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BIOENERGY AND BIOFUELS

Enhanced butanol production in *Clostridium acetobutylicum* ATCC 824 by double overexpression of 6-phosphofructokinase and pyruvate kinase genes

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Abstract This study elucidated the importance of two critical enzymes in the regulation of butanol production in *Clostridium acetobutylicum* ATCC 824. Overexpression of both the 6-phosphofructokinase (*pfkA*) and pyruvate kinase (*pykA*) genes increased intracellular concentrations of ATP and NADH and also resistance to butanol toxicity. Marked increases of butanol and ethanol production, but not acetone, were also observed in batch fermentation. The butanol and ethanol concentrations were 29.4 and 85.5 % higher, respectively, in the fermentation by double-overexpressed *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* than the wild-type strain. Furthermore, when fed-batch fermentation using glucose was carried out, the butanol and total solvent (acetone, butanol, and ethanol) concentrations reached as high as 19.12 and 28.02 g/L, respectively. The reason for improved butanol formation was attributed to the enhanced NADH and ATP concentrations and increased tolerance to butanol in the double-overexpressed strain.

Keywords 6-phosphofructokinase · Pyruvate kinase · ATP · NADH · *Clostridium acetobutylicum*

Abbreviations

hsdR	Host-specific restriction deficient
mcrA	Methylcytosine-specific restriction deleted
recA1	Homologous recombination deleted
endA1	Endonuclease deleted
p15A *ori*	p15 origin of replication
Cm^r	Chloramphenicol resistance
*Φ3*TI	*B. subtilis* phage Φ3TI methyltransferase gene
ColE1	ColE1 origin of replication
pIM13	pIM13 gram-positive origin of replication
Ap^r	Ampicillin resistance
MLS^r	Macrolide-lincosamide-streptogramin B resistance
P-*ptb*	Clostridial phosphate butyryltransferase promoter
ctfA/B	Acetoacetyl-CoA transferase genes
adc	AADC gene
pfkA	6-phosphofuctokinase gene
pykA	Pyruvate kinase gene

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Introduction

Acetone–butanol–ethanol (ABE) fermentation started back in 1861 when Pasteur described the production of the 4-carbon alcohol using *Vibrion butyrique* (Dürre 2008). In 1876, the well-known *Clostridium acetobutylicum* was isolated by Albert Fitz (Dürre 2008). Since World War I, the *C. acetobutylicum* has been widely used for the ABE fermentation. The *C. acetobutylicum* is a gram-positive spore-forming bacterium that is able to metabolize a wide variety of sugars into acids (acetic and butyric acid) and solvents (acetone, butanol, and ethanol).

Recently, butanol is touted to be a renewable energy source in replacement for gasoline and can be used as a fuel additive. Similar to gasoline, butanol can be directly utilized in any existing gasoline engine. However, butanol production from clostridial fermentation is hampered by its low titer and productivity. Hence, there have been attempts to overcome this limit through manipulation of the gene structure of

the solventogenic clostridia. Some of these metabolic engineering approaches were discussed in the review papers of Papoutsakis (2008) and Lütke-Eversloh and Bahl (2011).

In the metabolic pathway of *C. acetobutylicum*, the production of acids and solvents is mainly catalyzed by acidogenic (e.g., acetate kinase, phosphate acetyltransferase, butyrate kinase, and phosphate butyltransferase) and solventogenic (e.g., aldehyde dehydrogenase, CoA transferase (CoAT), acetoacetate decarboxylase, and alcohol dehydrogenase) enzymes (Gottwald and Gottschalk 1985; Jones and Woods 1986; Green et al. 1996; Desai et al. 1999; Alsaker et al. 2004; Sillers et al. 2008; Lehmann et al. 2012; Cooksley et al. 2012; Jang et al. 2012). These acidogenic and solventogenic enzymes are encoded by their specific genes. Many researchers have tried to improve the overall butanol production of the *C. acetobutylicum* by gene manipulation (Gottwald and Gottschalk 1985; Green et al. 1996; Desai et al. 1999; Nair et al. 1999; Harris et al. 2000; Alsaker et al. 2004; Tomas et al. 2003; Sillers et al. 2008; Mao et al. 2010; Cooksley et al. 2012; Jang et al. 2012; Lehmann et al. 2012; Mann et al. 2012). These efforts were based on the ideas that deleting the genes responsible for acid productions, expressing the genes for solventogenesis, and increasing the tolerance of the cell to butanol may enhance the overall butanol production of the cell. For instance, deleting the butyrate kinase gene (*buk*) and/or inserting the alcohol/aldehyde dehydrogenase (*aad*) gene in *C. acetobutylicum* have been found to increase both butanol and ethanol formation (Harris et al. 2000). It was thought that the accumulation of metabolic precursors of butyrate (either butyryl-CoA or butyryl-P) was necessary for solvent production (Gottwald and Gottschalk 1985). The overexpression of the *aad* gene resulted in additional flux toward the AAD enzyme catalyzing a higher formation of ethanol and not butanol (Harris et al. 2000). The deletion of *buk* gene by Green et al. (1996) led to an increase in butanol production. Deletion of the phosphotransacetylase gene (*pta*) showed substantial improvement by the butanol production of *C. acetobutylicum* ATCC 824 (Green et al. 1996). Inactivation of the acid-forming *buk* and *pta* genes had been thought to redirect the carbon flow of the cell toward solvent formation. Non-sporulating and non-solventogenic *C. acetobutylicum* (without pSOL1 megaplasmid) overexpressing the *aad* and thiolase (*thl*) genes or deactivating the acetate kinase gene (*ack*) reactivated the solvent formation pathway of the cells (Sillers et al. 2008). The pSOL1 megaplasmid was thought to carry several oxidoreductase genes which were responsible for electron flow (Sillers et al. 2008). The overexpression of *Spo0A*, a promoter of the solventogenic genes including *aad*, CoA transferase A (*ctfA*), CoA transferase B (*ctfB*), and acetoacetate decarboxylase (*adc*) also accelerated the cell metabolism towards spore and solvent formation (Alsaker et al. 2004). Inactivating the suppressor *SolR* genes (*aad*, *ctfA*, *ctfB*, and *adc*) brought increased butanol and acetone formation with low acid production (Nair et al. 1999). Overexpressing the *groESL* operon in *C. acetobutylicum* ATCC 824, regulator of Class I heat shock genes, resulted in a similar improvement on the butanol production (Mann et al. 2012; Tomas et al. 2003). Heat shock proteins, which play important roles in protein biosynthesis and degradation, have also been shown to increase both butanol yield and butanol tolerance in *C. acetobutylicum* Rh8 (Mao et al. 2010). ClosTron-mediated mutations obtained by bacterial group II intron insertion were described by Lehmann et al. (2012) and Cooksley et al. (2012) to provide direct transformation of DNA into the defined sites of *C. acetobutylicum* ATCC 824 genome. However, little improvement on solvent production was found (Cooksley et al. 2012; Lehmann et al. 2012). Another approach was also directed on the improvement of butanol yields through the activation of hot channel or the direct route of acetyl-CoA condensation to butyryl-CoA and subsequent reduction to butanol (Jang et al. 2012). In conventional batch fermentation, the cold channel or the assimilation of organic acids (acetate and butyrate) into acetyl-CoA and butyryl-CoA are generated first. These CoAs are then used for ethanol formation or butyryl-CoA conversion. Butanol is produced from the reduction of butyryl-CoA. Comparing these two channels, the hot channel was suggested to have higher butanol production because it avoided yield losses from the acid re-assimilation steps (i.e., acetone and CO_2 formation) (Jang et al. 2012).

Although these studies promoted the development of a high-solvent-producing *C. acetobutylicum*, none have reported the overexpression of the genes of 6-phosphofructokinase (*pfkA*, NC_003030.1) and/or pyruvate kinase (*pykA*, NC_003030.1) in *C. acetobutylicum*. The abovementioned studies mainly focused on the solventogenic regulation of the metabolic pathway of clostridia and not on the upstream or the glycolytic pathway of the cell. A recent study regarding the metabolome remodeling of the acidogenic–solventogenic transition in *C. acetobutylicum* found that NAD(P)H decreased sharply during solventogenesis because of the large changes in the metabolic fluxes (Amador-Noguez et al. 2011). In this study, the overexpression of *pfkA* and *pykA* genes were investigated based on the idea that these genes might affect the ATP and NADH production in the Embden–Meyerhof–Parnas (EMP) pathway (Belouski et al. 1998) which are necessary for the solvent production in the cell. The phosphofructokinase (PFK) and pyruvate kinase (PYK) catalyze two of the three irreversible reactions in the EMP, and the PFK catalyzes the rate-limiting step of the pathway (Belouski et al. 1998). According to Meyer and Papoutsakis (1989), increased ATP and NADH levels in the *C. acetobutylicum* cell were well associated with the increased solvent production. The ATP is required by the cell to undergo several reduction processes after glucose breakdown and NADH is essential in the solvent formation pathways (Jones and Woods 1986). These energy sources, therefore, could enhance metabolic

flux to solventogenesis. This study developed three recombinant *C. acetobutylicum* strain bearing the *pfkA*, *pykA*, and both *pfkA* and *pykA* genes and investigated their metabolic profiles in batch and fed-batch fermentations. Furthermore, a butanol toxicity test was also carried out to examine if the increased levels of ATP and NADH contributed to higher butanol tolerance.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *C. acetobutylicum* ATCC 824 was purchased from the American Type Culture Collection (ATCC). *Escherichia coli* strains were obtained from commercial sources. Plasmids, pAN1 and pSOS94 were provided by Prof. Papoutsakis (University of Delaware, Newark, DE).

Growth conditions

E. coli strains were grown aerobically at 37 °C in Luria–Bertani (LB) medium supplemented with chloramphenicol (32 μg/mL) and/or ampicillin (50 μg/mL) as needed (Ausubel et al. 1987). The *C. acetobutylicum* ATCC 824 pre-culture was grown anaerobically at 37 °C in a 160-mL serum vial filled with 30 mL of Reinforced Clostridial Medium (RCM). The RCM was formulated using the following components per liter of distilled water: 10 g tryptose, 10 g beef extract, 3 g yeast extract, 5 g NaCl, 1 g soluble starch, 0.5 g cysteine hydrochloride, 3 g sodium acetate, and 15 g glucose. The main batch fermentation was carried out using Clostridium Growth Medium (CGM). The composition of CGM per liter of distilled water was as follows: 1 g NaCl, 2 g $(NH_4)_2SO_4$, 5 g yeast extract, 0.75 g KH_2PO_4, 0.75 g K_2HPO_4, 2 g asparagine, 0.70 g $MgSO_4{\cdot}7H_2O$, 0.01 g $MnSO_4{\cdot}H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, and 80 g glucose. Anaerobic conditions were maintained by sparging the serum vial with nitrogen prior to inoculation. Colonies of *C. acetobutylicum* and *E. coli* were grown on agar-solidified RCM and LB medium, respectively. For recombinant strains, 100 μg/mL and 40 μg/L of erythromycin were supplemented for liquid and solid medium, respectively.

Plasmid DNA isolation and construction

The genomic DNA of *C. acetobutylicum* ATCC 824 were isolated using the GeneJET™ Genomic DNA Purification Kit (Fermentas K0721, EU) and the purified DNA was dissolved in ddH_2O. The kits for plasmid isolation were purchased from Fermentas (GenJET™ Plasmid Miniprep Kit K502, EU), and the isolated plasmid was dissolved in TE buffer (pH 8.0). All genes and plasmid were stored at −20 °C prior to usage. Restriction enzymes were purchased from Fermentas (Fermentas FastDigest®, EU) and the restriction enzyme mapping was performed using either single- or double-enzyme digestions. The agarose gel electrophoresis was run using 1 % SeaMatrix™ Agarose LE (GenoSapiens, Daejon, Korea) and 1× TAE (Bio-Rad Laboratories, Hercules, CA) running buffer (pH 8.0) at 100 V for 15 to 30 min. The gene or plasmid DNA on the agarose gel was stained with trace amount of ethidium bromide. Standard DNA ladder (Fermentas O'GeneRuler™ Express DNA Ladder, EU) was used to compare the isolated DNA after illuminating the electrophorized gel under UV light (312 nm).

Amplification of the genes *pfkA*, *pykA*, and *pfkA+pykA* from *C. acetobutylicum* ATCC 824 was achieved by completing 35 PCR cycles (120 s denaturation at 95 °C; annealing at 72 to 48 °C for 160 s (*pfkA*), 42 °C for 240 s (*pykA*), and 43 °C for 400 s (*pfkA+pykA*); and 10-, 12-, and 15-min elongation at 72 °C for *pfkA*, *pykA*, and *pfkA+pykA*, respectively). Corresponding primers were listed in Table 2. Resulting gene fragment of *pfkA*, *pykA*, and *pfkA+pykA* with their natural ribosome binding site and *Bam*HI and *Avr*II sites on both ends had an approximate sizes of 1, 1.5, and 2.5 kb, respectively. The plasmid pSOS94 isolated from *E. coli*

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Clostridium acetobutylicum ATCC824	Wild type strain	ATCC
Escherichia coli		
ER2275	*hsdR mcrA recA1 endA1*	New England Biolabs
Top 10	*hsdR mcrA recA1 endA1*	Invitrogen
pAN1	p15A *ori* Cm^r *Φ3*TI	Mermelstein and Papoutsakis (1993)
pSOS94	ColE1 pIM13 Ap^r MLS^r P-*ptb ctfA/B adc*	Soucaille and Papoutsakis (unpublished data)
pSOS94/*pfkA*	ColE1 pIM13 Ap^r MLS^r P-*ptb pfkA*	This study
pSOS94/*pykA*	ColE1 pIM13 Ap^r MLS^r P-*ptb pykA*	This study
pSOS94/*pfkA+pykA*	ColE1 pIM13 Ap^r MLS^r P-*ptb pfkA pykA*	This study

Table 2 PCR primers used for the amplification of *pfkA*, *pykA*, and *pfkA+pykA* genes in *Clostridium acetobutylicum* ATCC 824

Genes	Primers
pfkA	Forward primer: 5′-GAC GGATCC ACAAATTAAGTTCCCT-3′
	Reverse primer: 5′-GCA CCTAGG CTATTTTGAAAGCATATT-3′
pykA	Forward primer: 5′-GAC GGATCC TATATAAAAGATGAGGAGA-3′
	Reverse primer: 5′-GCA CCTAGG TTACTCAACAACTTGAAC-3′
pfKA+pykA	Forward primer: 5′-GAC GGATCC ACAAATTAAGTTCCCT-3′
	Reverse primer: 5′-GCA CCTAGG TTACTCAACAACTTGAAC-3′

Top 10/pSOS94 was double digested with *Bam*HI and *Ehe*I. The digested PCR product and pSOS94 were purified using GenJET™ Plasmid Miniprep Kit (Fermentas K0691, EU) and dissolved in 30 μL ddH_2O after gel excision from the electrophorized DNA. The pSOS94 was also treated with Klenow fragment (Fermentas EP0051, EU) to correct the DNA sequence provided by Soucaille and Papoutsakis (unpublished data).

For the ligation of pSOS94 and PCR products, T4 DNA ligase (Fermentas EL0011, EU) was used. The ligation mixtures containing 4-μL digested pSOS94, 6-μL digested PCR product, 2 μL T4 DNA ligation buffer, 1 μL T4 DNA ligase, and 5 μL ddH_2O were kept at 22 °C for 6 h. For the blunt end ligation, 50 % polyethylene glycol (PEG 4000, Sigma P-3640, St. Louis, MO) solution was added to the ligation mixtures to obtain a 5 % concentration.

The ligated products were reproduced by transforming into *E. coli* Top 10 using the vendor provided protocol (One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen, Carlsbad, CA) with slight modification; 2-μL ligation product was added to 50-μL competent cell and incubated on ice for 30 min to allow DNA saturation. After which, the mixture was heat shocked for 90 s at 42 °C and incubated again on ice for 5 min. A pre-warmed 250 μL Super Optimal broth with Catabolite repression (Invitrogen, Carlsbad, CA) medium was added in each vial. The mixture was cultured at 37 °C for 60 min and spread on the LB plate containing 50 μg/mL ampicillin. After cultivation at 37 °C overnight, colonies were picked up and analyzed by plasmid isolation.

For the transformation of the recombinant plasmid into *C. acetobutylicum* ATCC 824, it was methylated first in *E. coli* ER2275/pAN1 to avoid the attack of natural restriction system (*Cac*8241) of *C. acetubutylicum* (Mermelstein and Papoutsakis 1993). Then, 10 μL of methylated plasmids were purified and electrotransformed at an electric pulse of 5 kV/cm in 200 μL of 0.3 M sucrose. All electroporation procedures were carried out using a Gene Pulser apparatus (Bio-Rad Laboratories) and cuvette with 0.1 cm electrode gap (Bio-Rad Laboratories). After electrotransformation, recombinant C. *acetobutylicum* ATCC 824 was cultured in 1 mL RCM for 4 h at 37 °C and spread on an RCM agar plate containing erythromycin. The three recombinants were colony picked, grown in liquid culture, confirmed through plasmid isolation, restriction, and electrophoresis and designated as ATCC 824/*pfkA*, ATCC 824/*pykA*, and ATCC 824/*pfkA+pykA*, respectively. The recombinant strains were deposited in the Korean Collection for Type Cultures (KCTC) and given an accession number of KCTC12406BP, KCTC12407BP, and KCTC12408BP for the *C. acetobutylicum* ATCC 824/*pfkA*, *C. acetobutylicum* ATCC 824/*pykA*, and *C. acetobutylicum* ATCC 824/*pfkA+pykA*, respectively.

Fermentation studies

Colonies of the recombinant strains of *C. acetobutylicum* ATCC 824 were pre-cultured in RCM medium with 100 μg/mL erythromycin for 24 h at 37 °C. A working volume of 20 mL in 160 mL serum vial was used for batch fermentation studies. For the main culture, CGM was used with the addition of 100 μg/mL erythromycin.

For fed-batch fermentations, a 5-L fermentor (KF 5-L Kobiotech, Incheon, Korea) containing 2-L CGM medium with 100 μg/mL erythromycin was used. For maintaining pH in fed-batch fermentation above pH 5.5, 4 M NaOH or 1 M HCl was used. Glucose solution containing 100 μg/mL erythromycin was fed when the glucose level in the medium was almost completely exhausted. Glucose concentration was adjusted to be approximately 30 g/L after every feeding. Samples were withdrawn periodically to monitor the cell growth and solvent production.

Toxicity tests

Butanol was added to the main culture when OD_{600} was 1.2±0.1 to determine the effect of butanol on the growth of the recombinant strains. The butanol concentration was set at 0, 0.5, 1.0, 1.5, and 2.0 % (*v*/*v*), and growth was monitored after butanol addition.

Analytical methods

Cell growth was monitored at 600 nm (OD_{600}) using a spectrophotometer (Thermo Spectronic Genesis 20, Madison, WI). To determine the dry cell weight, cells were harvested by centrifugation at 12,000 rpm for 5 min, washed twice with distilled water, and dried to a constant weight at 80 °C. Glucose concentration was assayed using the dinitrosalicylic colorimetric method (Miller 1959).

The concentrations of butanol, acetone, ethanol, acetate, and butyrate were analyzed by a gas chromatograph (GC, M600D, Young-Lin, Seoul, Korea) consisting of an injector (220 °C), a fused-silica capillary column (HP-FFAP, 30 m × 0.2 mm × 0.33 μm), an oven (50 °C for 1 min, 50 to 80 °C at 5 °C/min, 80 to 200 °C at 15 °C/min, and 200 °C for 15 min), and a detector (flame ionization detector, 250 °C) with helium carrier at 1.5 mL/min.

NADH (Biovision K337, Milpitas, CA) and ATP (PerkinElmer 6016941, Boston, MA) were assayed following the procedures provided with the kits. For the NADH, the absorbance at 450 nm was measured after adding the NAD cycling enzyme mix. The ATP assay system was based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. Light intensity was measured using the TD-20/20 Luminometer (Turner Biosystems, Sunnyvale, CA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The recombinant strains were identified by SDS-PAGE using the Mini-Protein 3 Electrophoresis Cell (Bio-Rad Laboratories). The 12.5 % (*v*/*v*) gel was used with 80 (spacer gel) and 150 V (separation gel), respectively. The assayed protein was identified using a standard marker (Fermentas SM0971, EU).

Results

Plasmid construction and electrotransformation

Sequences of the *pfkA* (CAC0517) and *pykA* (CAC0518) were accessed through the NCBI database (www.ncbi.nlm.nih.gov) using the accession number of NC003030. These two genes consecutively converged in a single orientation. Upon accession, primers were designed using the Primer-BLAST tool of the NCBI website. The PCR-amplified products of the *pfkA*, *pykA*, and *pfkA*+*pykA* genes from the genomic DNA of *C. acetobutylicum* ATCC824 were approximately 1, 1.5, and 2.5 kb, respectively.

PCR products were purified and ligated with the pSOS94 vector to create pSOS94/*pfkA*, pSOS94/*pykA*, and pSOS94/*pfkA*+*pykA* under the control of phosphate butyltransferase (*ptb*) promoter. Upon ligation, the recombinant plasmids, pSOS94/*pfkA*, pSOS94/*pykA*, and pSOS94/*pfkA*+*pykA*, had total sizes of approximately 6.0, 6.6, and 7.5 kb, respectively (Fig. 1). These recombinant plasmids were transformed into the chemically competent *E. coli* Top 10 and verified by digesting with *Bam*HI and *Avr*II.

To protect from natural restriction in the cell of *C. acetobutylicum*, the recombinant plasmids were isolated from *E. coli* Top 10 and transformed into *E. coli* ER2275/pANI for methylation. Final transformation into the *C. acetobutylicum* ATCC 824 was achieved by electroporation of the methylated plasmids. The recombinant strains were picked from the colonies appeared on the RCM plate containing 40 μg/mL erythromycin.

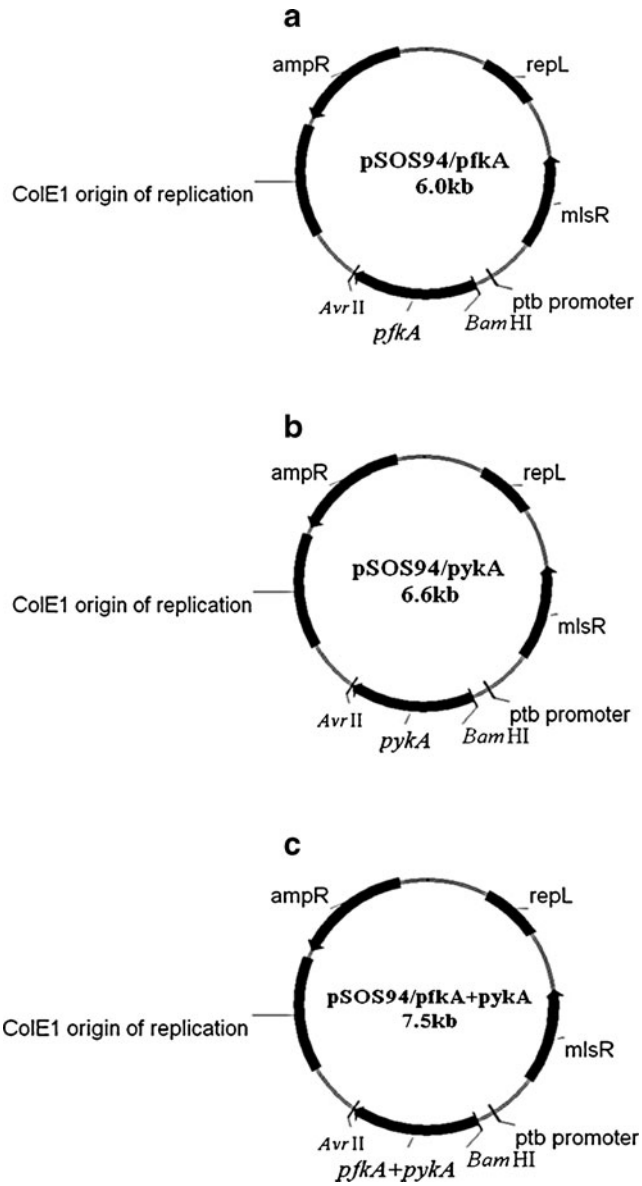

Fig. 1 Plasmid maps. **a** pSOS94/*pfkA*. **b** pSOS94/*pykA*. **c** pSOS94/*pfkA*+*pyKA*

SDS-PAGE analysis

The expression of PFK and PYK in the recombinant *C. acetobutylicum* ATCC 824 strains were analyzed by SDS-PAGE. Compared with *C. acetobutylicum* ATCC 824 (Fig. 2, lane 2), the SDS-PAGE assay showed that the 6-phosphofructokinase (PFK) and pyruvate kinase (PYK) were successfully over-expressed in the recombinant strains of ATCC 824/*pfkA* (Fig. 2, lane 3), ATCC 824/*pykA* (Fig. 2, lane

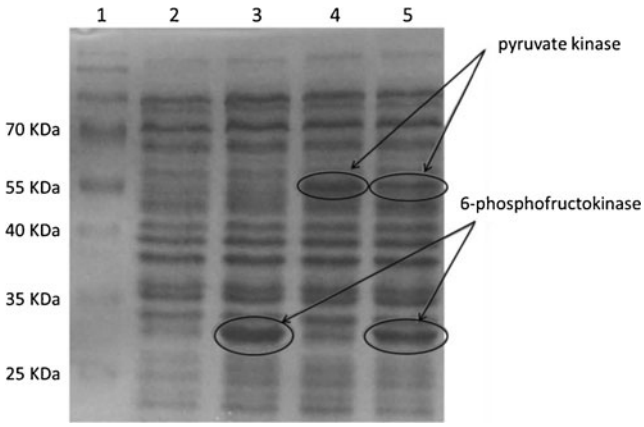

Fig. 2 SDS-PAGE analysis for the recombinant *C. acetobutylicum* ATCC 824 strains. *Lanes 1*, protein marker; *2*, *C. acetobutylicum* ATCC824 (wild type); *3*, ATCC 824/*pfkA*; *4*, ATCC 824/*pykA*; and *5*, ATCC 824/*pfkA*+*pykA*

4), and ATCC 824/*pfkA*+*pykA* (Fig. 2, lane 5). PFK and PYK were found to be approximately 55 and 30 kDa (Fig. 2), respectively.

Batch fermentation

For solvent production, it was observed that the butanol and ethanol concentrations of the three recombinant strains were higher than those of the wild-type strain. As shown in Table 3, *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* produced 13.07 g/L butanol and 2.17 g/L ethanol from the batch fermentation with 80 g/L of initial glucose. These concentrations of butanol and ethanol were 29.4% and 85.5% higher compared with the wild type. *C. acetobutylicum* ATCC 824/*pfk*A, and *C. acetobutylicum* ATCC 824/*pyk*A strain also produced more butanol and ethanol than the wild-type strain. Correspondingly, butanol yield and butanol productivity were also higher for the recombinant strains than the wild-type strain (Table 3).

NADH and ATP concentrations

In order to examine if intracellular concentrations of these energy materials were enhanced by the overexpression of PFK and PYK in *C. acetobutylicum*, ATCC 824/*pfkA*+*pykA* compared with the wild type, NADH and ATP were extracted from cells grown in RCM with 80 g/L initial glucose and measured. As shown in Fig. 3, the NADH and ATP concentrations of wild type and *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* were almost identical at 12 h of fermentation. After 24 h of fermentation, NADH concentration decreased while ATP concentration reached the highest peak. Although the NADH and ATP concentrations decreased after 24 h of fermentation, *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* showed higher concentrations of both NADH and ATP than the wild type, which indicated that overexpression of *pfkA* and *pykA* resulted in higher ATP and NADH production until 72 h.

Butanol toxicity tests

In order to examine if the over-expression of *pfkA* and *pykA* genes imparts resistance to butanol toxicity, the recombinant strain *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* was grown in RCM with butanol that was added at various levels when cell density (OD_{600}) was 1.2 (at approximately 12 h). As shown in Fig. 4, added butanol decreased the specific growth rate and maximum cell density, which were thought to be caused by the toxicity of butanol. When butanol was added at 1.5 % (*v*/*v*), the specific growth rate of the wild-type strain was only 45 % of that of the control (no butanol added). In case of the recombinant strain, when the butanol concentration was lower than 1.5 % (*v*/*v*), the maximum specific growth rate and OD_{600} were significantly higher than the wild-type strain. This showed that the double-overexpressed strain was more tolerant to butanol than the wild type.

Fed-batch fermentation studies

Since the double-overexpressed *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* maintained higher concentrations of NADH and ATP (Fig. 3), was more resistant to butanol (Fig. 4), and produced more butanol and ethanol than the wild type in batch fermentation (Table 3), fed-batch fermentation was carried out for further increase of butanol production. As a

Table 3 End-product concentrations, butanol yield, and butanol productivity after 72 h of the batch fermentations in *Clostridium acetobutylicum* ATCC 824, *C. acetobutylicum* ATCC 824/*pfkA*, *C. acetobutylicum* ATCC 824/*pykA*, and *C. acetobutylicum* ATCC 824/*pfkA*+*pykA*

Strain	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Butanol yield (g/g)	Butanol Productivity (g L^{-1} h^{-1})
ATCC 824	4.09±0.14	10.10±0.08	1.17±0.12	0.97±0.04	0.23±0.02	0.1265	0.1403
ATCC824/*pfkA*	4.06±0.05	10.60±0.15	1.30±0.09	0.90±0.02	0.20±0.02	0.1326	0.1472
ATCC824/*pykA*	3.85±0.05	11.89±0.24	1.96±0.06	0.83±0.05	0.20±0.03	0.1489	0.1651
ATCC824/*pfkA*+*pykA*	3.61±0.08	13.07±0.10	2.17±0.09	0.83±0.02	0.18±0.02	0.1637	0.1815

Note: "±" average of three replicates

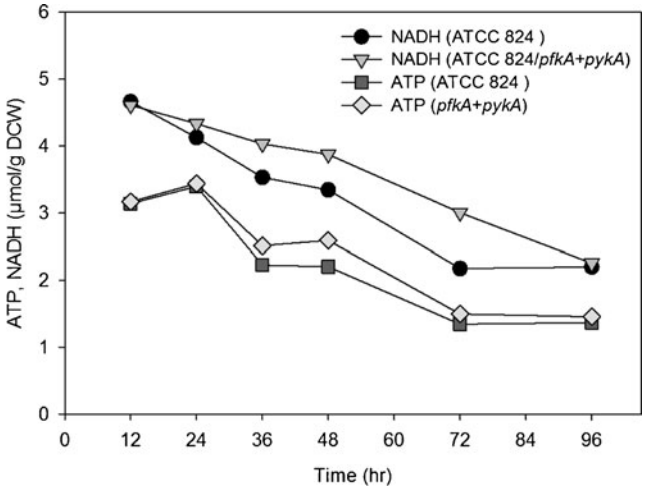

Fig. 3 Profiles of NADH and ATP concentrations in *C. acetobutylicum* ATCC824 and *C. acetobutylicum* ATCC 824/*pfkA+pykA* during 96 h of batch fermentation in the RCM medium with 80 g/L initial glucose

feeding material, glucose was added into the fermentor to adjust its concentration to be 30 g/L after feeding event.

Fed-batch fermentation using the wild-type strain is shown in Fig. 5. The glucose was added when glucose was depleted and the first feeding was provided after 24 h of fermentation; the subsequent feeding event was followed at 36 h. Only two events of glucose feeding were carried out because of the perceived sluggish growth and slow glucose consumption rate after 48 h. Based on Fig. 5, the total glucose consumed was 70.24 g/L with butanol production of 13.25 g/L, which was nearly similar to those found in batch fermentation of the *C. acetobutylicum* ATCC 824/*pfkA+pykA* (Table 3).

In case of the double-overexpressed *C. acetobutylicum* ATCC 824/*pfkA+pykA* (Fig. 6), three events of glucose feeding were carried out because the second glucose fed was consumed smoothly. Thus, 91.24 g/L glucose was consumed in total. The butanol concentration produced from this

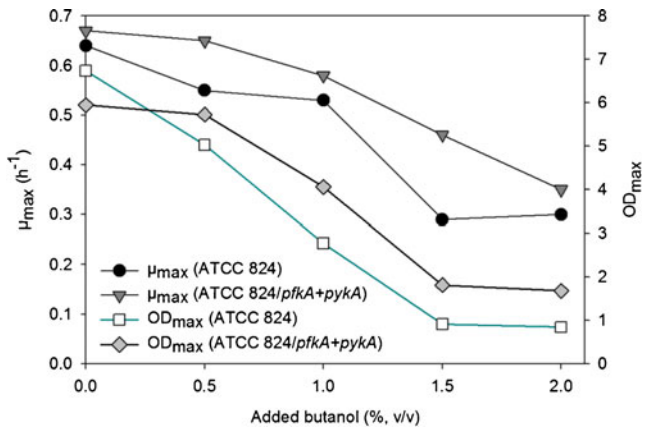

Fig. 4 Maximum specific growth rate and cell density (OD_{600}) *of C. acetobutylicum* ATCC 824 and ATCC 824/*pfkA+pykA* in the presence of different concentration of butanol

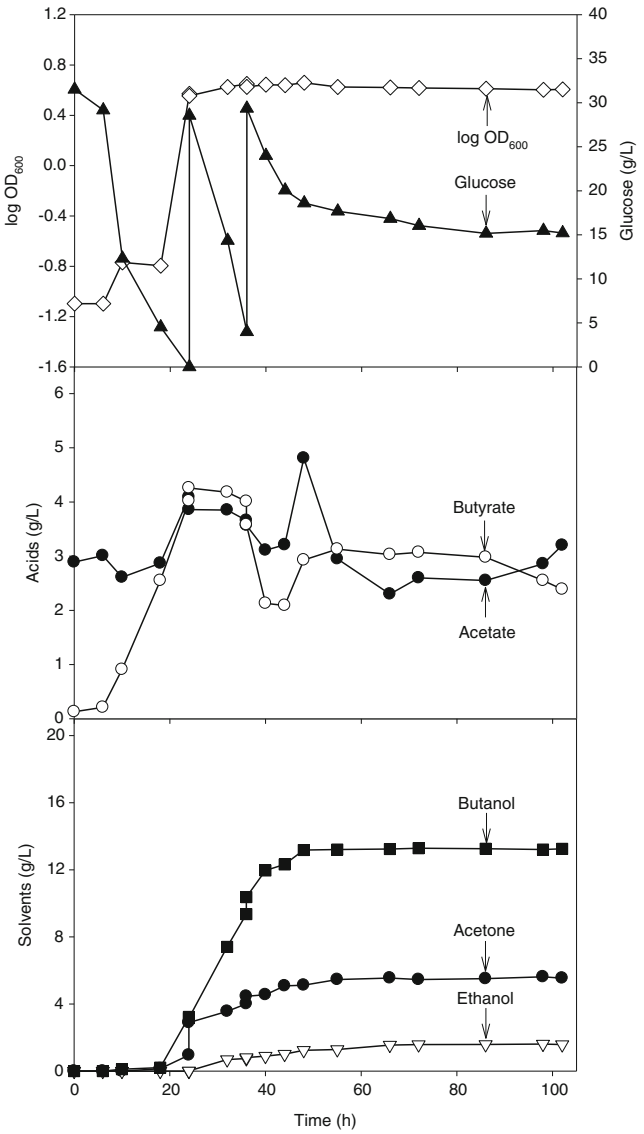

Fig. 5 Fed-batch fermentation profiles of *C. acetobutylicum* ATCC 824 during 102 h of cultivation in RCM medium at constant pH 5.5

fermentation reached 19.12 g/L, showing a significant increase from the previous batch fermentation.

Discussion

Batch fermentation

Batch fermentation studies revealed that the three recombinant strains (ATCC 824/*pfkA*, ATCC 824/*pykA*, and ATCC 824/*pfkA+pykA*) showed a slightly longer lag phase compared with the wild-type strain (Fig. 7). The slow growth of the recombinant strains could be attributed to metabolic burden caused by overexpression of PFK and PYK. Despite the longer lag phase, the maximum OD_{600} was observed to almost the same as that of the wild type. Glucose consumption also

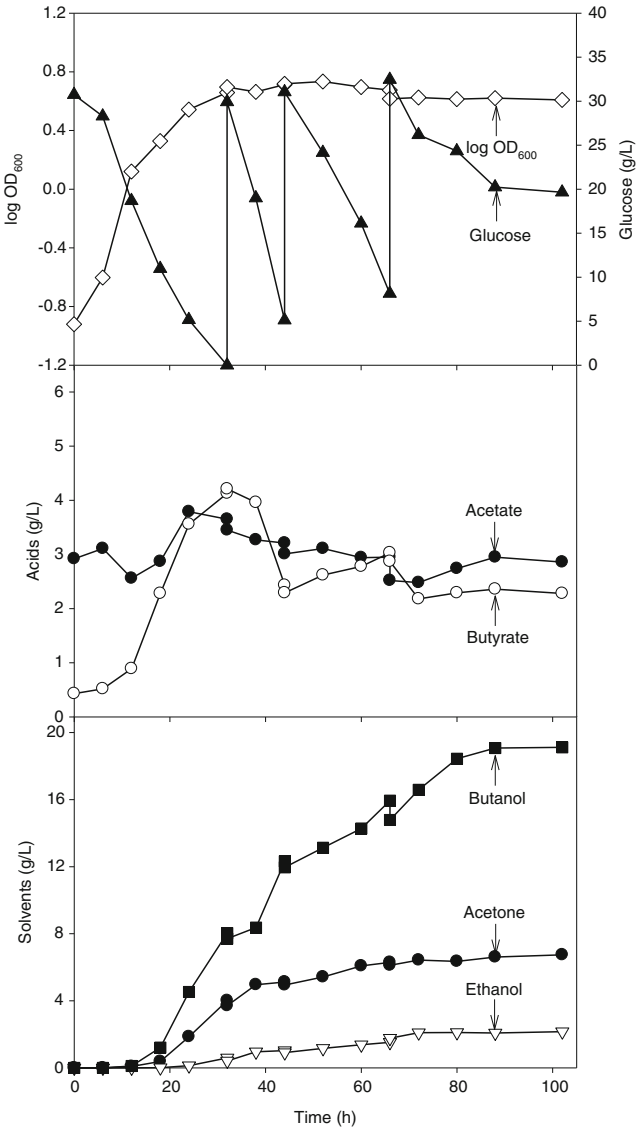

Fig. 6 Fed-batch fermentation profiles of *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* during 102 h of cultivation in RCM medium at constant pH 5.5

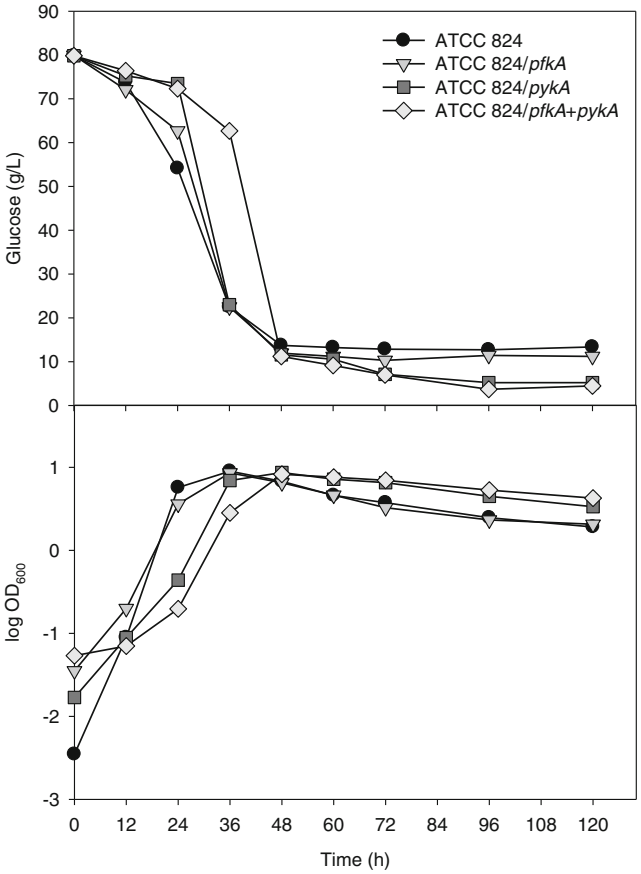

Fig. 7 Time course of OD_{600} and glucose during 120 h batch fermentation in RCM medium of *C. acetobutylicum* ATCC824 (control), ATCC 824/*pfkA*, ATCC 824/*pykA*, and ATCC 824/*pfkA*+*pykA*

appeared similar among strains except that glucose degradation rate and residual glucose concentration were lower in *C. acetobutylicum* ATCC 824/*pykA* and *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* than the wild type and *C. acetobutylicum* ATCC 824/*pfkA* (Fig. 7). Thus, the over expression of PYK and double overexpression of PFK and PYK seemed to enhance the metabolic rate of EMP pathway.

Interestingly, the acetone concentration of the three recombinant strains was lower than that of the wild-type strain. As shown in the metabolic pathway of *C. acetobutylicum* (Fig. 8), the formation of acetone does not require NAD(P)H. Therefore, the electron flow must be directed towards butanol formation. Previous studies showed that acetone production was directly proportional to butanol formation (Jones and Woods 1986). However, there could be another factor which drives butanol formation. Recent study on rex (transcriptional regulator of solventogenesis)-negative mutant *C. acetobutylicum* rex::int(95) demonstrated that increased *adhE2* gene and high NADH-dependent alcohol dehydrogenase activities produced high concentrations of ethanol and butanol, however acetone was significantly reduced (Wietzke and Bahl 2012). Similarly, the results of the chemically mutagenized, butanol exposed, and metabolically engineered *C. acetobutylicum* ATCC 824 showed an excellent butanol but not acetone production (Scheel and Lütke-Eversloh 2013). They found that the redox-dependent homologous genes, *adhE1* and *adhE2*, could be responsible for higher butanol and ethanol production (Sillers et al. 2009). Furthermore, a thiolase-mediated carbon flux also improved the ethanol and butanol titers in *C. acetobutylicum*, while the final acetone concentration remained similar to the control strain (Mann and Lütke–Eversloh 2013). The thiolase gene might influence the *adhE2*-encoded NADH-dependent aldehyde/alcohol dehydrogenase, which was linked to alcohologenic phenotype (high butanol and ethanol formation) (Mann and Lütke–Eversloh 2013). As observed by Hüsemann and Papoutsakis (1989), the high ATP level inhibited the formation of butyryl phosphate from butyryl-CoA, by which the butyrate synthesis

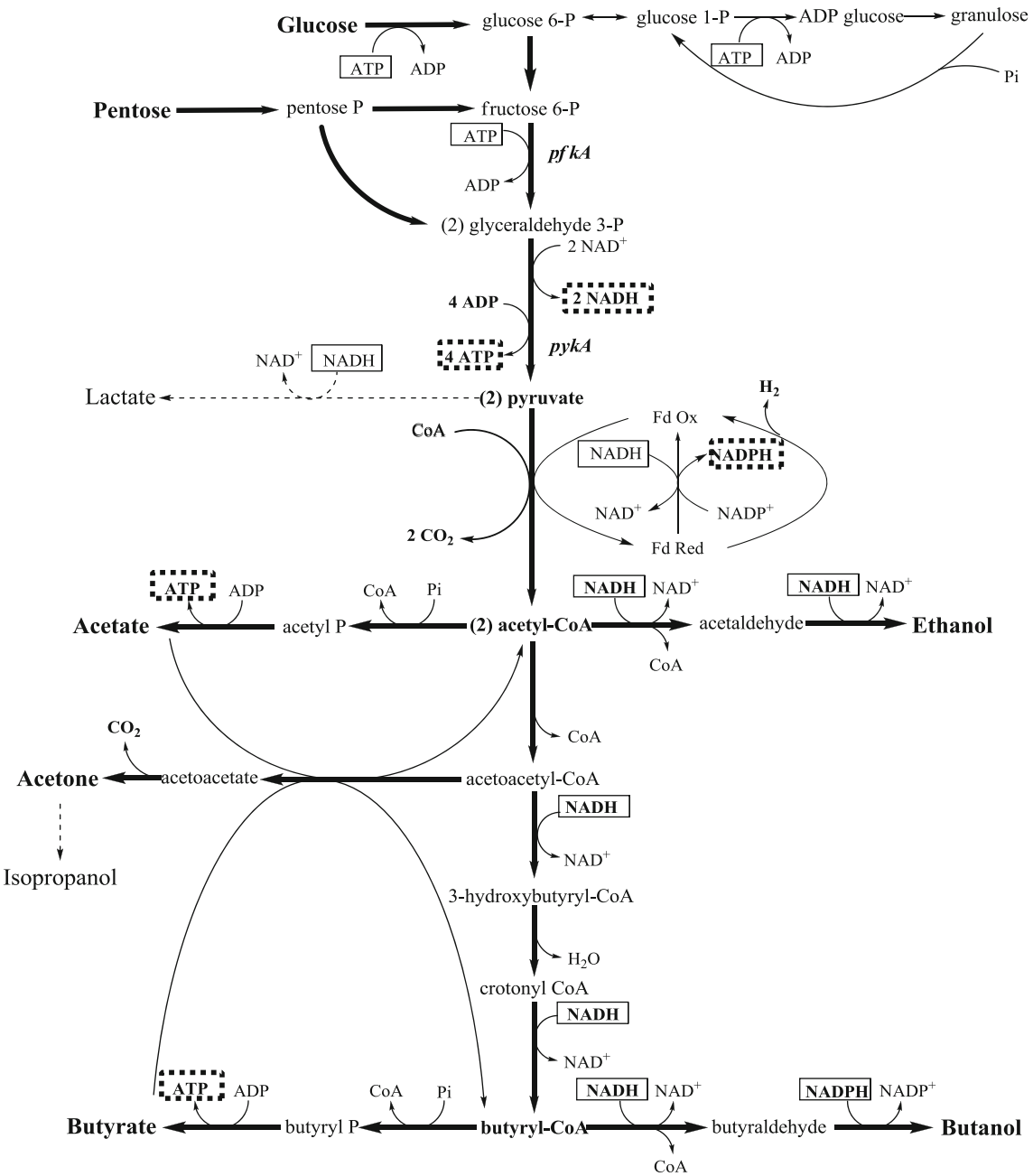

Fig. 8 Metabolic pathways of *C. acetobutylicum*. *Thick and dashed arrows* indicate major and side metabolic reactions, respectively. *Words in plain and dotted boxes* indicate the consumption and generation of intracellular NAD(P)H and ATP, respectively. Abbreviations: *ADP* adenosine diphosphate, *Fd* ferredoxin, *Ox* oxidation, *P* phosphate, *Pi* inorganic phosphate, *Red* reduction (modified from Jones and Woods 1986)

was effectively blocked and consequently the carbon flow was directed into the butanol pathway. Alternatively, an increase in ethanol formation decreases the level butyryl-CoA, which is a precursor for butanol synthesis (Sillers et al. 2009). In this context, the excess ATP and NADH production might lead the carbon flux towards ethanol and butanol production in order to maintain electron balance of the metabolic system (Fig. 8). The enzymes involved in the solventogenic phase of *C. acetobutylicum* were found to be not limited at the condition of sufficient reducing power during batch fermentation (Hartmanis and Gatenbeck 1984). Thus, the metabolic flux toward butanol synthesis could spontaneously occur during the event of fermentation without the inhibiting effects on the solventogenic enzymes.

Final acid concentrations also showed obvious differences among the four strains. As shown in Table 3, the double-overexpressed *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* produced about 15 and 22 % less acetic and butyric acids,

respectively, than the wild-type strain. This might indicate that the *pfkA* and *pykA* double overexpression in *C. acetobutylicum* ATCC 824 affected the acid re-uptake process of the cell. In such case, these acids are reutilized more effectively in the solventogenic stage of the recombinant strains than the wild type. It was stated that the NADH formation and its use are balanced with butyrate reutilization (Sillers et al. 2008). Furthermore, this might also indicate that because of the ATP surplus, the acid pathway was less necessary hence acid production in the recombinant strain became lower.

NADH and ATP concentrations

ATP and NADH levels had been proposed as factors that induce solvent production (Meyer and Papoutsakis 1989). Since the acidogenesis pathway produces more ATP than solventogenesis (Jones and Woods 1986), a limited supply of ATP or high demand of ATP switches the clostridial metabolism to acidogenesis. Thus, a high ATP level is needed to maintain a high flux towards solvent formation. Moreover, the high NADH level might force the cell to oxidize this cofactor in the butanol and ethanol pathways (Fig. 8). Therefore, the *pfkA* and *pykA* which regulate the NADH and ATP production are of great importance for obtaining a high butanol and ethanol yields. As shown in Fig. 3, the *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* yielded higher concentrations of both NADH and ATP than the wild type, which corresponded to the increase in butanol concentration of the recombinant strain (Table 3).

Butanol toxicity tests

It was found that the presence of about 12 g/L of butanol can cause severe inhibition in the metabolic activity of the clostridial cell (Lee et al. 2008). Besides gene manipulations, random mutagenesis was also performed addressing the toxicity problem in solventogenic clostridia (Annous and Blaschek 1991; Qureshi and Blaschek 2001). A hyper-butanol-producing *C. beijerinckii* BA 101 was an example of successful chemical mutation which produced elevated levels of butanol (Qureshi and Blaschek 2001). Although it was not fully understood, in this study it was suspected that the *C. acetobutylicum* strain carrying the double-over expressed genes (*pfkA*+*pykA*) enhanced its tolerance to butanol. At a high butanol concentration, it was reported that the cellular energy status was severely affected wherein the intracellular pH and ATP concentration decreased (Tomas et al. 2003; Lee et al. 2008). The ATP concentration in *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* was higher than *C. acetobutylicum* ATCC 824 (Fig. 3). Therefore, it could be concluded that the increased ATP concentration by the double overexpression of *pfkA* and *pykA* genes brought the cell to be more resistant to butanol, which led to an increased butanol formation. The NADH concentration also increased in the double-overexpressed strain, which could be another reason for the improved butanol production. Furthermore, the study of hyperamylolytic mutants created by random mutagenesis had also exhibited enhanced glycolytic flux, which could be related to the higher ATP and NAD(P)H production (Annous and Blaschek 1991; Qureshi and Blaschek 2001). Recently, it was shown that ATP and NADH drove the photosynthetic production of butanol in cyanobacteria (Lan and Liao 2012) and fermentative production of butanol in a recombinant *E. coli* (Shen et al. 2011). It was observed that the accumulation of NADH was necessary to induce the production of butanol in *E. coli* (Shen et al. 2011). Thus, these findings manifested the importance of ATP and NADH for inducing both product formation and chemical conversion in some microbial cells including butanol-producing clostridia such as *C. acetobutylicum*.

Fed-batch fermentation studies

Compared with the fed-batch fermentation of wild-type strain (Fig. 5), 30.7 % increase in butanol concentration was achieved in the double-overexpressed *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* (Fig. 6). A low final butyrate level was also observed, indicating that higher butyrate assimilation occurred, which might have directly corresponded to the increased activity of the CoAT pathway. The assimilation of butyrate and gradual supply of glucose might have activated a higher acetone formation. As seen in Fig. 6, the final acetone concentration reached 6.74 g/L, which was almost twofold higher than the previous batch fermentation. The final butanol and ethanol concentrations were 2.16 and 19.12 g/L, respectively, yielding a total solvent concentration of 28.02 g/L, which was 27.3 % higher than the wild-type fed-batch fermentation. From these results, we concluded that butanol and ethanol production was significantly enhanced in the PFK and PYK overexpressed strain by combined effects of increased levels of ATP and NADH and increased resistance to butanol toxicity.

In conclusion, the new recombinant *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* demonstrated both an increased butanol and ethanol production. Batch fermentation using the double-overexpressed *C. acetobutylicum* ATCC 824/*pfkA*+*pykA* produced 29.4 and 85.5 % higher butanol and ethanol (Table 3), respectively, than the wild-type strain. Moreover, the highest butanol and ethanol concentrations were achieved at 19.03 and 2.93 g/L (Fig. 6), respectively, in the fed-batch fermentation. The improved butanol production was identified to be brought by the higher production of ATP and NADH from the overexpression of the *pfkA* and *pykA* genes. The overexpression of these EMP genes also increased butanol tolerance. These results manifested the importance of the EMP cofactors, NADH and ATP, which are needed in the product formation of the *C. acetobutylicum* strain.

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