Erratum to: Production of isobutanol from crude glycerol by a genetically-engineered *Klebsiella pneumoniae* strain

Baek-Rock Oh · Sun-Yeon Heo · Sung-Mok Lee · Won-Kyung Hong ·
Jang Min Park · You Ree Jung · Dae-Hyuk Kim · Jung-Hoon Sohn ·
Jeong-Woo Seo · Chul Ho Kim

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Abstract *Klebsiella pneumoniae* was engineered to produce isobutanol from crude glycerol as a sole carbon source by expressing acetolactate synthase (*ilvIH*), keto-acid reducto-isomerase (*ilvC*) and dihydroxy-acid dehydratase (*ilvD*) from *K. pneumoniae*, and α -ketoisovalerate decarboxylase (*kivD*) and alcohol dehydrogenase (*adhA*) from *Lactococcus lactis*. Engineered *K. pneumoniae*, Δ *ldhA*/pBR-iBO (*ilvIH-ilvC-ilvD-kivD-adhA*), produced isobutanol (160 mg l⁻¹) from crude glycerol. To increase the yield of isobutanol, we eliminated the 2,3-butanediol pathway from the recombinant strain by inactivating α -acetolactate

decarboxylase (*adc*). This further engineering step improved the yield of isobutanol from 160 to 320 mg l⁻¹. This represents the first successful attempt to produce isobutanol from crude glycerol.

Keywords Crude glycerol · Genetic engineering · Isobutanol · *Klebsiella pneumoniae*

Introduction

During biodiesel production, crude glycerol is formed in bulk as a major by-product and can represent as much as 10 % (w/w) of the biodiesel generated (Johnson et al. 2007). This surplus of crude glycerol has had a deleterious effect on the

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The original paper contained an inadvertent error. Regretfully, the compound named as “2-butanol” was incorrect and should have been given as “isobutanol”. We apologise to the authors for making this error. The article re-published in the following pages is therefore the correct version of this paper.

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B.-R. Oh · S.-Y. Heo · S.-M. Lee · W.-K. Hong ·
J. M. Park · Y. R. Jung · J.-W. Seo · C. H. Kim (✉)
Applied Microbiology Research Center, Bio-Materials
Research Institute, Korea Research Institute of Bioscience
and Biotechnology (KRIBB), Jeongeup,
Jeonbuk 580-185, South Korea
e-mail: kim3641@kribb.re.kr

D.-H. Kim
Institute for Molecular Biology and Genetics, Research
Center of Bioactive Materials, Chonbuk National
University, Jeonju, Jeonbuk 561-756, South Korea

J.-H. Sohn
Systems and Synthetic Biology Research Center, Korea
Research Institute of Bioscience and Biotechnology
(KRIBB), Taejeon 305-333, South Korea

traditional market for glycerol and has created a significant environmental problem because glycerol cannot be discharged directly into the environment without treatment (da Silva et al. 2009). Glycerol has been considered a low-cost feedstock for industrial value-added products. In addition to being cheap and abundant, glycerol is more reduced than sugars, and thus can be used to obtain reduced, value-added products (Dharmadi et al. 2006).

Isobutanol is a valuable, advanced biofuel with superior performance and economics. In addition, it has a higher octane number, lower hygroscopicity and higher energy density than ethanol, and the same capabilities as gasoline (Connor and Liao 2011; Lee et al. 2012). Notably, when isobutanol is converted to 2-butylene, it can serve as a precursor for a number of value-added products, such as terephthalic acid, used in the syntheses of polyester and other bioplastics (Potera 2009; Curran et al. 2013).

Metabolic engineering is used to enhance the microbial production of value-added products (Strohl 2001). The general design approach for enhancing the yield of such products is to overexpress elements of the corresponding biosynthetic pathway and delete components of competing pathways (Gonzalez et al. 2008; Durnin et al. 2009; Smith and Liao 2011; Curran et al. 2013). Isobutanol production from glucose has been reported using genetically-engineered microorganisms: an *Escherichia coli* strain was engineered by deleting several genes involved in competing pathways and overexpressing acetolactate synthase (AlsS) from *Bacillus subtilis*, ketol-acid reductoisomerase-dihydroxyacid dehydratase (IlvC–IlvD) from *E. coli*, alpha-ketoisovalerate decarboxylase (kivd) from *Lactococcus lactis*, and alcohol dehydrogenase (adh2p) from *Saccharomyces cerevisiae*; the resulting strain produced 22 g isobutanol l⁻¹ from glucose (Atsumi et al. 2008). Lee et al. (2012) reported the production of isobutanol (151 mg l⁻¹) from glucose by *S. cerevisiae* through overexpression of Kivd and valine biosynthetic enzymes (acetolactate synthase, acetohydroxyacid reductoisomerase, and dihydroxyacid dehydratase).

Here, we describe the first construction of a microbial system for the production of isobutanol from glycerol using *K. pneumoniae*, a well-known glycerol-fermenting microorganism. The genetic stability of the engineered *K. pneumoniae* was also investigated.

Materials and methods

Bacterial strains, plasmids, and media

Klebsiella pneumoniae Δ ldhA mutant, derived from ATCC 200721, has been described (Oh et al. 2012a). *Escherichia coli* DH5 α was used for DNA manipulation. Lambda Red and FLP recombinases were expressed by helper plasmids pKD46 (Datsenko and Wanner 2000) and pCP20 (Cherepanov and Wackernagel 1995), respectively; replication of these plasmids is temperature-sensitive and the plasmids can thus be easily eliminated. The pIJ773 vector was the source of the apramycin-resistance gene. Microbial cells were grown in LB medium [yeast extract (Difco), 0.5 % (w/v); Bacto-tryptone (Difco), 1 % (w/v); and NaCl, 1 % (w/v)] or Germ medium (Oh et al. 2011), supplemented with antibiotic (10 μ g tetracycline ml⁻¹). Germ medium contained 20 g crude glycerol l⁻¹, 2 g (NH₄)₂SO₄ l⁻¹, 3.4 g K₂HPO₄ l⁻¹, 1.3 g KH₂PO₄ l⁻¹, 0.2 g MgSO₄ l⁻¹, 0.02 g CaCl₂·2H₂O l⁻¹, 1 g yeast extract l⁻¹, 1 ml Fe solution [5 g FeSO₄·7H₂O l⁻¹ and 4 ml HCl (37 %, w/v) l⁻¹], and 1 ml trace element solution [70 mg ZnCl₂ l⁻¹, 100 mg MnCl₂·4H₂O l⁻¹, 60 mg H₃BO₃ l⁻¹, 200 mg CoCl₂·4H₂O l⁻¹, 20 mg CuCl₂·2H₂O l⁻¹, 25 mg NiCl₂·6H₂O l⁻¹, 35 mg Na₂MoO₄ l⁻¹·2H₂O, and 4 ml l⁻¹ HCl (37 %, w/v)]. Crude glycerol (purity 80 %, w/w) was obtained from a biodiesel-producing company (GS Bio, Yeosu, Korea).

Construction of recombinant plasmids

A schematic representation of the strategy used to construct pBR-iBO is shown in Supplementary Fig. 1. The lacZ promoter sequence (P_{lacZ}-ilvIH) was amplified using the primer pair P_{lacZ}-ilvIH-F (5'-AGC GGG CAG TGA GCG CAA-3') and P_{lacZ}-ilvIH-R (5'-ACT AGT AGC TGT TTC CTG TGT GAA ATT G-3'); underlined bases indicate a *Spe*I site. The open reading frames of the following genes were amplified from chromosomal DNA of *K. pneumoniae* MGH78578 (ATCC 200721) and *L. lactis* using the primers shown in Supplementary Table 1: acetolactate synthase (*K. pneumoniae* ilvIH; ABR75542.1 and ABR75543.1), keto-acid reductoisomerase (*K. pneumoniae* ilvC; ABR79645.1), dihydroxyacid dehydratase (*K. pneumoniae* ilvD; ABR79642.1), alpha-ketoisovalerate decarboxylase (*L. lactis* kivD; CAG34226.1), and alcohol dehydrogenase (*L. lactis*

adhA; AAK05905.1). Amplified DNA fragments were cloned into the pGEM TEasy vector (Promega, Madison, WI, USA), followed by nucleotide sequencing to confirm that no errors were introduced during cloning. A *SpeI*-*XbaI* fragment containing the *ilvIH* gene was next inserted into the corresponding restriction sites downstream of the *lacZ* promoter sequence (pGEM-*ilvIH*). pGEM-*P_{lacZ}-ilvIH-ilvC-ilvD-kivd-adh* was generated by sequentially inserting *NheI-SpeI* fragments containing *ilvC* (pGEM-*ilvC*), *ilvD* (pGEM-*ilvD*), *kivd* (pGEM-*kivd*), *adhA* (pGEM-*adh*) into *SpeI* sites of pGEM-*ilvIH*. Finally, pBR322 was cleaved with *ScaI*, treated with alkaline phosphatase, ligated with a DNA fragment obtained by digestion of pGEM-*P_{lacZ}-ilvIH-ilvC-ilvD-kivd-adh* with *NotI*, and then treated with Klenow fragment to yield plasmid pBR-iBO (*ilvIH-ilvC-ilvD-kivd-adh*). The final plasmid was transformed into *K. pneumoniae* strains by electroporation (Fournet-Fayard et al. 1995).

Deletion of the 2,3-butanediol (2,3-BD) biosynthesis pathway

The chromosomal acetolactate decarboxylase gene (*adc*) involved in 2,3-BD biosynthesis (Supplementary Fig. 2) was deleted by first amplifying 0.3-kb DNA sequences upstream and downstream of *adc* by PCR using the primer pairs P1 (5'-ATC GAA AAC GTC TCA AAC CAG C -3') and P2 (5'-GAT CGT CGA GGA CGT CGG TCG TTA ACA TAG ACC TGA CTG CTG AAG G-3') for the upstream region and P3 (5'-CCT TCA GCA GTC AGG TCT ATG TTA ACG ACC GAC GTC CTC GAC GAT C-3') and P4 (5'-CCT TAA CTT TCT ACG GAA CGG A-3') for the downstream region (bases in italics indicate a *HpaI* site). The PCR conditions included an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, with a final incubation at 72 °C for 7 min. The PCR products were annealed using P1 and P4, and the resultant product was cloned into pGEM-T Easy. Next, an apramycin-resistance gene, *aac(3)IV*, obtained from pIJ773 by digestion with *EcoRI* and *HindIII*, was inserted into the *HpaI* site of the PCR product following treatment with Klenow fragment. The resultant plasmid, designated pT-*adc*-Apra, was used as a template for PCR amplification of the deletion cassette, which was next introduced into

K. pneumoniae Δ *ldhA* by electroporation (Fournet-Fayard et al. 1995). Correct integration of the DNA fragment by homologous recombination was confirmed by Southern hybridization using the upstream regions of *adc* and *aac(3)IV* to probe *PstI*-digested chromosomal DNA; the probes were labeled using the DIG (digoxigenin-dUTP) system (Roche Diagnostics GmbH, Mannheim, Germany).

Cultivation of *K. pneumoniae* strains

Seed cells for fermentation were prepared in 1 l flasks containing 200 ml Germ medium. Flasks were incubated at 37 °C for 12 h, and cultures were subsequently inoculated into growth vessels at 10 % (v/v). Batch fermentations were conducted in 5 l stirred-vessel systems (Kobiotech Co. Ltd., Incheon, Korea) containing 2 l germ medium; all fermentation experiments were at 37 °C with stirring at 200 rpm and an airflow rate of 0.5 vvm. IPTG was added to cultures at 0.5 mM to induce the expression of genes. All data are average of three independent experiments.

Analytical methods

Concentrations of crude glycerol, 1,3-propanediol (1,3-PD), ethanol, acetate, lactate, succinate, and 2,3-BD were determined using HPLC equipped with a refractive index detector and an organic acid analysis column (300 × 78 mm, Aminex HPX-87H; Bio-Rad). The mobile phase was 5 mM H₂SO₄ at 0.8 ml min⁻¹. Column and cell were at 65 °C and 45 °C, respectively (Oh et al. 2012a). Isobutanol was determined using GC equipped with a ZB-WAX plus column (Phenomenex). The column was controlled according to the following gradient program: 50 °C for 1 min; then increasing at 15 °C min⁻¹; 225 °C for 5 min. Biomass concentration was determined from the OD₆₀₀ value.

Results and discussion

Isobutanol production from crude glycerol by *K. pneumoniae* Δ *ldhA* harboring pBR-iBO

To produce isobutanol from glycerol, we constructed the plasmid pBR-iBO containing genes constituting

Fig. 1 Genetic engineering steps used to construct a microbial system for producing isobutanol from glycerol. *Gly* glycerol, *DHA* dihydroxyacetone, *DHAP* dihydroxyacetone phosphate, *G3P* glycerol 3-phosphate, *PEP* phosphoenolpyruvate, *PYR* pyruvate, *Lac* lactate, *AcLac* acetolactate, *Act* acetoin, *2,3BD* 2,3-butanediol, *DHIV* dihydroxy isovaleric acid, *KIV* α -

ketoisovalerate, *IBA* isobutyric acid, *iBuOH* isobutanol, *3-HPA* 3-hydroxypropionaldehyde, *1,3PDO* 1,3-propanediol, *ldhA* lactate dehydrogenase, *adc* acetolactate decarboxylase, *ilvIH* acetolactate synthase, *ilvC* keto-acid reductoisomerase, *ilvD* dihydroxy-acid dehydratase, *kivd* α -ketoisovalerate decarboxylase, *adh* alcohol dehydrogenase

Table 1 Analysis of glycerol fermentation metabolites in *K. pneumoniae* strains

Metabolites (g l ⁻¹)	$\Delta ldhA$ / pBR322	$\Delta ldhA$ / pBR-iBO	$\Delta ldhA$ Δadc / pBR322	$\Delta ldhA$ Δadc / pBR-iBO
Acetate	0.5	0.5	0.8	0.4
Ethanol	1	1.1	1.4	1.3
Isobutanol	0	0.16	0	0.32
1,3-Propanediol	7.8	7.7	8.3	8
Succinate	0.4	0.5	0.6	0.6
2,3-Butanediol	2.3	1.9	0	0

the biosynthesis pathway (*ilvIH*–*ilvC*–*ilvD*–*kivd*–*adhA*) and introduced it into the *K. pneumoniae* strain (Fig. 1), which is a typical glycerol-fermenting bacterium. Unfortunately, simply inserting this gene cassette into the wild-type *K. pneumoniae* strain was insufficient and isobutanol production was not detected (data not shown). When the recombinant plasmid pBR-iBO was introduced into the lactate dehydrogenase-deficient *K. pneumoniae* $\Delta ldhA$ mutant, production of isobutanol from crude glycerol was detected (Table 1). Interestingly the production of isobutanol began upon cessation of growth of the mutant cells (Fig. 2). This stationary stage-specific production might be related to the high toxicity to isobutanol. Consistent with this, we found that the growth of *K. pneumoniae* cells was

Fig. 2 Fermentation of *K. pneumoniae* $\Delta ldhA$ mutant harboring pBR322 (a) and pBR-iBO (b). Closed circles, residual glycerol levels; open circles, OD₆₀₀ values; closed squares, isobutanol; closed triangles, 1,3-PD levels

inhibited in medium containing more than 1.3 % (10.4 g l⁻¹) isobutanol (data not shown). Li et al. (2011) reported that growth of *E. coli* and *C. glutamicum* were also inhibited at this concentration of isobutanol. Although the isobutanol titer was low

Fig. 3 Fermentation of *K. pneumoniae* $\Delta ldhA$ Δadc mutant harboring pBR322 (a) and pBR-iBO (b). Closed circles, residual glycerol levels; open circles, OD_{600} values; closed squares, isobutanol; closed triangles, 1,3-PD levels

($0.16\ g\ l^{-1}$), to the best of our knowledge, this is the first report of microbial system constructed to produce isobutanol from glycerol, especially crude glycerol derived from the biodiesel industry.

Enhancement of isobutanol production by elimination of the 2,3-BD biosynthesis pathway

Further genetic engineering was attempted to enhance the yield of isobutanol. Because 2,3-BD is another major product of the oxidative pathway of glycerol metabolism (Table 1), we constructed a 2,3-BD pathway-deficient $\Delta ldhA$ mutant strain harboring pBR-iBO by inactivating the acetolactate decarboxylase gene (*adc*) via homologous recombination. Southern blotting confirmed correct insertion of the apramycin-resistance gene into the chromosomal *adc* gene (Supplementary Fig. 2). Consistent with the results of molecular analyses, 2,3-BD production was eliminated in the $\Delta ldhA$ Δadc mutant strain harboring pBR-iBO. Notably, isobutanol yield was improved twofold, from 160 to $320\ mg\ l^{-1}$ (Fig. 3), demonstrating the ability of metabolic engineering to enhance isobutanol production from glycerol.

Genetic stability of the isobutanol-production trait

The stability of the plasmid pBR-iBO in the *K. pneumoniae* $\Delta ldhA$ $\Delta budA$ mutant strain was examined. After batch fermentation of the recombinant strain for 20 h, the cells were collected and cultivated on LB plates containing antibiotic. Colonies were then tested for maintenance of plasmid DNA by replica plating on medium with and without antibiotic. These experiments showed that pBR-iBO was maintained in 96 % of cells of the mutant strain (data not shown), indicating good genetic stability.

In the present work, a microbial system for producing of isobutanol from crude glycerol was constructed by genetic engineering *K. pneumoniae*, which can produce 2,3-BD from glycerol (Oh et al. 2012b). The alteration of 2,3 BD biosynthesis pathway is promising and can lead to effective production of isobutanol from glycerol. This was accomplished by deleting competing pathways leading to biosynthesis of lactate and 2,3-BD and expressing genes (*ilvIH*, *ilvC*, *ilvD*, *kivd* and *adh*) constituting the isobutanol biosynthesis pathway. This is first report of the production of isobutanol from a waste carbon source rather than from glucose.

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