation was carried out in 165 mL serum bottle with a working volume of 50 mL of initial OD, 0.05–0.15. To create anaerobic conditions, the serum bottles were flushed with argon gas (99.9%) for 10 min and sealed with butyl rubber septum and aluminum caps. All Erlenmeyer flask and serum bottles were incubated at 37 °C in an orbital incubator shaker at 200 rpm. Samples were periodically withdrawn to determine cell growth, glucose consumption, and metabolite production. For analysis of the gas content, gas in the head space was also withdrawn with gas-tight syringe.

In silico *metabolic flux analysis (MFA)*

The glucose metabolic network model was developed for *in silico* MFA [16,17]. The model contains 61 reactions: glycolysis, 14; pentose phosphate pathway, 8; Entner-Doudoroff pathway (EDP), 2; tricarboxylic acid cycle, 11; pyruvate metabolism, 6; energy metabolism 12; transport reaction, 7; and growth flux, 1. The metabolic demands of the precursors and cofactors for the biomass formation were adapted from Kim et al. [16]. The uptake rates of glucose, production rate of metabolites and growth flux obtained from the fermentation experiments were used in the MFA to predict external secretion rates and internal net fluxes of various metabolites. Maximization of the specific growth rate was used as the objective function for simulations. The underdetermined system was solved by linear optimization using the program package MetaFluxNet [18].

Real-time PCR

E. coli SH5, SH6 and SH8AE mutants were cultivated in a modified M9 medium anoxically at 37 °C, with 250 rpm in an orbital incubator shaker. The cells were harvested during the exponential growth phase. Approximately 2×10^8 cells were collected in vials containing two volumes of RNA protect reagent (Qiagen, Inc., USA). Total RNA extraction, cDNA synthesis and RT-PCR were performed as described previously [19].

Analytical methods

Bacterial growth was measured using a spectrophotometer (Lambda 20, Perkin Elmer, USA) at 600 nm. H_2 and CO_2 were measured by GC using the method described by Seol et al. [5]. All the experiments were conducted in duplicate. Glucose consumption and all metabolite measurements were determined using high performance liquid chromatography (Agilent Technologies, HP, 1160 series) equipped with refractive index (RI) and photodiode array (PDA) detectors. The assay for the activity of soluble transhydrogenase (UdhA) was carried out as described by Boonstra et al. [20].

Results and discussion

Estimation of theoretical yields for the co-production of hydrogen and ethanol

Glycolysis of glucose in *E. coli* can occur through three pathways, i.e., the EMP pathway, PPP and EDP (Fig. 1). The EMP

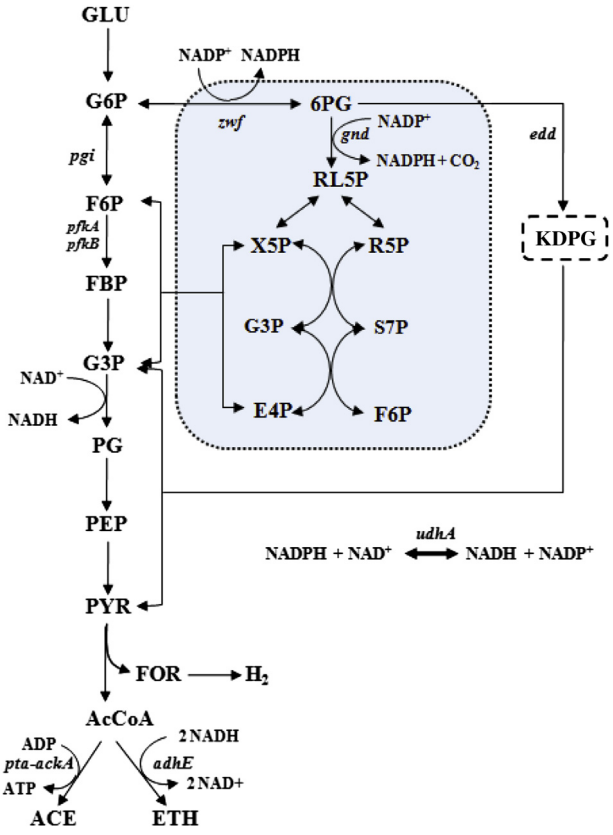

Fig. 1 – Glucose metabolisms in *E. coli* strains used in this study. Pentose phosphate pathwaywas represented in the box. Abbreviations: GLU, glucose; G6P, glucose-6-phosphate; F6P,fructose-6-phosphate; 6PG, 6-phosphogluconate; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; PG, phosphoglycerate; PEP, phosphoenolpyruvate; PYR,pyruvate; AcCoA, acetyl-CoA; FOR, formate; ACE, acetate; ETH, ethanol; RL5P, Ribulose-5-phosphate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate.

pathway is the best-studied route, cleaving 1 mol glucose into 2 mol of 3-carbon pyruvate. Two moles of net ATP and NADH are generated during this glycolysis. The PPP starts with the conversion of glucose-6-phosphate (G6P) to 6-phosphoglu conolactone by glucose-6-phosphate dehydrogenase (encoded by *zwf*) at the G6P branch and plays an important role in supplying NADPH and 5-carbon sources. PPP can operate in several different modes to meet the cellular demands, which result in the production of different amount of NADPH and 5-carbon compounds [21]. When the PPP runs in the non-cyclic mode maximizing NADPH production, the overall supplement of reducing power is increased compared to that of the EMP pathway by producing 2 mol of NADPH in the upper PPP and 5/3 mol of NADH in the lower glycolysis [22]. Meanwhile, the pyruvate yield is less than that from the EMP pathway because of the loss of one carbon as CO_2. In the EDP, the conversion of 6-carbon into each glyceraldehyde-3-phosphate and pyruvate makes the net ATP yield of 1 mol mol^{-1} glucose.

The reducing power supplied in the EDP is the same as that in the EMP pathway by producing each 1 mol of NADH and NADPH. The conversion of pyruvate to fermentative metabolites depends on the availability of NADH. Ethanol production increases if enough NADH is available in the cell, while acetate production increases at low NADH supply. The maximum theoretical yields of H_2 and ethanol for each pathway can be calculated as follows:

EMP: Glucose $\rightarrow$ 2 H_2 + 1 Ethanol + 2 ATP

PPP: Glucose $\rightarrow$ 1.67 H_2 + 1.67 Ethanol + 1.67 ATP

EDP: Glucose $\rightarrow$ 2 H_2 + 1 Ethanol + 1 ATP

This calculation shows that the theoretical maximum yields for the co-production of H_2 and ethanol are obtained by the PPP as 1.67 mol mol^{-1} glucose each. Compared to the EMP pathway and EDP, H_2 production in PPP is slightly lower and this is due to the sacrifice of 1 carbon among the 6 carbons of glucose for the production of extra reducing power. If we assume that only H_2 and ethanol are valuable, the energy recovery in both the EMP pathway and the EDP is 69.6% (2 mol H_2 plus 1 mol ethanol per mol glucose) while 99.3% (1.67 mol H_2 plus 1.67 mol ethanol per mol glucose) for the PPP. The co-production of H_2 and ethanol should be considered a good strategy for improving energy recovery.

Comparison of cell growth

To stimulate the PPP, the EMP pathway was perturbed by deleting *pgi* or *pfkA* [23,24]. The deletion of *pgi* is expected to force all carbon flux to the PPP and/or EDP because the EMP pathway is completely blocked. On the other hand, deleting the *pfkA* gene cannot block the EMP completely because there are 2 Pfk isozymes. Considering that *pfkA* encodes the major isozyme, however, the deletion of PfkA was expected to significantly reduce the carbon flux through the EMP pathway and increase the carbon fluxes to other pathways. Consequently, the yields of pyruvate, ATP and the reducing power, which depend upon the ratio of the carbon flows through the three glycolytic pathways, should be altered.

Before analyzing carbon fluxes and co-production yields, cell growth of the developed strains was examined under aerobic and anaerobic conditions (Fig. 2A–B). All strains developed, except the SH7 which is defective in *pgi* gene, showed similar growth patterns as the parental strain, SH5, under aerobic conditions. Under anaerobic conditions, on the other hand, the deletion of *ackA-pta* affected cell growth significantly. The final biomass yields and specific growth rate were reduced by ~50%. This suggests the importance of the acetate production pathway for ATP production under anaerobic condition [25]. The additional deletion of *pgi* or *pfkA* to *ackA-pta* completely stopped anaerobic cell growth. Enhancing yeast extract concentration in the culture medium from 0.3 to 3.0 g L^{-1} could not recover the cell growth (data not shown). This suggests that, differently from aerobic conditions, anaerobic cell growth without the EMP pathway is very challenging. Not many reports are available in the literatures

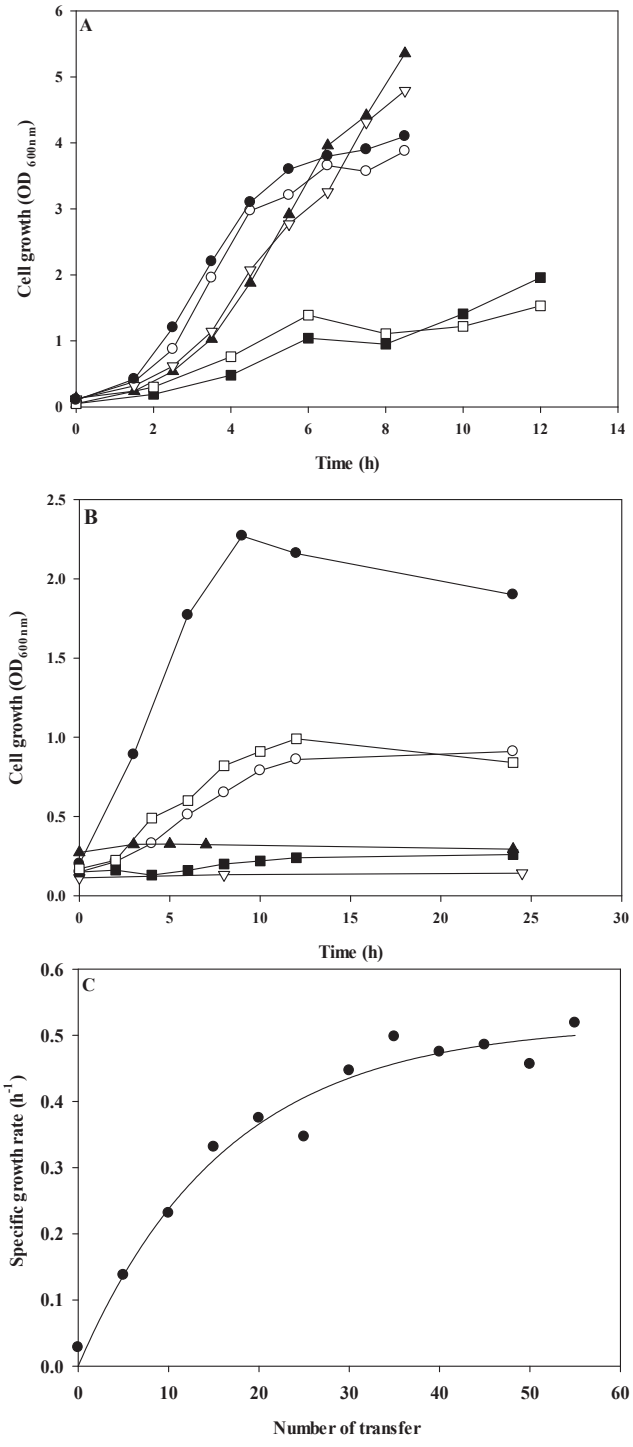

Fig. 2 – Comparison of cell growths under aerobic (A) and anaerobic (B) conditions, and the adaptive evolution of SH8 (C). Symbols: ●, SH5; ○, SH6; ▲, SH7; ▽, SH7U; ■, SH8; □, SH8AE.

on the growth of Δ*pgi* mutants and the contribution of the PPP or EDP to carbon metabolism in the Δ*pgi* mutants under anaerobic conditions. Recently, Kim et al. have reported growth disability of Δ*pgi* mutant under anaerobic conditions but without detailed explanation [26]. Because no experiment is possible without cell growth, the strains SH7 and SH8 were

subjected to successive transfer in the M9 medium for adaptive evolution (Fig. 2C). As the transferring numbers increased, the strain SH8 showed improved cell growth and finally a higher growth rate than SH6 under anaerobic conditions. However, with SH7, adaptive evolution was not successful. Consequently, the adapted SH8 strain, designated as SH8AE, was employed for the further studies.

Comparison of co-production yields of H_2 and ethanol from glucose

Co-production of H_2 and ethanol in SH5, SH6 and SH8AE were compared under anaerobic conditions (Table 2). The base strain SH5 consumed all of the glucose in 12 h and produced H_2 and ethanol with the yields of 1.42 ± 0.03 and 0.84 ± 0.02 mol mol^{-1}, respectively. Acetate of 0.73 mol mol^{-1} and formate of 0.12 mol mol^{-1} were also produced. SH6 did not produce acetate because it lacks the *ackA-pta* gene. On the other hand, the ethanol production increased to 0.95 ± 0.02 mol mol^{-1} (which is higher than that of SH5 by 10%) and the H_2 production decreased to 0.69 ± 0.03 mol mol^{-1}. One significant change by the *ackA-pta* deletion was the large excretion of pyruvate (0.73 ± 0.02 mol mol^{-1}). This is attributed to limited NADH supply required for the conversion of acetyl-CoA to ethanol in the *ackA-pta* deletion mutant; without sufficient NADH, acetyl-CoA is not fully converted to ethanol and results in the accumulation of acetyl-CoA and pyruvate. For successful co-production of H_2 and ethanol, it seems critical to supply enough NADH. The strain SH8AE, constructed for the improved supply of NAD(P)H by diverting carbon flow from the EMP pathway to the PPP, produced more H_2 (1.03 ± 0.02 mol mol^{-1}) and ethanol (1.04 ± 0.05 mol mol^{-1}) compared to SH6. However, the yields of both products were still much lower than the theoretical ones and, furthermore, a comparable amount of pyruvate as the one by SH6 was excreted. This indicates that the simple deletion of the *pfkA* gene from SH6 is not sufficient for supplying enough reducing power for ethanol production. Carbon distributions and reduction degree balances were also estimated for all strains, based on the yields of major metabolites (Table 2). Carbons were well recovered with the SH5 and SH8AE strains, but only 94% of carbon was recovered with SH6. This suggests that some other unknown metabolites have been produced by SH6.

Table 2 – Comparison of metabolite yields, carbon distribution and reduction degree balance.

Strains	SH5 (Glucose)	SH6 (Glucose)	SH8AE (Glucose)	SH8AE (Sorbitol)
Yield (mol/mol glucose)				
H_2	1.42 ± 0.03	0.69 ± 0.03	1.03 ± 0.02	1.25 ± 0.03
Ethanol	0.84 ± 0.02	0.95 ± 0.02	1.04 ± 0.05	1.49 ± 0.06
Acetate	0.73 ± 0.02	0.02 ± 0.05	0.04 ± 0.00	0.05 ± 0.00
Pyruvate	0.02 ± 0.00	0.73 ± 0.02	0.74 ± 0.04	0.24 ± 0.01
Formate	0.12 ± 0.05	0.19 ± 0.05	0.11 ± 0.01	0.25 ± 0.01
Carbon distribution (%)[a]				
Pyruvate	1.07	37.67	39.17	11.31
Ethanol	28.58	31.25	34.64	51.60
Acetate	24.86	2.45	1.47	1.68
Formate	2.09	2.47	1.80	4.22
Biomass	18.39	8.74	8.15	5.96
CO_2	24.23	11.50	17.25	19.79
Recovery (%)	99.22	94.10	101.44	94.56
Reduction degree balance				
Reactant				
Glucose or sorbitol	24	24	24	26
Product				
Pyruvate	0.17	6.15	6.27	1.92
Ethanol	10.28	11.26	12.47	17.88
Acetate	5.97	0.59	0.35	0.40
Formate	0.25	0.30	0.09	0.50
Cell mass	4.49	2.13	1.99	1.46
H_2	2.10	1.38	2.07	2.50
CO_2				
Total	23.26	21.81	23.24	24.26
Errors (%)	−3.07	−9.31	−3.15	−5.17

[a] Carbon distribution (%) = product-carbon moles/substrate-carbon moles × 100.

Effect of carbon source on co-production

The unsuccessful co-production of H_2 and ethanol by SH8AE was attributed to insufficient supply of reducing power for the conversion of acetyl-CoA to ethanol. This can be due to either the low production of NADPH (through the PPP) or the slow conversion of NADPH to NADH. The deletion of the *pfkA* gene from SH6 might not fully divert the carbon flux from the EMP pathway to the PPP and, thus, not enough NADPH might be produced. Alternatively, despite sufficient NADPH production through the PPP, the conversion of NADPH to NADH, which is the cofactor for alcohol dehydrogenase (AdhE) (see Fig. 1), could not be enough owing to the low activity of soluble transhydrogenase (encoded by *udhA* and converting NADPH to NADH) in *E. coli* [27]. To examine the effect of transhydrogenase, *udhA* was overexpressed in SH8AE using a multi-copy plasmid (data not shown). The transhydrogenase activity in the SH8AE host was almost negligible, while the recombinant showed a significantly increased activity of 0.93 ± 0.12 U mg protein^{-1}. Nevertheless, there were no significant changes in the amount of pyruvate excretion and co-production yields (data not shown).

To examine the effect of the NAD(P)H supply, co-production experiment was carried out with sorbitol as carbon source (Table 2). Sorbitol is more reduced than glucose, and produces 1 more NADH than glucose when converted to pyruvate. With sorbitol as a carbon source, the cell growth rate and the carbon consumption rate were reduced, and some of sorbitol was detected in the media even after 24 h of cultivation (data not shown). However, the ethanol and H_2 yields were greatly increased to 1.49 ± 0.06 and 1.25 ± 0.03 mol mol^{-1}, respectively (Table 2). In addition, pyruvate was considerably reduced to 0.24 ± 0.01 from 0.74 ± 0.04 mol mol^{-1}. These results suggest that the deletion of *pfkA* could not fully divert glucose metabolism from the EMP pathway to the PPP, and not enough reducing power for the ethanol production was generated in SH8AE.

Comparison of gene expression

Deletion of the *ackA-pta* and *pfkA* genes did not supply enough NAD(P)H for ethanol production, most probably due to the

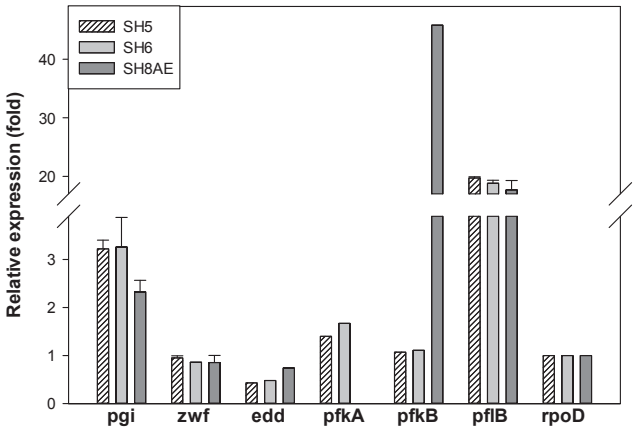

Fig. 3 – Relative mRNA levels of genes of SH5, SH6 and SH8AE. Error bars represent standard deviations of the mRNA levels. The mRNA levels were compared with those of thereference gene, *rpoD*.

insufficient diversion of carbon flux from the EMP pathway to the PPP. To investigate whether these are related to each other, and thus it can be confirmed by gene expression levels for the enzymes in the glycolytic pathways, quantitative PCR experiments were conducted (Fig. 3). The genes chosen for comparison were *pgi* (the first gene of the EMP pathway), *pfkA* and *pfkB* (the two isozymes of phosphofructokinase), *zwf* (PPP), and *edd* (the first genes of the EDP). The expression of *pflB* was also examined to investigate its role in the pyruvate excretion. If the activity of pyruvate formate lyase (PFL), one of whose component is encoded by *pflB*, is very low compared to that of glycolytic pathways, pyruvate should get excreted. The housekeeping *rpoD* gene, encoding the σ^{70} in RNA polymerase, was used as the reference gene for mRNA quantification.

In the base strain of SH5, the expressions of *zwf* and *edd* were much lower than that of *pgi*. In the *ackA-pta* deletion mutant (SH6), similar expression profiles of the three genes (*zwf*, *edd* and *pgi*) were obtained. These results indicate that, among the three glycolytic pathways shown in Fig. 1, the contribution of the EMP pathway to glucose catabolism

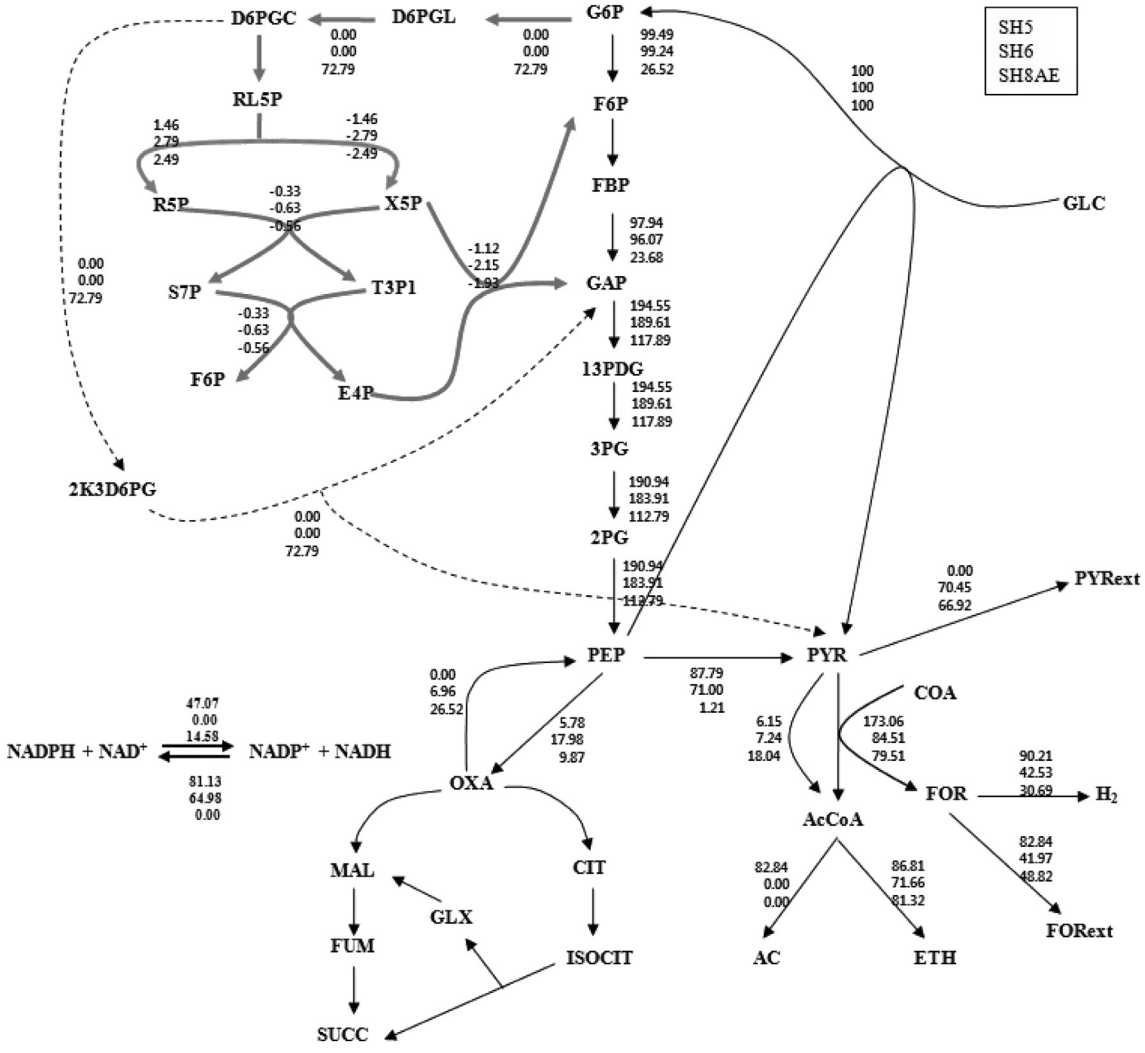

Fig. 4 – All fluxes were normalized to the specific glucose uptake of 100. Metabolic flux distributions of SH5, SH6, and SH8AE were represented upper, middle and bottom, respectively. See the abbreviations in Fig. 1.

should be the highest for both the SH5 and SH6 strains. Also, the results suggest that the massive pyruvate excretion observed with SH6 has not been caused by the change in the rate of pyruvate production through glycolysis or its dissimilation by PFL (see below). With SH8AE, notable changes in the expression of the three genes were observed. The *pfkA* gene was not expressed (confirming its deletion), whereas the *pfkB* expression was drastically increased [28]. The expression of *pgi* was also reduced in the Δ*pfkA* mutant. This suggests that the tendency of *E. coli* to rely on the EMP pathway is very high although deletion of the major isozyme, *pfkA*, most likely constricted the EMP pathway. It should be noticed that the deletion of *pfkA* did not enhance the expression of *zwf*, but enhanced the expression of *edd* by ~70%. This indicates that, contrary to our intention, the deletion of *pfkA* has not activated the PPP but has the EDP instead. Because the activation of the EDP cannot boost the additional supply of NADH, the deletion of *pfkA* might not be a promising strategy. In all three strains, expression of *pflB* did not change. This suggests that the change in glycolytic pathways or accumulation of excessive pyruvate has not affected the PFL activity.

In silico *metabolic flux analysis*

The RT-PCR analysis has suggested that, when the EMP pathway is disturbed by the *pfkA* deletion, the EDP rather than the PPP is activated. *In silico* MFA was conducted to get further evidence for this speculation. As shown in Fig. 4, MFA indicated that, in both SH5 and SH6 strains, glucose was metabolized through the EMP pathway almost exclusively. In SH8AE, on the other hand, the flux through the EMP pathway was reduced while the flux through the EDP significantly was increased. This supports the result from RT-PCR that the deletion of *pfkA* activates the EDP, instead of the PPP. The reason for the activation of the EDP in the *E. coli* cells having the reduced EMP pathway activity and growing anaerobically is not clear. It is likely that the fast-growing cell selected by the adaptive evolution was the one which produces sufficient NADPH through the EDP. Alternatively, bypassing PEP by activating the EDP could confer growth advantage for cells. Recently, Flamholz et al. discussed the merits of the EDP over the EMP pathway in prokaryotes [29]. They suggested that the EDP is thermodynamically more favorable because it avoids the unfavorable reactions in the EMP pathway, such as the reaction by PFK. More studies to elucidate the reason for the undesirable shift of carbon metabolism in the Δ*pfkA* mutants are under progress.

Conclusions

This study focused on the co-production of H_2 and ethanol from glucose using recombinant *E. coli*. Increasing ethanol production was attempted by blocking acetate producing pathway (Δ*pta-ackA*) and perturbing the EMP pathway (Δ*pgi* or Δ*pfkA*). Ethanol production yield was increased by 20% (1.04 ± 0.05 mol mol^{-1}) after disrupting *pta-ackA* and *pfkA*. On the other hand, pyruvate excretion, due to the insufficient supply of NADH, and consequent reduction of H_2 production were noticed. Quantitative gene expression (RT-PCR) and MFA suggested that these undesirable results should be related with the activation of the EDP rather than the PPP after perturbation of the EMP pathway. For successful co-production of H_2 and ethanol, extensive studies for activating the PPP and thus enhancing the system's reducing power are under progress.

Acknowledgments

This study was supported by the Development of Biohydrogen Production Technology using the Hyperthermophilic Archea Program of the Ministry of Ocean and Fisheries in the Republic of South Korea (D10906313H320000120) and by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP, 2012K1A3A1A19036612).

REFERENCES

[1] Rittmann S, Herwig C. A comprehensive and quantitative review of dark fermentative biohydrogen production. Microb Cell Fact 2012;11:115–23.
[2] Kim JK, Nhat L, Cheun YN, Kim SW. Hydrogen production conditions form food waste by dark fermentation with *Clostridium beijerinckii* KCTC 1785. Biotechnol Bioproc Eng 2008;13:499–504.
[3] Kim S, Seol E, Oh Y-K, Wang GY, Park S. Hydrogen production and metabolic flux analysis of metabolically engineered *Escherichia coli* strains. Int J Hydrogen Energy 2009;34:7417–27.
[4] Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. Enhanced hydrogen production from formic acid by formate hydrogen lyase-overproducing *Escherichia coli* strains. Appl Environ Microbiol 2005;71:6762–8.
[5] Seol E, Jang Y, Kim S, Oh Y-K, Park S. Engineering of formate-hydrogen lyase gene cluster for improved hydrogen production in *Escherichia coli*. Int J Hydrogen Energy 2012;37:15045–51.
[6] Ghosh D, Bisaillon A, Hallenbeck PC. Increasing the metabolic capacity of *Escherichi coli* for hydrogen production through heterologous expressionof the *Ralstonia eutropha* SH operon. Biotechnol Biofuels 2013;6:122–31.
[7] Veit A, Akhtar MK, Mizutani T, Jones PR. Constructing and testing the thermodynamic limits of synthetic NAD(P)H: H_2 pathways. Mocrobial Biotechnol 2008;1:382–94.
[8] Foglia D, Wukovits W, Friedl A, Vrije T, Classen P. Fermentative hydrogen production: influence of application of mesophilic and thermophilic bacteria on mass and energy balances. Chem Eng Trans 2011;25:815–20.
[9] Hema R, Pushpa A. Production of clean fuel from waste biomass using combined dark and photofermentation. IOSR J Comp Eng 2012;1:39–47.
[10] Smolinski A, Howaniec N. Sustainable production of clean energy carrier-hydrogen. Ecol Chem Eng 2009;16:335–45.
[11] Peixoto G, Pantoja-Filho JLR, Agnelli JAB, Barboza M, Zaiat M. Hydrogen and methane production, energy recovery, and organic matter removal from effluents in a two-stage fermentative process. Appl Biochem Biotechnol 2012;168:651–71.
[12] Forster AH, Gescher J. Metabolic engineering of *Escherichia coli* for production of mixed-acid fermentation end products. Front Bioeng Biotechnol 2014;2:1–12.

[13] Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 2009;97:6640–5.
[14] Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of *Escherichia coli* K-12 in-frame single-gene knockout mutants: the Keio collection. Mol Syst Biol 2006. http://dx.doi.org/10.1038/msb4100050.
[15] Ashok S, Sankaranarayana M, Ko Y, Jae K-E, Ainale S, Kumar V, et al. Production of 3-hydroxypropionic acid from glycerol by recombinant *Klebsiella pneumoniae* Δ*dhaT*Δ*yqhD* which can produce vitamin B12 naturally. Biotechnol Bioeng 2012;110:511–24.
[16] Schillin CH, Edwards JS, Letscher D, Palsson BØ. Combining pathway analysis with flux balance analysis for the comprehensive study of metabolic systems. Biotechnol Bioeng 2001;7:286–309.
[17] Reed JL, Vo TD, Schilling CH, Palsson BØ. An expanded genome-scale model of *Escherichia coli* K-12 (ijR904 GSM/GPR). Genome Biol 2003;4:R54.
[18] Lee DY, Yun H, Park S, Lee SY. MetaFluxNet: the management of metabolic reaction information and quantitative metabolic flux analysis. Bioinformatics 2003;19:2144–6.
[19] Ainala S, Seol E, Sekar B, Park S. Improvement of carbon monoxide-dependent hydrogen production activity in *Citrobacter amalonaticus* Y19 by over-expressing the CO-sensing transcriptional activator, CooA. Int J Hydrogen Energy 2014;39:10417–25.
[20] Bonnstra B, French CE, Wainwright I, Bruce NC. The *udhA* gene of *Escherichia coli* encodes a soluble pyridine nucleotide transhydrogenase. J Bacteriol 1999;181:1030–4.
[21] Stryer L. Biochemistry, 4th edition, Pentose phosphate pathway and gluconeogenesis: 563–565.
[22] Siedler S, Lindner SN, Bringer S, Wendisch VF, Bott M. Reductive whole-cell biotransformation with *Corynebacterium glutamicum*: improvement of NADPH generation from glucose by a cyclized pentose phosphate pathway using *pfkA* and *gapA* deletion mutants. Appl Microbiol Biotechnol 2013;97:143–52.
[23] Usui Y, Hirasawa T, Furusawa C, Zhiral T, Yamamoto N, Mori H, et al. Investigating the effects of perturbations to *pgi* and *eno* gene expression on central carbon metabolism in *Escherichia coli* using ^{13}C metabolic flux analysis. Microb Cell Fact 2012;11:87–102.
[24] Yipeng W, Ka-Yiu S, George NB. Improvement of NADPH bioavailability in *Escherichia coli* through the use of phosphofructokinase deficient strains. Appl Microbiol Biotechnol 2013;97:6883–93.
[25] Hasona A, Kim Y, Healy FG, Ingram LO, Shanmugam KT. Pyruvate formate lyase and acetate kinase are essential for anaerobic growth of *Escherichia coli* on xylose. J Bacteriol 2004;186:7593–600.
[26] Kim S, Lee CH, Nam SW, Kim P. Alteration of reducing powers in an isogenic phosphoglucose isomerase (*pgi*)-disrupted *Escherichia coli* expressing NAD(P)- dependent malic enzymes and NADP-dependent glyceraldehydes 3-phosphate dehydrogenase. Lett Appl Microbiol 2011;52:433–40.
[27] Ailen MS, Jared A, Unsiya H, George NB, Ka-Yiu S. Effect of overexpression of a soluble pyridine nucleotide transhydrogenase (UdhA) on the production of poly(3-hydroxybutyrate) in *Escherichia coli*. Biotechnol Prog 2006;22:420–5.
[28] Hua Q, Joyce AR, Fong SS, Palsson BØ. Metabolic analysis of adaptive evolution for *in silico*-designed lactate-producing strains. Biotechnol Bioeng 2006;59:992–1002.
[29] Flamholz A, Noor E, Bar-Even A, Liebermeister W, Milo R. Glycolytic strategy as a tradeoff between energy yield and protein cost. Proc Natl Acad Sci USA 2013;110:10039–44.