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Original Article

Disruption of the acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio

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ABSTRACT

A possible way to improve the economic efficacy of acetone–butanol–ethanol fermentation is to increase the butanol ratio by eliminating the production of other by-products, such as acetone. The acetoacetate decarboxylase gene (*adc*) in the hyperbutanol-producing industrial strain *Clostridium acetobutylicum* EA 2018 was disrupted using TargeTron technology. The butanol ratio increased from 70% to 80.05%, with acetone production reduced to approximately 0.21 g/L in the *adc*-disrupted mutant (2018adc). pH control was a critical factor in the improvement of cell growth and solvent production in strain 2018adc. The regulation of electron flow by the addition of methyl viologen altered the carbon flux from acetic acid production to butanol production in strain 2018adc, which resulted in an increased butanol ratio of 82% and a corresponding improvement in the overall yield of butanol from 57% to 70.8%. This study presents a general method of blocking acetone production by *Clostridium* and demonstrates the industrial potential of strain 2018adc.

1. Introduction

Butanol is an important industrial chemical and an alternative fuel currently produced via petrochemical processes. The bioproduction of butanol, typically referred to as acetone–butanol–ethanol (ABE) fermentation, is performed by solventogenic *Clostridium* species, such as *C. acetobutylicum*, and was one of the largest industrial fermentation processes in the world during the early part of the 20th century. However, this industrial process was completely shut down at the end of the 20th century in China with the rapid rise of the petrochemical industry. Recently, with the tremendous national demand for solvents, this old technology is undergoing regeneration in China (Chiao and Sun, 2007). 1-Butanol production through engineered microorganisms, such as *Escherichia coli*, *Saccharomyces cerevisiae*, was also reported, but had low titers (Atsumi et al., 2008a, b; Shen and Liao, 2008; Inui et al., 2008; Steen et al., 2008). Recent advances in molecular biology and metabolic engineering techniques of butyric acid clostridia offer an opportunity to re-establish the ABE fermentation as an economically viable process (Sillers et al., 2009).

Acetone, ethanol, and butanol are the dominant products of the ABE process, of which butanol constitutes 60–70% (w/w) of the total solvents, acetone 20–30%, and ethanol about 10%. Butanol is the most valuable of these solvent products. Because the total solvent yield of *C. acetobutylicum* accounts for 35–37% of the starch provided, close to the maximum theoretical yield (Chiao and Sun, 2007), a possible way to improve the economic efficacy of biobutanol production is to increase the butanol ratio by eliminating the production of other solvent by-products, such as acetone. In this way, the cost of product separation can also be reduced in the industrial ABE process. Two key enzymes are responsible for acetone formation in *C. acetobutylicum*, acetoacetate decarboxylase (AADC, encoded by the *adc* gene) and coenzyme-A transferase (CoAT, encoded by *ctfAB*). The mutant strain *C. acetobutylicum* 2-BrBu1, which was generated by chemical mutation, is deficient in acetone production but also has a reduced butanol ratio (Janati-Idrissi et al., 1987). Tummala et al., downregulated the expression of *adc* with an antisense RNA strategy to repress acetone production. However, the acetone titer did not decrease, and there was a slight reduction of 17% in the normalized (based on the maximum absorbance at 600 nm, A_{600}) acetone concentration. This reduction did not correlate with the much larger reductions in enzyme levels. This research team then downregulated CoAT expression by the same method, and although acetone production was successfully repressed, the butanol titer was also significantly reduced, suggesting that *aad* (which encodes alcohol/aldehyde dehydrogenase, AAD) expression might also be altered by *ctfB* antisense RNA, because *aad*, *ctfA*, and *ctfB* comprise a polycistronic operon (Tummala et al., 2003b). To overcome this problem, the overexpression of *aad* was

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combined with antisense RNA targeted against *ctfB* in *C. acetobutylicum* ATCC 824. Although the butanol titer was restored, the butanol ratio of the mutant strain decreased, because the bacterium still produced a small amount of acetone and an increased amount of ethanol (Tummala et al., 2003a). Further attempts to improve the butanol selective production by promoter optimization of *aad* or *ctfB* asRNA, or coexpression of *thl* (encoding thiolase) led to increased alcohol (butanol plus ethanol) titers. However, the efforts to exercise control over the ratio of butanol to ethanol produced was not so successful, which indicated the necessity of a more complex metabolic engineering strategy (Sillers et al., 2009). The *solR* (a gene identified upstream *aad*) disrupted strain of *C. acetobutylicum* ATCC 824, 824sloR, was reported to have increased butanol ratio compared to the wild-type strain ATCC 824 under fermentation pH $\geq$ 4.5 (Nair et al., 1999), and later report on 824solR fermentation under pH $\geq$ 5.0 (Harris et al., 2001) or without pH control (Shao et al., 2007) did not show that improvement. The successful research on butanol ratio improvement could be achieved by expression of *aad* in the megaplasmid pSOL1 (containing *aad* and the acetone formation genes) deficient strain M5 or DG1 (Nair and Papoutsakis, 1994; Cornillot et al., 1997; Sillers et al., 2008). Other publication for acetone elimination was reported in patent WO2008052596 and WO2008052973, but no fermentation data was provided for acetone-deficient mutant strain (Soucaille, 2008a, b)

Early attempts to increase the butanol ratio by rounds of culture and chemical mutagenesis in our laboratory were successful. *C. acetobutylicum* EA 2018 (CCTCC M 94061), produced in our laboratory, has a higher butanol ratio comprising up to 70% of the total solvents, compared with 60% in *C. acetobutylicum* ATCC 824 (Zhang et al., 1997). The *C. acetobutylicum* genome has been sequenced and annotated (Nolling et al., 2001), and methods for genetic deletion (Harris et al., 2002; Heap et al., 2007; Shao et al., 2007) and gene overexpression (Mermelstein and Papoutsakis, 1993) have been developed. However, no genetic modification of *C. acetobutylicum* EA 2018 to further increase the butanol ratio has yet been reported.

In this study, we focused on the genetic modification of *C. acetobutylicum* EA 2018 to further increase the butanol ratio by eliminating acetone production. We used TargeTron technology (Shao et al., 2007) to completely disrupt the *adc* gene in *C. acetobutylicum* EA 2018, to overcome the problem of leaky expression during antisense RNA downregulation. The *adc*-disrupted mutant strain was further analyzed for changes in its major metabolic flux, pH control, and the regulation of *in vivo* electron flow. The butanol ratio was increased with a reduction in acetone production.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids are listed in Table 1.

2.2. Growth conditions

E. coli was grown aerobically at 37 °C in Luria–Bertani (LB) medium or LB solidified with agar (1.5% w/v) supplemented with the appropriate antibiotics (100 μg/mL ampicillin or 50 μg/mL spectinomycin). *C. acetobutylicum* strains were grown anaerobically at 37 °C in an anaerobic chamber (Thermo Forma Inc., Waltham, MA). Cultures of *C. acetobutylicum* were grown in liquid or solid CGM medium (Wiesenborn et al., 1988) plus agar (1.5% w/v), supplemented with antibiotics (40 μg/mL erythromycin or 34 μg/mL thiamphenicol) as necessary. Frozen stocks were made from 100 mL of 8% (w/v) corn mash culture supplemented with 1% (w/v) calcium carbonate fermented for 36 h, resuspended in 1 mL of 20% glycerol, and stored at −70 °C.

Table 1
Bacterial strains and plasmids used in the study.

Strains or plasmids	Relevant characteristics[a]	Source or reference
Bacterial strains		
C. acetobutylicum		
2018	EA 2018 (CCTCC M94061), characterised by an improved butanol ratio of 7:2:1 (Butanol:Acetone:Ethanol) compared to the ratio of 6:3:1 exhibited by the Weizmann strain, ATCC 824.	Zhang et al. (1997)
2018p	EA 2018/pIMP1-Pptb	This study
2018adc	EA 2018 *adc::intron*/pSY6-adc	This study
2018adc^R	EA 2018 *adc::intron*/pSY6-adc/pIMP1-adc	This study
Escherichia coli		
DH5a	Commercial transformation host	GIBCO BRL, Life Technologies)
ER2275	Commercial transformation host	New England Biolabs
Plasmids		
pAN1	Cm^r, Φ3T I gene, p15A origin	Offered by Prof. Eleftherios T. Papoutsakis (Mermelstein and Papoutsakis, 1993)
pIMP1	Ap^r, MLSr; *repL*, ColE1 origin	Offered by Prof. Eleftherios T. Papoutsakis (Mermelstein and Papoutsakis, 1993)
pIMP1-Pptb	derived from pIMP1 with *ptb* promoter insertion for gene expression in *Clostridium* spp.	Offered by Prof. Eleftherios T. Papoutsakis
pSY6	*E. coli*/*Clostridium* shuttle vector, derived from pIMP1-Pptb by inserting the group II intron and *ltrA* ORF from pMTL20lacZT-TErmBtdRAM2	Shao et al. (2007)
pSY7	derived from pSY6, with Cm^r gene to replace MLS^r gene	Shao et al. (2007)
pSY8	derived from pIMP1-ptb, with Cm^r gene to replace MLS^r gene	This study
pSY6-adc	derived from pSY6 for intron insertion in *adc* at 180/181	This study
pSY8-adc	derived from pIMP1 by insertion of *adc* gene with its original promoter.	This study

[a] Cm^r, chloramphenicol/thiamphenicol-resistance gene; MLS^r, erythromycin-resistance gene; *ptb*, phosphotransbutyrylase gene; *adc*, acetoacetic acid decarboxylase gene; Ap^r, ampicillin-resistance gene; *repL*, pIM13 Gram-positive origin of replication.

Table 2
Primers used in the study.

Primer name	Sequence (5′–3′)	Description
adc180/181s-IBS	AAAACTCGAGATAATTATCCTTATTAGTCAGGTTTGTGCGCCCAGATAGGGTG	*adc* targetron primer[a]
adc180/181s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAGGTTTGATAACTTACCTTTCTTTGT	*adc* targetron primer[a]
adc180/181s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTACTAATCGATAGAGGAAAGTGTCT	*adc* targetron primer[a]
adc-P	CCCGGATCCTTGGAATTGTTTATAGTG	*adc* promoter forward primer
adc-C	TTACTTAAGATAATCATATA	*adc* reverse primer
adc_163-177	GAGCCCTTAGTCAGG	forward primer inside *adc* from 163 to 177 base
adc_714-699	AACTTCAGCTCTAGGC	reverse primer inside *adc* from 714 to 699 base
MLS-N	ACCGTGTGCTCTACGACC	MLS^r forward primer
MLS-C	CCCCCATGCTCCAACAGC	MLS^r reverse primer
cat-N	AAACATCTGTTTATGTTACAGTAATATTG	Cm^r forward primer from its promoter
cat-C	TTTATCGATTTATAAAAGCCAGTCATTA	Cm^r reverse primer

[a] Designed using the InGex Intron Prediction Program that accompanies the TargetTron products (www.Sigma-Aldrich.com/Targetronaccess).

2.3. Strain construction

The TargeTron plasmid pSY6–adc was constructed for disruption of the *adc* gene. The *adc*–targetron target sequence of 350 bp was amplified by primers adc180/181s-IBS, adc180/181s-EBS1d, and adc180/181s-EBS2, according to the protocol of the TargeTron™ Gene Knockout System Kit (Sigma-Aldrich, St Louis, MO, USA) to retarget the RNA portion of the intron. All primers and oligonucleotides used are listed in Table 2. pSY6–adc was then constructed by the insertion of the *adc*–targetron fragment into the *Xho*I and *Bsr*GI sites of pSY6 (Shao et al., 2007). The primers for the *adc*–targetron were designed using the InGex Intron Prediction Program accompanying the TargetTron products (www.Sigma-Aldrich.com/Targetronaccess), which introduced nucleotide substitutions into the IBS, EBS2, and EBS1d regions of the L1.LtrB intron originating from *Lactococcus lactis*. pSY6–adc was electroporated into strain EA2018, and the transformants were isolated on CGM plates containing 40 μg/mL erythromycin. The intron insertions were identified by colony PCR, using primers adc_163–177 and adc_714–699, which flank the insertion site at nucleotides (nt) 180/181 of *adc*. Colonies grown for 48 h were picked into the 50 μL PCR reaction mixture without template. To confirm the intron insertion site, PCR identification was performed using the genomic DNA of these mutants as templates. The amplified fragments were cloned into pMD19-T (TaKaRa Biotechnology Co. Ltd., Dalian, Liaoning, China) for sequencing. The *adc*-disrupted mutant was designated "2018adc". 2018adc still contains pSY6–adc, because the replicon of pSY6 is that of pIMP1 (Shao et al., 2007), which is very stable in *C. acetobutylicum* EA 2018. It could not be cured by successive transfer cultures for a number of generations in antibiotic-free medium as indicated in Section 3.

To calculate the stability of the insertion, 2018adc was tested in successive transfer cultures (every 24 h, 0.5% inoculum) for 9 days (~100 generations) in antibiotic-free CGM medium, and was plated on solid CGM medium to isolate individual colonies. Thirty colonies were tested with colony PCR using primers adc_163–177 and adc_714–699 to determine how many clones retained the intron.

To confirm whether the phenotype of 2018adc was solely caused by *adc* disruption, pSY8 was constructed by removing the *cat* fragment from pSY7 by *Pvu*II and *Cla*I digestion, and inserting it into the corresponding sites of pIMP1. pSY8–adc was constructed by the insertion of the *adc* gene with its original promoter, amplified with primers adc-P and adc-C, into pSY8 at the *Bam*HI and *Sma*I sites. pSY8–adc was then electroporated into strain 2018adc, and the transformants were isolated on CGM plates containing 34 μg/mL thiamphenicol. The positive transformants were identified by colony PCR amplification of the *cat* gene with primers cat-N and cat-C. The positive mutant strain was designated "$2018adc^R$". The phenotype of the strain was then confirmed by fermentation in P2 medium.

pIMP1–ptb was transformed into *C. acetobutylicum* EA 2018, and the cells were isolated on CGM plates containing 40 μg/mL erythromycin. The positive transformants were confirmed by colony PCR, amplifying the erythromycin-resistance gene with primers MLS-N and MLS-C. The positive strain was designated "2018p". 2018p was used as the control strain for 2018adc.

Before electroporation, all plasmids were methylated using *E. coli* ER2275 (pAN1) to avoid the natural restriction system of *C. acetobutylicum* (Mermelstein and Papoutsakis, 1993). Once the plasmids were methylated, the electroporation protocol was performed as described previously (Mermelstein et al., 1992).

2.4. Genomic and plasmid DNA isolation

Plasmid DNA from *E. coli* and *C. acetobutylicum* was isolated with a Doupson plasmid extraction kit. Chromosomal DNA from *C. acetobutylicum* was extracted as previously described (Nakotte et al., 1998).

2.5. PCR

The PCR amplification of specific DNA sequences was performed according to the Bio-Rad manufacturer's instructions (PTC-200, Bio-Rad Laboratories Inc., Hercules, CA, USA). Genes and DNA fragments were all amplified using KOD-plus DNA polymerase (Fermentas International Inc., Burlington, Ontario, Canada) with primers as indicated (Table 2).

2.6. Fermentation

Fermentation experiments were performed anaerobically in P2 medium (glucose 60 g/L; K_2HPO_4 0.5 g/L; KH_2PO_4 0.5 g/L; CH_3COONH_4 2.2 g/L; $MgSO_4 \cdot 7H_2O$ 0.2 g/L; $MnSO_4 \cdot H_2O$ 0.01 g/L; NaCl 0.01 g/L; $FeSO_4 \cdot 7H_2O$ 0.01 g/L; ρ-aminobenzoic acid 1 mg/L; thiamine 1 mg/L; biotin 0.01 mg/L) (Baer et al., 1987) at 37 °C with 1% (w/v) calcium carbonate.. Erythromycin (20 μg/mL) or thiamphenicol (17 μg/mL) was added to the medium when necessary. Methyl viologen was prepared as a sterile stock solution in the oxidized state (colorless) and added as necessary to the medium before fermentation, at a final concentration of 6 mg/L. Fermentation was carried out in a 150 mL working volume in 250 mL serum bottles. Inocula were typically 10% (v/v) of a CGM preculture at $A_{600} = 1.0–2.5$. Samples were removed with sterilized injectors, and immediately cooled on ice to stop growth before further analysis.

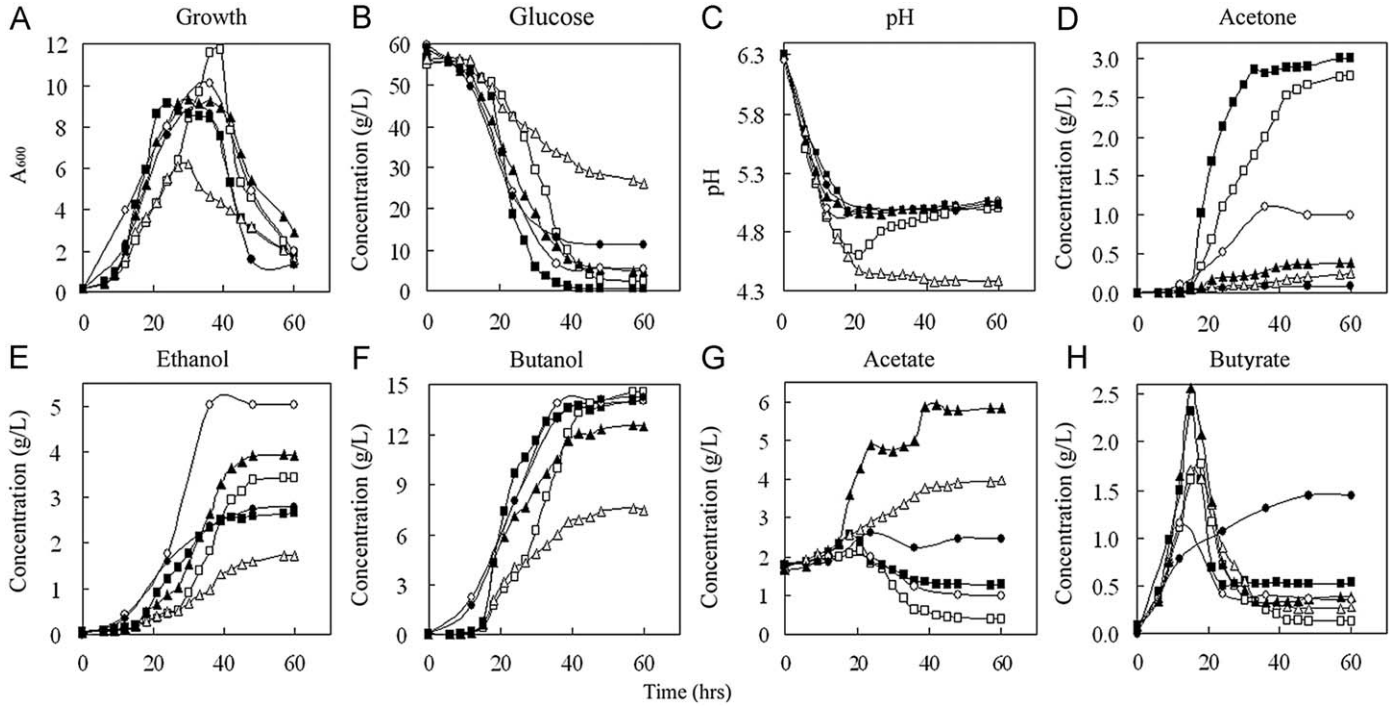

Fig. 1. Batch fermentations of 2018p in P2 medium (open squares), P2 medium supplemented with 1% calcium carbonate (closed squares), or P2 medium supplemented with 1% calcium carbonate and 6 mg/L methyl viologen (open circles); and 2018adc in P2 medium (open triangles), P2 medium supplemented with 1% calcium carbonate (closed triangles), or P2 medium supplemented with 1% calcium carbonate and 6 mg/L methyl viologen (closed circles). The media were supplemented with 20 μg/mL erythromycin. Fermentations were performed in duplicate, although the results for one fermentation are shown. Differences in product formation between the duplicate fermentations were less than 5%.

To confirm the genotypes of the recombinant strains 2018adc and 2018p at the end of fermentation, the genomic DNA of both strains was isolated. The genotype of strain 2018adc was identified by PCR amplification of the adc–targetron fragment using primers adc_163-177 and adc_714-699. To confirm strain 2018p, the erythromycin-resistance gene was amplified with primers MLS-N and MLS-C.

2.7. Analytical techniques

Cell density was measured at A_{600} using a DU730 spectrophotometer (Beckman Coulter). The samples were diluted as necessary to an optical density of $A_{600} = 0.02–0.8$. The supernatant concentrations of acetone, acetic acid, butyric acid, butanol, and ethanol were determined using a gas chromatograph (7890A, Agilent, Wilmington, DE, USA). Glucose concentrations were determined using a high-pressure liquid chromatography system (1200 series, Agilent).

3. Results

3.1. Disruption of the adc gene in C. acetobutylicum EA 2018 dramatically reduces acetone formation

More than 500 transformants were obtained from the electroporation of strain EA 2018 with pSY6–adc. Nine of 18 picked colonies had intron insertions in the *adc* gene fragment amplified and analyzed on agarose gel (*adc*–intron, 1.5 kb), whereas the rest showed the wild-type *adc* fragment (0.5 kb). All nine colonies were contaminated with the 0.5-kb wild-type *adc* fragment. The strain was further purified by streak plating, with strain verification by colony PCR. This indicated that intron retrohoming was initiated at different times depending on the cells' regeneration state: if retrohoming occurred during the colony growth stage, only some of the cells from one transformant showed the intron insert. More than 66% of the total colonies isolated from one transformant had the *adc*–intron fragment, detected on agrose gel after colony PCR. The total insertion frequency of the intron at nt 180/181 of *adc* was calculated to be about 33%. The sequencing results for the amplified *adc*–intron fragments verified the presence of the L1.LtrB intron integrated into the sense strand at the site between nt 180 and 181 of *adc*, numbered from its translation start codon.

During fermentation in P2 medium, the acetone level in strain 2018adc (0.21 g/L, ~5 mM) was dramatically reduced to one tenth the level of that in strain 2018p (2.83 g/L, ~50 mM; Fig. 1D, Table 3) and the butanol ratio increased to $80.05 \pm 0.07\%$, whereas that of strain 2018p was $71 \pm 1\%$ (Table 3).

In $2018adc^R$, acetone production was restored to that of the control (2018p), and the titers of the other products (butanol, ethanol, acetic acid, and butyric acid) were almost the same as those of the control (Table 3), which confirms that the phenotype of 2018adc was solely caused by the disruption of *adc*.

At the end of fermentation, the 1.5 kb *adc*–intron fragment was amplified from the genomic DNA of strain 2018adc. No bands of 0.5 kb were isolated, which would have indicated the presence of the wild-type *adc* gene. This result verifies the presence of the intron insertion in the *adc* gene after the completion of fermentation. For strain 2018p, the MLS fragment was amplified, showing the expected 1-kb fragment, which confirmed the presence of pIMP1–ptb in the culture.

To test the stability of the intron insertion into 2018adc, the cells were successively transfer cultured (~100 generation) for 9 days in antibiotic-free CGM. About 90% of the colonies were found to contain the 1.5-kb *adc*–intron fragment, which indicates rather instable insertion of the intron.

Table 3
Final product concentrations in batch fermentations of recombinant strains of *C. acetobutylicum* EA 2018[a].

Strain	Medium[b]	Fermentation characteristics									
		Max A_{600}	Doubling time (h)	Butanol (g/L)	Ethanol (g/L)	Acetone (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Total solvent (g/L)	Butnaol ratio of total solvent %	Butanol titer/ max A_{600}
2018p	P2	11±1	3.4±0.2	13.6±0.4	2.7±0.5	2.83±0.2	0.50±0.06	0.50±0.06	19.1±0.7	71±1	1.25±0.08
2018p	P2/C	8.9±0.3	3.0±0.3	13.6±0.2	2.5±0.3	2.86±0.05	1.5±0.2	0.6±0.2	18.9±0.4	71.8±0.6	1.53±0.05
2018p	P2/C/M	12.8±0.2	2.87±0.07	14.4±0.8	5.2±0.2	1.09±0.08	1.1±0.1	0.41±0.07	21±1	71.2±0.1	1.14±0.06
2018adc	P2	6.0±0.4	3.3±0.5	7.4±0.2	1.66±0.08	0.21±0.01	3.62±0.01	0.26±0.01	9.3±0.2	80.05±0.07	1.24±0.09
2018adc	P2/C	9.6±0.3	2.51±0.05	12.2±0.2	3.86±0.06	0.34±0.06	5.82±0.05	0.36±0.01	16.4±0.2	74.3±0.4	1.27±0.06
2018adc	P2/C/M	9.6±0.2	2.8±0.1	13.6±0.8	2.8±0.1	0.10±0.01	2.6±0.2	1.4±0.1	16.5±0.6	82±2	1.43±0.08
2018adc^R	P2/C	9.1±0.6	2.5±0.3	14.1±0.3	2.2±0.3	3.8±0.1	1.1±0.2	0.47±0.09	20.06±0.08	70.1±0.9	1.55±0.07

[a] Batch fermentation was performed in 250 mL serum bottles containing 150 mL of medium, and cultivated for 48 h at 37 °C. The experiments were performed with triplicate fermentations.
[b] P2/C, P2 medium supplemented with 1% calcium carbonate; P2/C/M, P2 medium supplemented with 1% calcium carbonate and 6 mg/L methyl viologen. The media were supplemented with 20 μg/mL erythromycin.

3.2. Butanol titer of strain 2018adc was increased by the regulation of the culture pH with the addition of calcium carbonate

The growth of strain 2018adc was inhibited to half that of the control strain 2018p, measured at A_{600} (Fig. 1A). Consequently, the ethanol and butanol titers were all reduced to approximately half the concentrations produced by the control strain (Fig. 1E, F), with half the glucose remaining unutilized (Fig. 1B).

To identify the reasons for this discrepancy, we analyzed the pH and acid concentrations throughout the fermentation of both the control and the modified strain. The control strain showed the typical biphasic fermentation profile that is characteristic of the solventogenic *Clostridium* species. During the exponential growth phase, the control strain displayed the typical synthesis of organic acids, with acetic acid and butyric acid as the main products (Fig. 1G, H). At the transition to the stationary phase, the production of acids decreased, and acetone and butanol became the dominant products. Parts of the previously produced acids were reassimilated and converted into solvents (Fig. 1G, H). As a consequence of this, the pH of the culture dropped initially from 6.3 to between 4.5 and 4, and then increased again upon the initiation of solventogenesis, with the final pH rising to about 5.0 (Fig. 1C). However, strain 2018adc did not undergo an acetic acid reassimilation phase, with the final concentration reaching approximately seven times that of strain 2018p (Fig. 1G, Table 3). This accumulation of acetic acid resulted in a continuous drop in pH to about 4.3 from the mid-exponential growth phase (20 h) until the end of fermentation (Fig. 1C).

This accumulation of acetic acid posed a severe threat to the growth of the modified strain. To optimize its growth, 1% calcium carbonate was added to the P2 medium to buffer the pH. This additional buffering capacity allowed the pH to be maintained at about pH 5.0 when the initial fermentation pH dropped to that level (Fig. 1C). Under these conditions, the growth of strain 2018adc recovered to that of the control strain (Fig. 1A). The addition of calcium carbonate promoted the cell growth of both strains and reduced their doubling times (Table 3). The acetone titer of strain 2018adc was still below 10 mM (0.34 g/L; Fig. 1D), and the butanol titer increased from 7.4±0.2 to 12.2±0.2 g/L, whereas that of the control strain did not change (Fig. 1F, Table 3). The ethanol titer of strain 2018adc was about 1.5 times that of the control strain (Fig. 1E, Table 3). More acetic acid was produced during the fermentation of strain 2018adc when calcium carbonate was added to the medium, and acetic acid production showed double peaks (Fig. 1G). The acetic acid accumulated by strain 2018adc at the end of fermentation with calcium carbonate was 3.88 times higher than that of the control strain, and 1.6 times higher than that of the modified strain without the addition of calcium carbonate (Fig. 1G, Table 3). Butyric acid did not accumulate in either strain 2018adc or 2018p when calcium carbonate was added (Fig. 1H, Table 3). The butanol ratio of strain 2018adc was 74.3±0.4%, whereas that of strain 2018p was 71.8±0.6% (Table 3).

3.3. Butanol yield of strain 2018adc was increased by the addition of methyl viologen through the regulation of the in vivo electron flow

Although acetone production was inhibited in strain 2018adc and the butanol ratio was increased from 71.8% (2018p) to about 74.3%, the butanol titer did not increase in P2 medium supplemented with 1% calcium carbonate (Table 3). To simulate the major metabolic flux of the two strains 2018p and 2018adc under two different culture conditions, P2 medium with or without 1% calcium carbonate, we renormalized the titers in Table 3 to report the molar yields of glucose, and the original acetic acid contained in the medium was deducted (Fig. 2). The inhibition of the acetone production flux did not contribute to an increase in butanol yield, but to an increase in acetic acid production under both culture conditions. The acetic acid molar yield of strain 2018adc was 25.7±0.2% or 25±3% with or without calcium carbonate, while that of the control strain 2018p was calculated of negative value, which indicated the consumption of the original acetic acid contained in the medium. Furthermore, the ethanol yield of strain 2018adc increased to 1.7 times that of strain 2018p in P2 medium supplemented with calcium carbonate (Fig. 2).

To solve the problem of acetic acid accumulation, we added methyl viologen to the medium, which was expected to down-regulate acetic acid production by regulating the electron flow. As a result, the acetic acid titer decreased in both the control strain 2018p and 2018adc in P2 medium supplemented with calcium carbonate. The acetic acid produced by 2018p decreased from 1.5±0.2 g/L (without methyl viologen) to 1.1±0.1 g/L (with methyl viologen), whereas that of strain 2018adc decreased from 5.82±0.05 to 2.6±0.2 g/L (Table 3). The ethanol titer of strain 2018p increased from 2.5±0.3 to 5.2±0.2 g/L when methyl viologen was added, but that of 2018adc decreased from 3.86±0.06 to 2.8±0.1 g/L. The butanol titer of strain 2018p did not show much change with the addition of methyl viologen (increased by 0.8 g/L), whereas that of strain 2018adc increased by 1.4 g/L (Fig. 1, Table 3). The butanol ratio was further increased from 74.3±0.4% (without methyl viologen) to 82±2% (with methyl viologen), whereas that of 2018p did not show any change (Table 3). The major metabolic flux of the two strains 2018p and 2018adc in P2 medium with or without methyl viologen are

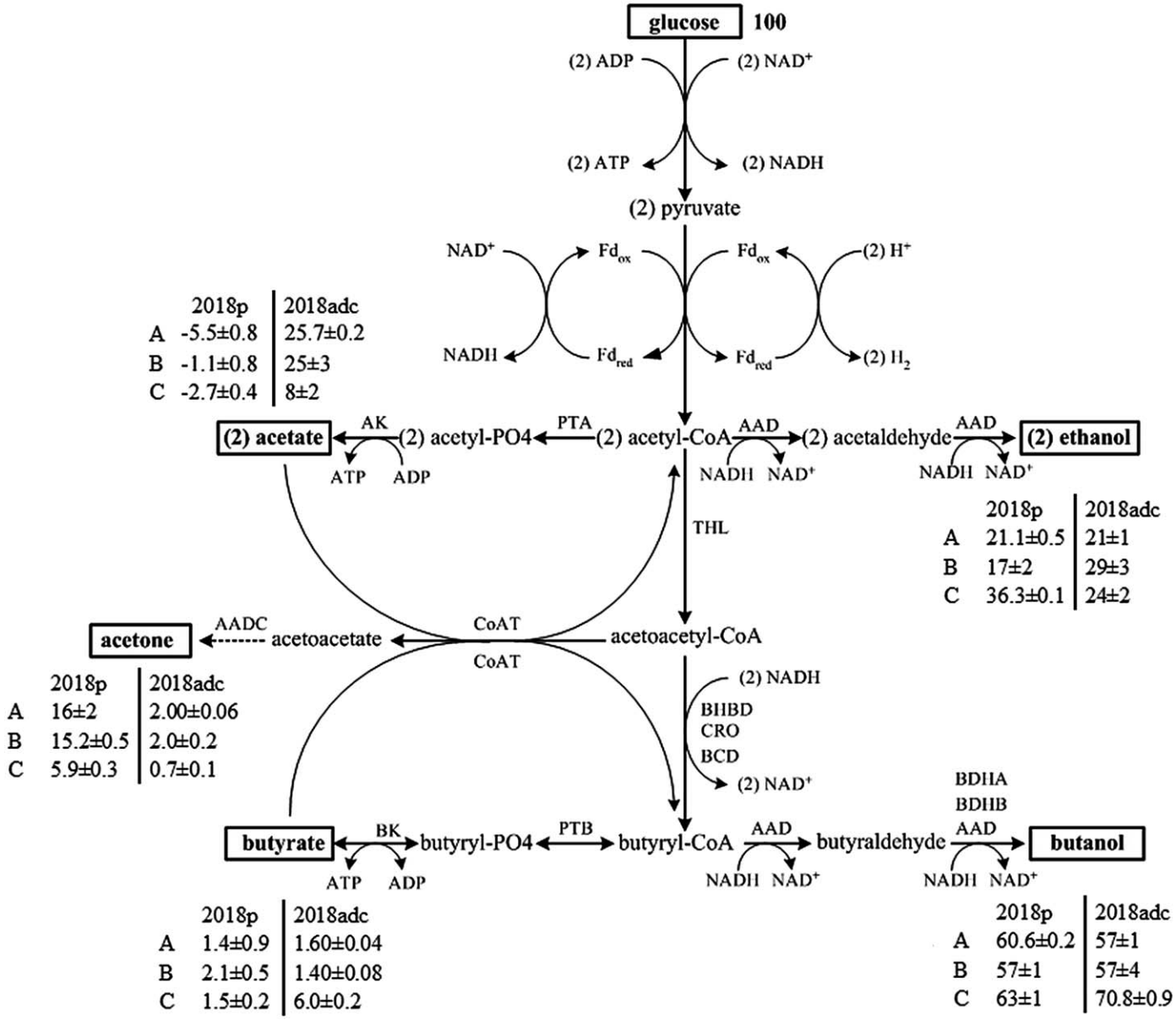

Fig. 2. Simulation of major metabolic fluxes in the two strains 2018p and 2018adc grown in medium A: P2 medium; B: P2 supplemented with 1% calcium carbonate; or C: P2 medium supplemented with 1% calcium carbonate and 6 mg/L methyl viologen. All fluxes are relative to a glucose uptake rate of 100 moles (arbitrary number). Enzymes are abbreviated as follows: phosphotransacetylase (PTA); acetic acid kinase (AK); thiolase (THL); β-hydroxybutyryl dehydrogenase (BHBD); crotonase (CRO); butyryl-CoA dehydrogenase (BCD); CoA transferase (CoAT); acetoacetic acid decarboxylase (AADC); butyric acid kinase (BK); phophotransbutyrylase (PTB); alcohol/aldehyde dehydrogenase (AAD); butanol dehydrogenase I (BDHA); butanol dehydrogenase II (BDHB). Dashed arrow indicates that AADC was disrupted in this pathway in 2018adc.

presented in Fig. 2. Compared with the molar yield without methyl viologen, the acetic acid yield of strain 2018adc dropped to about one third of the original level (25±3% to 8±2%), the butanol yield increased by approximately 20% (57±4% to 70.8±0.9%), and the acetone yield decreased to one third of the previous level (2.0±0.2% to 0.7±0.1%). However, in strain 2018p, the reduced acetone yield did not contribute to a large increase in the butanol yield (57±1% to 63±1%), but instead to the ethanol yield, which increased more than twofold (17±2% to 36.3±0.1%). The addition of methyl viologen did not affect the cell doubling time in either strain 2018p or strain 2018adc (Table 3).

4. Discussion

This study is the first to report an engineered increase in the butanol ratio of *C. acetobutylicum* by *adc* disruption. The elimination of acetone synthesis was chosen as the target because it is the next major solvent by-product after butanol. In *C. acetobutylicum*, two key enzymes, AADC and CoAT, are responsible for acetone formation. CoAT is suggested to be the rate-limiting enzyme for acetone production (Tummala et al., 2003b). CoAT is also responsible for acid reassimilation (Fig. 2), and the downregulation of its activity or its complete inactivation by gene disruption may cause acid accumulation. AADC does not limit acetone formation because the significant downregulation of AADC expression by antisense RNA strategy had no concomitant effect on acetone production (Tummala et al., 2003b). To overcome the problem of transcriptional leakage that can occur during antisense RNA downregulation, this study used TargeTron technology to disrupt the *adc* gene. Acetone production was reduced to approximately 0.21 g/L (about 5 mM) in strain 2018adc when the butanol ratio increased from 70% (EA 2018 wild type) to 80.05% (Table 3) in P2 medium. This small amount of residual acetone

might be produced by 10% revertants as presented in Section 3 of intron insertion stability test. The other possibility might be that the acetoacetate accumulated at higher concentrations by the cells of 2018adc was a very unstable molecule, and led to small amount of acetone production.

Disruption of the *adc* gene to eliminate acetone production can also be performed in other solventogenic *Clostridium* species, because the solvent production pathway is conserved in different species (Prof. David Jones, University of Otago, Dunedin, New Zealand, personal communication). To generate a homobutanol-producing strain, the next target for metabolic engineering should be the ethanol-synthesis pathway. Other clostridia with naturally low ethanol ratios are the obvious candidates for similar *adc* gene disruptions, such as *Clostridium beijerinckii* (Ezeji et al., 2007; Qureshi et al., 2007). Another method for eliminating ethanol production is to alter the substrate specificity of the enzymes that catalyze the conversion of both acetyl-CoA and butyryl-CoA to acetaldehyde and butyrlaldehyde, respectively, such as AAD.

Clostridia have a relatively complex physiology (Paredes et al., 2005; Papoutsakis, 2008), and even one gene disruption can cause large changes in the metabolic flux. This is supported by the results for strain 2018adc, in which acetone production was nearly abolished and acetic acid production was increased (Fig. 1D, G). The accumulation of acetic acid resulted in a continuous decline in pH, potentially destroying the transmembrane pH gradient (Gottwald and Gottschalk, 1985; Huang et al., 1985) and inhibiting cell growth. As a result, the butanol and ethanol titers were reduced to half the levels of those in the control strain (Fig. 1E, F). The acetic acid and butyric acid produced are reassimilated by the reaction catalyzed by CoAT, by the transfer of the CoA group of acetoacetyl-CoA to either acetate or butyrate to form acetyl-CoA or butyryl-CoA, respectively, leading to acetoacetate formation. AADC then catalyzes the irreversible decarboxylation of acetoacetate, producing acetone and CO_2 (Fig. 2). In strain 2018adc, the second step in acetone formation from acetoacetate was blocked, so the accumulation of acetic acid might be attributed to the *in vivo* accumulation of acetoacetate, which possibly caused the inhibition of CoAT activity, whereupon the transfer of CoA from acetoacetyl-CoA to acetate or butyrate would not be favored. The other hypothesis explaining the accumulation of acetic acid is the reverse reaction, in which CoA is transferred from acetoacetyl-CoA to acetate or butyrate (acetyl-CoA/butyl-CoA+acetoacetate = acetic acid/butyric acid+acetoacetyl-CoA), which has been reported in *Clostridium* species (Barker et al., 1978) when acetoacetyl-CoA had accumulated to a certain level. Butyric acid did not accumulate, which could be explained by the reverse reaction of the PTB–BK (phophotransbutyrylase–butyric acid kinase) pathway (Cary et al., 1988), whereas the reverse reaction of the PTA–AK (phosphotransacetylase–acetic acid kinase) pathway, which would take up acetic acid, has not been identified.

In this study, pH control was demonstrated to be a critical factor in the growth of strain 2018adc. This was achieved by the addition of calcium carbonate to the medium as a pH buffer, which restored the growth of strain 2018adc to the level observed in the control. Acetic acid production showed double peaks in P2 medium supplemented with calcium carbonate and may reflect the complex competition between the *in vivo* production and the reassimilation of acetic acid (Fig. 1G).

It has previously been reported that the acetic acid synthesis pathway can be inhibited or blocked, which could potentially overcome the problem of acetic acid overflow seen in this study (Green et al., 1996; Zhu et al., 2005; Liu et al., 2006) and may also allow the regulation of the metabolic flux, improving the butanol yield. However, we failed to produce *ack* (encoding acetate kinase)-disrupted mutants of strain 2018adc using TargeTron technology. Other methods have demonstrated that altered electron flow can be used to direct the carbon flow from acid to alcohol production (Rao and Mutharasan, 1987). This is because butanol and ethanol formation require the reducing equivalents in the form of NADH, whereas acid and acetone production do not (Fig. 2). In this study, reducing equivalents were regulated by the addition of the artificial electron acceptor methyl viologen, because the addition of viologen increases the NADH throughput in the alcohol-dependent pathways and possibly increases the NADH–NAD turnover rate (Rao and Mutharasan, 1987). The addition of methyl viologen to strain 2018adc and strain 2018p resulted in very different fermentation profiles, which were both analyzed for changes in metabolic flux (Fig. 2). In strain 2018adc, the yield of butanol increased from $57 \pm 4\%$ (without methyl viologen) to $70.8 \pm 0.9\%$ (with methyl viologen), whereas the butanol in strain 2018p did not change. The yield of acetic acid by strain 2018adc was halved by the addition of methyl viologen. The ethanol yield did not increase in strain 2018adc compared with that of cultures without methyl viologen, whereas that of strain 2018p almost doubled. This may have been because 2018p could not tolerate the higher butanol titer under such conditions. It has previously been reported that *C. acetobutylicum* fermentation rarely produces more than 13 g/L butanol, a level generally considered the toxic limit (Jones and Woods, 1986). Consequently, most NADH can only be oxidized in the ethanol formation pathway when methyl viologen is added. In strain 2018adc, the increased butanol titer and butyric acid concentration (Table 3) may be attributable to the increased NADH oxidized through the pathway from acetyl-CoA to butanol. The accumulation of butyric acid in strain 2018adc supplemented with methyl viologen (Fig. 1H) may also be attributable to the toxic effects of butanol, which prevent its reassimilation. At the end of fermentation with strain 2018adc (supplemented with calcium carbonate and methyl viologen), 10 g/L glucose remained unutilized and yet the strain had reached a maximum butanol titer equivalent to that seen in the other experiments. This could have industrial implications for the use of this strain, whereby the initial feedstock concentration can be reduced while maintaining favorable butanol titers, leading to improved yields and reduced feedstock costs. The addition of methyl viologen may also affect the hydrogen evolution rate, which can possibly be reduced as described previously (Rao and Mutharasan, 1987).

Gene modification strategies that regulate electric flow might be used to inhibit acid production. One method is to inhibit clostridial hydrogenase. It has been demonstrated that carbon monoxide sparging reduces hydrogenase activity, because it causes the transfer of electrons from FdH_2 to NAD^+ (mediated by NAD:Fd oxidoreductase) and thus increases the availability of NADH (Kim et al., 1984; Datta and Zeikus, 1985; Chou et al., 2008). Antisense RNA strategies have also been used to reduce hydrogenase activity (Nakayama et al., 2008). Another possible way to alter the redox balance of cells is to overexpress genes leading to an increase in *in vivo* NADH levels, such as the gene encoding formate dehydrogenase, as was performed in *E. coli* (Berrios-Rivera et al., 2002b; Berrios-Rivera et al., 2002a; Sanchez et al., 2005; Zhang et al., 2009). pSY6/pSY7 targetron plasmids offer an efficient tool for multiple gene modifications, allowing a number of these knockout targets to be evaluated simultaneously within one strain (Shao et al., 2007). This study has shown the potential of strain 2018adc for use in industrial applications.

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References

Atsumi, S., Cann, A.F., Connor, M.R., Shen, C.R., Smith, K.M., Brynildsen, M.P., Chou, K.J., Hanai, T, Liao, J.C., 2008a. Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab. Eng. 10, 305–311.

Atsumi, S., Hanai, T., Liao, J.C., 2008b. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451, 86–89.

Baer, S.H., Blaschek, H.P., Smith, T.L., 1987. Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 53, 2854–2861.

Barker, H.A., Jeng, I.M., Neff, N., Robertson, J.M., Tam, F.K., Hosaka, S., 1978. Butyryl-CoA: acetoacetate CoA-transferase from a lysine-fermenting Clostridium. J. Biol. Chem. 253, 1219–1225.

Berrios-Rivera, S.J., Bennett, G.N., San, K.Y., 2002a. The effect of increasing NADH availability on the redistribution of metabolic fluxes in *Escherichia coli* chemostat cultures. Metab. Eng. 4, 230–237.

Berrios-Rivera, S.J., Bennett, G.N., San, K.Y., 2002b. Metabolic engineering of *Escherichia coli*: increase of NADH availability by overexpressing an NAD(+)-dependent formate dehydrogenase. Metab. Eng. 4, 217–229.

Cary, J.W., Petersen, D.J., Papoutsakis, E.T., Bennett, G.N., 1988. Cloning and expression of *Clostridium acetobutylicum* phosphotransbutyrylase and butyrate kinase genes in *Escherichia coli*. J. Bacteriol. 170, 4613–4618.

Chiao, J., Sun, Z., 2007. History of the acetone–butanol–ethanol fermentation industry in China: development of continuous production technology. J. Mol. Microbiol. Biotechnol. 13, 12–14.

Chou, C.J., Jenney Jr., F.E., Adams, M.W., Kelly, R.M., 2008. Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels. Metab. Eng. 10, 394–404.

Cornillot, E., Nair, R.V., Papoutsakis, E.T., Soucaille, P., 1997. The genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. J. Bacteriol. 179, 5442–5447.

Datta, R., Zeikus, J.G., 1985. Modulation of acetone–butanol–ethanol fermentation by carbon monoxide and organic acids. Appl. Environ. Microbiol. 49, 522–529.

Ezeji, T., Qureshi., N., Blaschek, H.P., 2007. Butanol production from agricultural residues: impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. Biotechnol. Bioeng. 97, 1460–1469.

Gottwald, M., Gottschalk, G., 1985. The internal-pH of *Clostridium acetobutylicum* and its effect on the shift from acid to solvent formation. Arch. Microbiol. 143, 42–46.

Green, E.M., Boynton, Z.L., Harris, L.M., Rudolph, F.B., Papoutsakis, E.T., Bennett, G.N., 1996. Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiology 142, 2079–2086.

Harris, L.M., Blank, L., Desai, R.P., Welker, N.E., Papoutsakis, E.T., 2001. Fermentation characterization and flux analysis of recombinant strains of *Clostridium acetobutylicum* with an inactivated *solR* gene. J. Ind. Microbiol. Biotechnol. 27, 322–328.

Harris, L.M., Welker, N.E., Papoutsakis, E.T., 2002. Northern, morphological, and fermentation analysis of spo0A inactivation and overexpression in *Clostridium acetobutylicum* ATCC 824. J. Bacteriol. 184, 3586–3597.

Heap, J.T., Pennington, O.J., Cartman, S.T., Carter, G.P., Minton, N.P., 2007. The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J. Microbiol. Methods 70, 452–464.

Huang, L., Gibbins, L.N., Forsberg, C.W., 1985. Transmembrane pH gradient and membrane potential in *Clostridium acetobutylicum* during growth under aceto-genic and solventogenic conditions. Appl. Environ. Microbiol. 50, 1043–1047.

Inui, M., Suda, M., Kimura, S., Yasuda, K., Suzuki, H., Toda, H., Yamamoto, S., Okino, S., Suzuki, N., Yukawa, H., 2008. Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl. Microbiol. Biotechnol. 77, 1305–1316.

Janati-Idrissi, R., Junelles, A.M., Kanouni, A., Petitdemange, H., Gay, R., 1987. Selection of mutants of *Clostridium acetobutylicu*m defective in production of acetone. Ann. Inst. Pasteur. Microbiol. 138, 313–323.

Jones, D.T., Woods, D.R., 1986. Acetone–butanol fermentation revisited. Microbiol. Rev. 50, 484–524.

Kim, B.H., Bellows, P., Datta, R., Zeikus, J.G., 1984. Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl. Environ. Microbiol. 48, 764–770.

Liu, X., Zhu, Y., Yang, S.T., 2006. Construction and characterization of ack deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid and hydrogen production. Biotechnol. Prog. 22, 1265–1275.

Mermelstein, L.D., Papoutsakis, E.T., 1993. *In vivo* methylation in *Escherichia coli* by the Bacillus subtilis phage phi 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. 59, 1077–1081.

Mermelstein, L.D., Welker, N.E., Bennett, G.N., Papoutsakis, E.T., 1992. Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. Biotechnology 10, 190–195.

Nair, R.V., Green, E.M., Watson, D.E., Bennett, G.N., Papoutsakis, E.T., 1999. Regulation of the *sol* locus genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824 by a putative transcriptional repressor. J. Bacteriol. 181, 319–330.

Nair, R.V., Papoutsakis, E.T., 1994. Expression of plasmid-encoded aad in *Clostridium acetobutylicum* M5 restores vigorous butanol production. J. Bacteriol. 176, 5843–5846.

Nakayama, S., Kosaka, T., Hirakawa, H., Matsuura, K., Yoshino, S., Furukawa, K., 2008. Metabolic engineering for solvent productivity by downregulation of the hydrogenase gene cluster hupCBA in *Clostridium saccharoperbutylacetonicum* strain N1-4. Appl. Microbiol. Biotechnol. 78, 483–493.

Nakotte, S., Schaffer, S., Bohringer, M., Durre, P., 1998. Electroporation of, plasmid isolation from and plasmid conservation in *Clostridium acetobutylicum* DSM 792. Appl. Microbiol. Biotechnol. 50, 564–567.

Nolling, J. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J. Bacteriol. 183, 4823–4838.

Papoutsakis, E.T., 2008. Engineering solventogenic clostridia. Curr. Opin. Biotech-nol. 19, 420–429.

Paredes, C.J., Alsaker, K.V., Papoutsakis, E.T., 2005. A comparative genomic view of clostridial sporulation and physiology. Nat. Rev. Microbiol. 3, 969–978.

Qureshi, N., Saha, B.C., Cotta, M.A., 2007. Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. Bioprocess Biosyst. Eng. 30, 419–427.

Rao, G., Mutharasan, R., 1987. Altered electron flow in continuous cultures of *Clostridium acetobutylicum* induced by viologen dyes. Appl. Environ. Microbiol. 53, 1232–1235.

Sanchez, A.M., Bennett, G.N., San, K.Y., 2005. Effect of different levels of NADH availability on metabolic fluxes of *Escherichia coli* chemostat cultures in defined medium. J. Biotechnol. 117, 395–405.

Shao, L. Targeted gene disruption by use of a group II intron (targetron) vector in *Clostridium acetobutylicum*. Cell Res. 17, 963–965.

Shen, C.R., Liao, J.C., 2008. Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab. Eng. 10, 312–320.

Sillers, R., Chow, A., Tracy, B., Papoutsakis, E.T., 2008. Metabolic engineering of the non-sporulating, non-solventogenic *Clostridium acetobutylicum* strain M5 to produce butanol without acetone demonstrate the robustness of the acid-formation pathways and the importance of the electron balance. Metab. Eng. 10, 321–332.

Sillers, R., Al-Hinai, M.A., Papoutsakis, E.T., 2009. Aldehyde–alcohol dehydrogenase and/or thiolase overexpression coupled with CoA transferase downregulation lead to higher alcohol titers and selectivity in *Clostridium acetobutylicum* fermentations. Biotechnol. Bioeng. 102, 38–49.

Steen, E.J., Chan, R., Prasad, N., Myers, S., Petzold, C.J., Redding, A., Ouellet, M., Keasling, J.D., 2008. Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. Microb. Cell Fact. 7, 36–43.

Soucaille, P., 2008a. Producing n-butanol by culturing a microorganism in a culture medium comprising a carbon source and recovering n-butanol from the culture medium, where a gene involved in butyrate formation is deleted in the microorganism. WO 2008052596.

Soucaille, P., 2008b. Production of n-butanol for synthesis of acrylate/methacrylate esters, involves culturing microorganism in culture medium comprising source of carbon and recovering n-butanol, where at least one gene is deleted in the microorganism. WO 2008052973.

Tummala, S.B., Junne, S.G., Papoutsakis, E.T., 2003a. Antisense RNA downregulation of coenzyme A transferase combined with alcohol–aldehyde dehydrogenase overexpression leads to predominantly alcohologenic *Clostridium acetobutyli-cum* fermentations. J. Bacteriol. 185, 3644–3653.

Tummala, S.B., Welker, N.E., Papoutsakis, E.T., 2003b. Design of antisense RNA constructs for downregulation of the acetone formation pathway of *Clostridium acetobutylicum*. J. Bacteriol. 185, 1923–1934.

Wiesenborn, D.P., Rudolph, F.B., Papoutsakis, E.T., 1988. Thiolase from *Clostridium acetobutylicum* ATCC 824 and its role in the synthesis of acids and solvents. Appl. Environ. Microbiol. 54, 2717–2722.

Zhang, Y., Yang, Y., Chen, J., 1997. High-butanol ratio *Clostridium acetobutylicium*, culturing method and its use. Chinese Patent: CN 1063483C.

Zhang, Y., Huang, Z., Du, C., Li, Y., Cao, Z., 2009. Introduction of an NADH regeneration system into *Klebsiella oxytoca* leads to an enhanced oxidative and reductive metabolism of glycerol. Metab. Eng. 11, 101–106.

Zhu, Y., Liu, X., Yang, S.T., 2005. Construction and characterization of *pta* gene-deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid fermentation. Biotechnol. Bioeng. 90, 154–166.