Appl Microbiol Biotechnol (2008) 77:1219–1224
DOI 10.1007/s00253-007-1246-8

Production of isopropanol by metabolically engineered *Escherichia coli*

Toru Jojima · Masayuki Inui · Hideaki Yukawa

Received: 12 September 2007 / Revised: 8 October 2007 / Accepted: 9 October 2007 / Published online: 7 November 2007

Abstract A genetically engineered strain of *Escherichia coli* JM109 harboring the isopropanol-producing pathway consisting of five genes encoding four enzymes, thiolase, coenzyme A (CoA) transferase, acetoacetate decarboxylase from *Clostridium acetobutylicum* ATCC 824, and primary–secondary alcohol dehydrogenase from *C. beijerinckii* NRRL B593, produced up to 227 mM of isopropanol from glucose under aerobic fed-batch culture conditions. Acetate production by the engineered strain was approximately one sixth that produced by a control *E. coli* strain bearing an expression vector without the clostridial genes. These results demonstrate a functional isopropanol-producing pathway in *E. coli* and consequently carbon flux from acetyl-CoA directed to isopropanol instead of acetate. This is the first report on isopropanol production by genetically engineered microorganism under aerobic culture conditions.

Keywords Isopropanol · *Escherichia coli* · *Clostridium acetobutylicum* · *Clostridium beijerinckii* · Metabolic engineering

Introduction

Production of fuel and valuable chemicals from biomass-derived sugars has attracted much attention during last few decades due to the rising oil price and as an effort to reduce CO_2 emissions to tackle global warming. As a result, society's needs are gradually shifting from petroleum to renewable biomass for feedstock.

Solvent production from biomass is not a new concept. Large amounts of acetone and butanol were produced by fermentation (AB fermentation) early in the twentieth century, although a decline of the fermentation began as a result of economic competition from the petrochemical industry after the World War II (Dürre 1998). AB fermentation is achieved by microorganisms of the genus *Clostridium* under strictly anaerobic conditions. Biosynthetic pathways and relevant genes to AB fermentation in *Clostridium acetobutylicum* are well studied (Mitchell 1998). Figure 1a shows the metabolic pathways of acetone and butanol synthesis as well as related metabolic pathways. Another *Clostridium* bacterium, *C. beijerinckii*, produces isopropanol instead of acetone together with butanol (George et al. 1983). This microorganism possesses an additional primary–secondary alcohol dehydrogenase (ADH) that catalyzes the reduction of acetone (Ismaiel et al. 1993). Although several attempts have been made to overcome limitations of AB fermentation such as the loss of ability for solvent production (known as "degeneration") upon serial subculturing or in continuous culturing (Mitchell 1998) and growth inhibition by product solvents that cause low titer of products, acetone and butanol are currently produced from petrochemical processes.

Heterologous expression of clostridial genes for solvent production in industrial organisms, such as *Escherichia coli*, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae*, is a promising approach for industrial production of biosolvents competitive with petrosolvents because so much information on their physiology and well-established genetic tools are available. For example, an engineered strain of *E. coli* expressing four genes coding for three enzymes from *C. acetobutylicum*, thiolase (THL), coenzyme A (CoA)

T. Jojima · M. Inui · H. Yukawa (✉)
Research Institute of Innovative Technology for the Earth (RITE),
9-2 Kizugawadai,
Kizugawa, Kyoto 619-0292, Japan
e-mail: mmg-lab@rite.or.jp

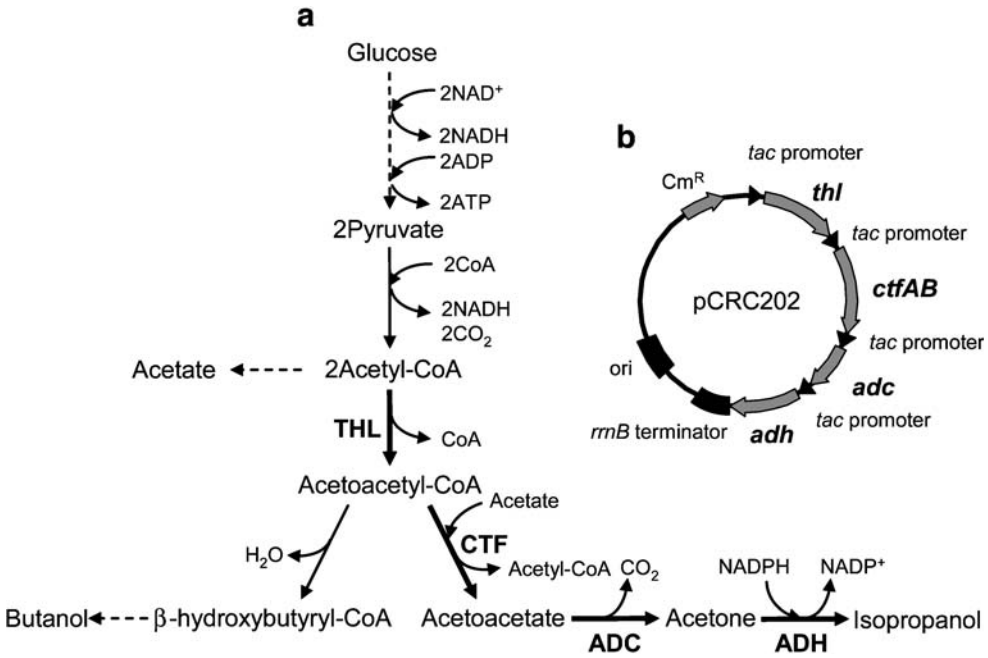

Fig. 1 Biosynthetic pathway of isopropanol and related metabolic pathways in *E. coli* (**a**) and plasmid for introduction of isopropanol-producing pathway into *E. coli* (**b**). Enzymes introduced into engineered *E. coli* are indicated in *bold* letters in **a**: thiolase (THL), CoA-transferase (CTF), acetoacetate decarboxylase (ADC), primary–secondary alcohol dehydrogenase (ADH). Abbreviations in **b**: *thl* thiolase gene, *ctfAB* genes for two subunits of CoA-transferase, *adc* acetoacetate decarboxylase gene, *adh* primary–secondary alcohol dehydrogenase gene, Cm^R chloramphenicol resistance gene

transferase (CTF), and acetoacetate decarboxylase (ADC), produced acetone at titers comparable to those obtained with *C. acetobutylicum* (Bermejo et al. 1998).

This study demonstrates production of isopropanol from glucose by genetically engineered *E. coli*. Isopropanol is used as a solvent in many applications, as a cleaner for electric devices, as a fuel additive for dissolving water in fuel lines, and as a feedstock for production of chemicals such as propylene. Most studies for isopropanol–butanol fermentation by clostridia so far relate to improvement of fermentation processes to prevent product inhibition, for example, with a continuous fermentation using Ca-alginate-entrapped clostridial cells (Krouwel et al. 1983), pervaporation (Matsumura et al. 1992), and product adsorption by XAD resin (Groot and Luyben 1986). This is the first report describing isopropanol production by metabolic engineering.

Materials and methods

Microorganisms and cultivation

E. coli strain JM109 (Takara, Shiga, Japan) was grown in Luria–Bertani (LB) medium. Antibiotics were included as appropriate at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml. *C. beijerinckii* NRRL B593 (synonym: DSM6423) was grown in reinforced clostridial medium (BD Difco, NJ, USA) at 30°C in an anaerobic atmosphere (95% nitrogen and 5% hydrogen). After 24-h incubation, clostridial cell was harvested by centrifugation (5,000×*g*, 10 min) and was used for genome extraction.

DNA amplification and plasmid construction

General techniques for DNA manipulation were performed according to the reference (Sarmbrook and Russell 2001). Three DNA fragments containing the genes *thl* (Locus tag of *C. acetobutylicum* genome: CAC2873), *ctfAB* (CA_P0163, CA_P0164), and *adc* (CA_P0165) were amplified by polymerase chain reaction (PCR) with primers 1 and 2, 3 and 4, 5 and 6, respectively (Table 1). Genomic DNA of *C. acetobutylicum* ATCC824 was purchased from American Type Culture Collection (ATCC 824D-5). PCR was performed using a GeneAmp PCR system 9700 (Applied Biosystems, CA, USA) and PrimeSTAR DNA polymerase (Takara). Thermal cycling consisted of 94°C for 3 min followed by 30 cycles of 95°C for 20 s and 58°C for 30 s and 72°C for 1 min. Genomic DNA of *C. beijerinckii* was extracted by the method (Marmur 1961). The gene (accession no. AF157307) for ADH was amplified from *C. beijerinckii* genome with primers 7 and 8. Primers were designed to fuse Shine–Dalgarno sequence into the 5′ end of each gene. DNA fragment containing *tac* promoter was amplified from a plasmid pKK223-3 as a PCR template. Primer set for the *tac* promoter amplification was used as follows: 9 and 10 for *ctfAB*, 10 and 11 for *adc*, and 10 and 12 for *adh*. The *tac* promoter and each gene (*ctfAB*, *adc*, and *adh*) were joined by a crossover PCR method (Horton et al. 1989), and resulting amplicons were purified from an agarose gel and subsequently were cloned into a pGEM-T vector (Promega, WI, USA) after addition of deoxyadenosine to the 3′ ends of the PCR products to ensure DNA sequences of the amplicons. The genes with the *tac* promoter were excised from the pGEM-T vectors

Table 1 Oligonucleotides used in this study

Primer	Sequence (5′-3′)
Primer 1	CGAATTCAAAGGAGGAGTGTGTTGATGAAAGAAGTTGTAATAGC
Primer 2	GGATCCCTAGCACTTTTCTAGCAAT
Primer 3	TTCACACAGGAAACAAAGGAGGAGTGTGTTGATGAACTCTAAAATAATTAGA
Primer 4	GCATGCTAAACAGCCATGGGTCTAAG
Primer 5	TTCACACAGGAAACAAAGGAGGAGTGTGTTGATGTTAAAGGATGAAGTAATTAA
Primer 6	CCCGGGTTACTTAAGATAATCATATATAAC
Primer 7	TTCACACAGGAAACAAAGGAGGAGTGTGTTGATGAAAGGTTTTGCAATGCTA
Primer 8	GTCGACTTATAATATAACTACTGCTTTA
Primer 9	GGATCCCCATCGGAAGCTGTGG
Primer 10	TGTTTCCTGTGTGAAATTG
Primer 11	GCATGCCATCGGAAGCTGTGG
Primer 12	CCCGGGCCATCGGAAGCTGTGG

The restriction site used in the cloning procedure has been underlined.

and sequentially inserted into expression vector pCRC200 that has a multiple cloning site between *tac* promoter and *rrnB* terminator (Yasuda et al. 2007) to obtain pCRC202 (Fig. 1b). The plasmid pCRC202 was introduced into *E. coli* JM109 according to a standard method (Sarmbrook and Russell 2001).

Isopropanol production

E. coli was inoculated in LB medium (2.5 ml in a test tube) and grown overnight at 30°C. The resulting culture was inoculated into a 500-ml baffled flask containing 50 ml of a complex (SD8) medium (Luli and Strohl 1990). The culture was incubated for isopropanol production in an agitation incubator (Tasaki Scientific Instruments, Saitama, Japan) at 250 rpm and 37°C. Glucose was occasionally supplemented to maintain glucose concentration more than 30 mM. Data represent average values in triplicate experiments. Standard deviations were within 5% of the mean.

Analyses

Isopropanol and acetone were determined by using a gas chromatography (GC-14B, Shimadzu, Kyoto, Japan) equipped with Thermon-1000 Sunpak-A 50/80 (Shinwa Chemical Industries, Kyoto, Japan). Organic acids were quantified by high-performance liquid chromatography (model-8020, Tosoh, Tokyo, Japan) equipped with an electric conductivity detector and a TSKgel OApak-A column (Tosoh) operating at 40°C with a 0.75 mM H_2SO_4 mobile phase at a flow rate of 1.0 ml/min. The concentration of glucose was measured by an enzyme electrode glucose sensor according to manufacture's protocol (BF-5, Oji Scientific Instruments, Hyogo, Japan). Optical density was measured at 610 nm using a Novaspec II spectrophotometer (Amersham Pharmacia biotech, UK), and dry weights of cells were determined by drying at 80°C.

Results

Engineering isopropanol productivity in *E. coli*

Isopropanol-producing pathway was designed by combining endogenous glycolytic pathway in *E. coli* with four enzymes, THL, CTF, ADC from *C. acetobutylicum* ATCC824, and ADH from *C. beijerinckii* NRRL B593. Although *C. acetobutylicum* produces acetone and butanol but not isopropanol, its acetone-producing pathway shares the same three enzymes (THL, CTF, and ADC) with isopropanol-producing pathway of *C. beijerinckii* (Fig. 1a). Although the genes for the enzymes are identified from *C. acetobutylicum* (Cary et al. 1990; Petersen and Bennett 1990; Petersen and Bennett 1991), no report has been described about gene identification of the three enzymes from *C. beijerinckii* NRRL B593. Thus, we employed the genes from *C. acetobutylicum* for construction of a synthetic isopropanol pathway in *E. coli*. Plasmid pCRC202 was constructed to express the five genes (*thl*, *ctfAB*, *adc*, and *adh*) under control of the *tac* promoter in *E. coli* (Fig. 1b). Theoretical yield using this pathway was 1 mol of isopropanol formed from 1 mol of glucose consumed. Isopropanol production was conducted in aerobic culture conditions because oxidation of NADH without formation of fermentative metabolites, such as lactate, was preferable.

Effect of metabolic engineering on growth rate and glycolytic flux

Specific growth rates were calculated to be 1.0 and 1.4 (h^{-1}) for isopropanol-producing *E. coli* bearing plasmid pCRC202 (*E. coli*-pCRC202) and control *E. coli* bearing an expression vector pCRC200 (*E. coli*-pCRC200), respectively, indicating that engineered *E. coli* grew slightly slower than *E. coli*-pCRC200 under the conditions tested

(Fig. 2a). In contrast, dry cell mass of *E. coli*-pCRC202 and *E. coli*-pCRC200 after 48-h cultivation was 5.7 and 2.6 g/l, respectively. *E. coli*-pCRC202 consumed glucose continuously, whereas *E. coli*-pCRC200 almost ceased consuming glucose at the onset of stationary phase (Fig. 2b).

Production of isopropanol by genetically engineered *E. coli*

Fermentation was conducted in a glucose fed-batch culture in a baffled flask. Glucose was occasionally supplemented at concentrations ranging from 30 to 200 mM. After 36-h cultivation, 227±6 mM of isopropanol was detected in the culture broth, and further incubation did not increase isopropanol concentration (Fig. 3). Acetone formation was also observed. No isopropanol and acetone were detected in a broth of the *E. coli*-pCRC200 strain, indicating that the clostridial genes were actively expressed in *E. coli*-pCRC202. Isopropanol yield was 51% after 36-h cultivation, and total solvent yield (isopropanol plus acetone) was 61%. Maximum volumetric productivity of isopropanol was 10.3 $mmol \cdot l^{-1} \cdot h^{-1}$ under the conditions.

Production of acetic acid

Acetate production by the isopropanol-producing strain, *E. coli*-pCRC202, was expected to be lower than that by *E. coli*-pCRC200 because isopropanol-producing pathway competes with acetate-formation pathway at the acetyl-CoA node (Fig. 1a). After 48-h fed-batch culture, *E. coli*-pCRC202 produced sixfold lower acetate than *E. coli*-pCRC200. The pH values of culture broths were 4.6 and 5.8 after 24 h in control and engineered *E. coli*, respectively. These results clearly indicated that carbon flux was redirected to isopropanol (and acetone) from acetate in the genetically engineered *E. coli*.

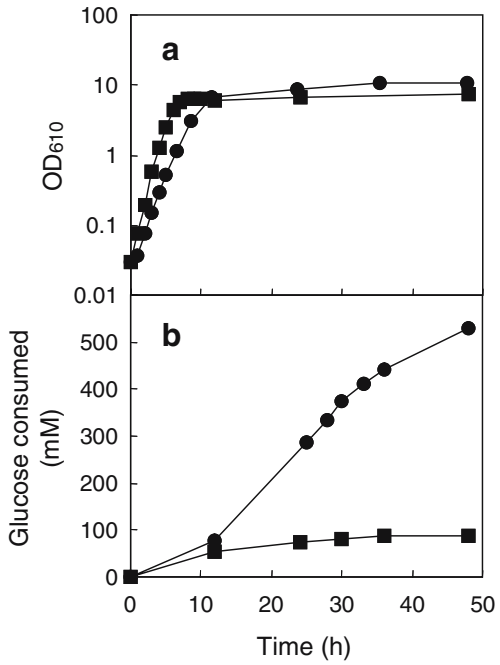

Fig. 2 Effect of metabolic engineering on growth (**a**) and glucose utilization (**b**) in glucose fed-batch cultures. *Circles* and *squares* indicate engineered *E coli*-pCRC202 and *E. coli*-pCRC200, respectively

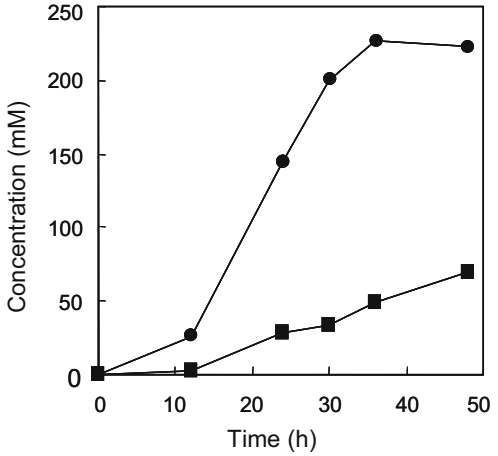

Fig. 3 Isopropanol production by engineered *E. coli* in glucose fed-batch culture. *Circles* and *squares* indicate concentrations of isopropanol and acetone in a culture broth, respectively

Discussion

This study demonstrated that *E. coli* with the five genes coding for four enzymes from two *Clostridium* species produced up to 227 mM of isopropanol under aerobic culture conditions (Fig. 3). Isopropanol titer and productivity of engineered *E. coli* were higher than those of clostridia in a batch culture. *C. isopropylicum* IAM 19239 immobilized with Ca-alginate has been demonstrated to produce

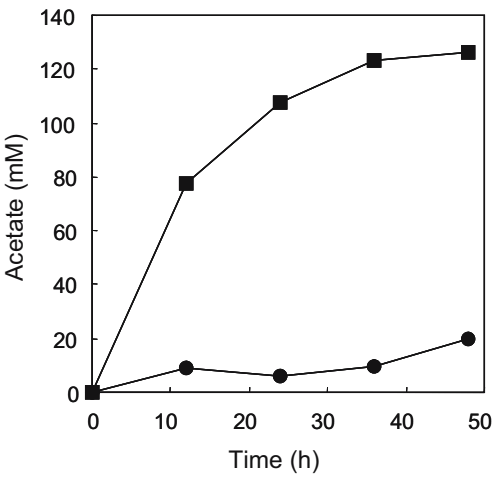

Fig. 4 Effect of metabolic engineering on acetate production in glucose fed-batch cultures. *Circles* and *squares* indicate acetate concentrations in culture broths of engineered *E coli*-pCRC202 and *E. coli*-pCRC200, respectively

77 mM of isopropanol for approximately 67 h of a batch fermentation (Matsumura et al. 1992). Employing XAD resin to adsorb solvent and repress fermentation inhibition, *C. beyerinckii* LMD 27.6 produces 67 mM for 100 h in a batch culture (Groot and Luyben 1986). Clostridia produce isopropanol together with butanol, which causes lower titer of isopropanol, while engineered *E. coli* developed in this study lacks butanol-producing pathway.

The biosynthetic pathway of isopropanol shares the three enzymes with the acetone-producing pathway (Fig. 1a). In particular, some strains of *C. beijerinckii* produce both acetone and isopropanol together with butanol (Chen and Hiu 1986). Acetone formation was also observed in the broth of isopropanol-producing *E. coli* in this study (Fig. 3). This result suggests that the reaction from acetone to isopropanol might be one of the rate-limiting steps for the biosynthetic pathway of isopropanol. Another possible explanation is reoxidation of isopropanol. Further studies on flux analyses and genetic and biochemical characterization of enzymes relevant to isopropanol metabolism are required to reduce acetone and increase isopropanol productivity.

Acetone-producing *E. coli* were constructed by introducing the genes encoding three enzymes, THL, CTF, and ADC from *C. acetobutylicum* (Bermejo et al. 1998). The *E. coli* produced 93 and 154 mM of acetone in shake flask and bioreactor cultivations, respectively, whereas the isopropanol titer was 227 mM in shake flask culture of *E. coli*-pCRC202 in this study. The yield of isopropanol and solvent (isopropanol plus acetone) was 51 and 61%, respectively, in the present study. These values were slightly higher than those of acetone-producing *E. coli* where acetone yield was below 50% (Bermejo et al. 1998). It is uncertain that isopropanol titer and yield were higher than those of acetone-producing *E. coli*. Possibly, differences in plasmid construction might affect productivity. In acetone-producing *E. coli*, one thiolase promoter from *C. acetobutylicum* is located at the 5′ upstream of a synthetic acetone operon, while the *tac* promoter fused with every gene for THL, CTF, ADC, and ADH in this study (Fig. 1b).

Engineered *E. coli* continued glucose consumption for a longer time than *E.coli*-pCRC200 (Fig. 2b). This result is most probably explained by difference in acetate productivity between engineered *E. coli* and *E. coli*-pCRC200 (Fig. 4). The control *E. coli*-pCRC200 strain excreted much more acetate than engineered strain, resulting in lower broth pH of *E. coli*-pCRC200. The pH values were 4.6 and 5.8 in *E. coli*-pCRC200 and engineered *E. coli*, respectively. *E. coli* accumulates acetate when growing at a high rate of glucose consumption, which is known as overflow metabolism (El-Mansi and Holms 1989; Luli and Strohl 1990). Detrimental effect of acetate on growth (Luli and Strohl 1990) and production of recombinant protein has been reported (Jensen and Carlsen 1990). Isopropanol-producing pathway competes with acetate-producing pathway in an acetyl-CoA node (Fig. 1a). Therefore, introduction of isopropanol-producing pathway led to low acetate productivity compared to *E. coli*-pCRC200 (Fig. 4). Consequently, inhibition of glucose consumption by acetate seems to be repressed in engineered *E. coli* in this study.

The results described above indicated that expression of the five genes from two *Clostridium* spp. led to production of isopropanol in *E. coli* under aerobic conditions. Further improvement of isopropanol titer and productivity can be achieved by combining existing technologies such as high-cell-density fermentation, continuous fermentation, and *in situ* product recovery.

Acknowledgment We thank C. A. Omumasaba (RITE) for helpful comments on the manuscript.

References

Bermejo LL, Welker NE, Papoutsakis ET (1998) Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. Appl Environ Microbiol 64:1079–1085

Cary JW, Petersen DJ, Papoutsakis ET, Bennett GN (1990) Cloning and expression of *Clostridium acetobutylicum* ATCC 824 acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A-transferase in *Escherichia coli*. Appl Environ Microbiol 56:1576–1583

Chen JS, Hiu SF (1986) Acetone-butanol-isopropanol production by *Clostridium beijerinckii* (synonym, *Clostridium butylicum*). Biotechnol Lett 8:371–376

Dürre P (1998) New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl Microbiol Biotechnol 49:639–648

El-Mansi EM, Holms WH (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. J Gen Microbiol 135:2875–2883

George HA, Johnson JL, Moore WEC, Holdeman LV, Chen JS (1983) Acetone, isopropanol, and butanol production by *Clostridium beijerinckii* (syn. *Clostridium butylicum*) and *Clostridium aurantibutyricum*. Appl Environ Microbiol 45:1160–1163

Groot WJ, Luyben K (1986) In situ product recovery by adsorption in the butanol/isopropanol batch fermentation. Appl Micro Biotech 25:29–31

Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61–68

Ismaiel AA, Zhu CX, Colby GD, Chen JS (1993) Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of *Clostridium beijerinckii*. J Bacteriol 175: 5097–5105

Jensen EB, Carlsen S (1990) Production of human growth hormone in *Escherichia coli*: expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnol Bioeng 36:1–11

Krouwel PG, Groot WJ, Kossen NWF, van der Laan WFM (1983) Continuous isopropanol–butanol–ethanol fermentation by immobilized *Clostridium beijerinckii* cells in a packed bed fermenter. Enzyme Microbial Technol 5:46–54

Luli GW, Strohl WR (1990) Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in

batch and fed-batch fermentations. Appl Environ Microbiol 56: 1004–1011

Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:208–218

Matsumura M, Takehara S, Kataoka H (1992) Continuous butanol/isopropanol fermentation in down-flow column reactor coupled with pervaporation using supported liquid membrane. Biotechnol Bioeng 39:148–156

Mitchell W (1998) Physiology of carbohydrate to solvent conversion by clostridia. Adv Microb Physiol 39:31–130

Petersen DJ, Bennett GN (1990) Purification of acetoacetate decarboxylase from *Clostridium acetobutylicum* ATCC 824 and cloning of the acetoacetate decarboxylase gene in *Escherichia coli*. Appl Environ Microbiol 56:3491–3498

Petersen DJ, Bennett GN (1991) Cloning of the *Clostridium acetobutylicum* ATCC 824 acetyl coenzyme A acetyltransferase (thiolase; EC 2.3.1.9) gene. Appl Environ Microbiol 57: 2735–2741

Sarmbrook J, Russell D (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, NY

Yasuda K, Jojima T, Suda M, Okino S, Inui M, Yukawa H (2007) Analyses of the acetate-producing pathways in *Corynebacterium glutamicum* under oxygen-deprived conditions. Appl Microbiol Biotechnol (in press). DOI 10.1007/s00253-007-1199-y