

In silico aided metabolic engineering of *Klebsiella oxytoca* and fermentation optimization for enhanced 2,3-butanediol production

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Abstract *Klebsiella oxytoca* naturally produces a large amount of 2,3-butanediol (2,3-BD), a promising bulk chemical with wide industrial applications, along with various byproducts. In this study, the in silico gene knockout simulation of *K. oxytoca* was carried out for 2,3-BD overproduction by inhibiting the formation of byproducts. The knockouts of *ldhA* and *pflB* genes were targeted with the criteria of maximization of 2,3-BD production and minimization of byproducts formation. The constructed *K. oxytoca* Δ *ldhA* Δ *pflB* strain showed higher 2,3-BD yields and higher final concentrations than those obtained from the wild-type and Δ *ldhA* strains. However, the simultaneous deletion of both genes caused about a 50 % reduction in 2,3-BD productivity compared with *K. oxytoca* Δ *ldhA* strain. Based on previous studies and in silico investigation that the agitation speed during 2,3-BD fermentation strongly affected cell growth and 2,3-BD synthesis, the effect of agitation speed on 2,3-BD production was investigated from 150 to 450 rpm in 5-L bioreactors containing 3-L culture media. The highest 2,3-BD productivity (2.7 g/L/h) was obtained at 450 rpm in batch fermentation. Considering the inhibition of acetoin for 2,3-BD production, fed-batch fermentations were performed using *K. oxytoca* Δ *ldhA* Δ *pflB* strain to enhance 2,3-BD production. Altering the agitation speed from 450 to 350 rpm at nearly 10 g/L of acetoin during the fed-batch fermentation allowed for the production of 113 g/L 2,3-BD, with a yield of 0.45 g/g, and for the production of 2.1 g/L/h of 2,3-BD.

Keywords 2,3-Butanediol · *Klebsiella oxytoca* ·
In silico aided metabolic engineering ·
Fermentation optimization

Introduction

The colorless, odorless, and transparent chemical 2,3-butanediol (2,3-BD), synthesized as one of the fermentation products, is a promising bulk chemical owing to its wide industrial application. It can be directly dehydrated into 1,3-butadiene, which is extensively used in the manufacture of many synthetic rubbers [6, 13]. Dehydration of 2,3-BD imparts methyl ethyl ketone (MEK), which is an effective fuel additive, and is commonly used as an industrial solvent for resins and lacquers [6, 13, 45, 48]. Acetoin and diacetyl, which serve as flavoring agents for numerous food products, giving a buttery taste, also can be easily manufactured by the dehydrogenation of 2,3-BD. In addition, 2,3-BD has potential applicability in a broad range of industries for producing moistening and softening agents, printing inks, cosmetics, plasticizers and explosives, fumigants, spandex, and pharmaceuticals [6, 13, 48].

In the face of rising petroleum prices and increasing environmental concerns, production methods of bio-based fuels and chemicals are getting more public attention [15, 42, 46]. 2,3-BD can be produced by microbial fermentation, and several bacteria are reported to synthesize 2,3-BD as the major fermentation product under microaerobic conditions. Among them, *Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter aerogenes*, *Paenibacillus polymyxa*, *Serratia marcescens*, and *Pseudomonas chlororaphis* produce large amounts of 2,3-BD [6, 48]. *Klebsiella* spp., especially *K. oxytoca*, show the best 2,3-BD production performance in terms of yield, productivity, and final titer

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[6, 9, 13, 17, 19–22, 39, 45, 48] as well as carbon-source utilization (glucose, galactose, lactose, xylose, and glycerol) [4, 7, 16–18, 21, 47]. Furthermore, *K. oxytoca* belongs to Risk Group 1 (RG 1), being categorized as a generally regarded as safe (GRAS) organism (National Institute of Health, Guidelines for Research Involving Recombinant DNA Molecules, 2002), which indicates that *K. oxytoca* is safe for industrial use.

With an aim to enhance 2,3-BD production by reducing the formation of byproducts, including formic acid, acetic acid, ethanol, lactic acid, succinic acid, and acetoin, various genetic modifications on *K. oxytoca* have been conducted [21, 24, 47]. In one example, Ji et al. [21] reported that the insertional inactivation of acetaldehyde dehydrogenase gene (*aldA*) in *K. oxytoca* led to a significantly improved 2,3-BD production by reducing the formation of ethanol. In other studies, Yang et al. [47] and Kim et al. [24] constructed a lactate-deficient mutant of *K. oxytoca*, in which the *ldhA* gene was disrupted, for preventing the formation of lactic acid. However, traditional metabolic engineering approaches have many limitations in developing ideal strains because they focus on local metabolism and do not consider the overall but complex metabolic network of cells. Accordingly, the approaches through systems metabolic engineering, which studies an organism at the systems level, became important [2, 3, 12, 25, 26, 34]. In particular, in silico genome-scale metabolic models and their applications to simulating metabolic behavior have been widely employed as a useful and promising tool to provide a system-level understanding of a cell, as well as systematic strategies for strain improvement [1, 10, 23, 27, 29, 33, 37].

We recently constructed a genome-scale metabolic model of *K. oxytoca*, KoxGSC1457, containing 1,457 reactions and 1,099 metabolites [38]. In that study, knockout candidates that can overproduce 2,3-BD and reduce byproduct formation at the same time were identified using the KoxGSC1457 model, and *K. oxytoca* mutant strains were developed based on simulation results. In particular, the effect of agitation speed during 2,3-BD fermentation was carefully studied in order to improve cell growth and 2,3-BD productivity. Then, optimization of agitation speed during fed-batch fermentation was conducted to enhance 2,3-BD production.

Materials and methods

Genome-scale metabolic model and constraints-based flux analysis

In order to predict the metabolic characteristics in response to genetic perturbations and to identify knockout

candidates for 2,3-BD overproduction, we used the genome-scale metabolic model of *K. oxytoca*, KoxGSC1457 for this study. The KoxGSC1457 model contains 1,457 reactions (229 transport reactions and 1,228 biochemical reactions) and 1,099 metabolites [38]. The model was thoroughly validated by comparing its output with various experimental data in the previous study. Constraints-based flux analysis, including flux balance analysis (FBA), was carried out under the assumption of a pseudo-steady state [11, 14, 35, 43, 44]. Mass balances in the stoichiometric model can be set up as $S_{ij} \cdot v_j = 0$, in which S_{ij} is a stoichiometric coefficient of a metabolite i in the j th reaction and v_j is the metabolic flux [mmol/g dry cell weight (DCW/h)] of the j th reaction. The in silico model, which is an underdetermined system due to insufficient constraints, can be simulated by optimization techniques, including linear programming (LP), subject to the constraints of mass balances, experimental measurements, and thermodynamics, as follows:

$$\text{Maximize/minimize } Z = \sum_{j \in J} c_j v_j$$

$$\text{Subject to } \sum_{j \in J} S_{ij} v_j = b_i, \forall i \in I$$

$$\alpha_j \leq v_j \leq \beta_j, \forall j \in J,$$

where Z indicates objective function, which is usually the maximization of cell growth rate, and c_j is the weight of the reaction j . b_i is the net transport flux of metabolite i , which is zero for intermediate metabolites as in $S_{ij} \cdot v_j = 0$. α_j and β_j are the lower and upper bounds of the metabolic flux of the j th reaction. For in silico simulations, glucose consumption rate and oxygen uptake rate were constrained to 16 and 5 mmol/g DCW/h, respectively. In order to simulate the in silico model more accurately, the limits of uptake and secretion rates for some metabolites, including amino acids, organic acids (acetic acid, formic acid, lactic acid, pyruvic acid, and succinic acid), and alcohols (ethanol and acetoin), were constrained by experimentally measured flux values. Likewise, the limits associated with secretion for some metabolites, which were not produced during fermentation as with amino acids, were constrained to zero [38].

To make a 3D mesh plot graph as a continuous surface for in silico flux solution spaces, the cell growth rate was maximized while gradually increasing the 2,3-BD production rate and byproducts formation rate, respectively, from their minimal flux values to maximal flux values. In order to compare between experiments and in silico predictions, flux variability analysis was carried out. Flux variability analysis computes the ranges of in silico metabolic fluxes by maximizing or minimizing the fluxes of intracellular reactions with additional constraints of 95 %

optimal cell growth rate, which is a value to relax the mathematically precise solution down to almost infinite places of decimals [28, 37].

Bacterial strains and mutant construction

K. oxytoca GSC 12206 strain (KCTC12133BP, Korean Collection for Type Cultures, Daejeon, Korea) was used in this study. In-frame deletions based on homologous recombination were carried out using overlap polymerase chain reaction (PCR) products to disrupt the target genes in chromosome. The overlap product of the two fragments, in which each ~500-bp upstream and downstream regions of the target gene was amplified and overlapped by PCR, was transformed in the competent cells of *K. oxytoca* strain by electroporation. The colonies were then selected in Luria–Bertani (LB) chloramphenicol (25 mg/L) plates at 42 °C, and the integrated cassette was cured by *sacB* expression under sucrose pressure. Integration and excision were confirmed in all mutants by PCR screening using genome-specific primers.

Fermentation

Culture medium used in batch and fed-batch fermentations contained (per liter): yeast extract (Becton Dickinson, Le Pont de Claix, France), 5 g; MgSO₄·7H₂O, 0.25 g; (NH₄)₂SO₄, 6.6 g; K₂HPO₄, 8.7 g; KH₂PO₄, 6.8 g; trace metal solution, 10 ml. The trace metal solution contained (per liter): FeSO₄·7H₂O, 5 g; ZnSO₄·7H₂O, 0.1 g; MnSO₄·H₂O, 0.1 g; CaCl₂·2H₂O, 0.1 g; HCl 10 ml. For inoculum preparation, the suspended cells from single colonies on LB agar (Difco Laboratories, Detroit, MI, USA) plates were precultured in 20 ml test tubes containing 5 ml culture medium (10 g/L D-glucose) at 37 °C for 5 h. Then, 1 ml of the preculture was transferred to a 500-ml Erlenmeyer flask containing 300 ml culture medium (10 g/L D-glucose) and cultivated to an optical density of 1.5–2.0 at 600 nm (OD₆₀₀). The tube and flask cultivations were conducted in a rotary shaker at 150 rpm and 37 °C (JEIO Tech. Co. SI-900R). Next, 300 ml of the seed culture was transferred to a 5-L bioreactor. Batch fermentations were performed in a 5-L BIOFLO® & CELLIGEN® 310 bioreactor (New Brunswick Scientific Co., Edison, NJ, USA) containing 3 L culture medium (90 g/L D-glucose). The bioreactor was continuously aerated using a 0.2-µm membrane filter at a flow rate of 1 vvm (air volume/working volume/min). The temperature was maintained at 37 °C, and pH was controlled at 6.5 ± 0.1 by the automatic feeding of NH₄OH. Foaming was controlled by the addition of Antifoam 289 (Sigma, St. Louis, MO, USA). All bioreactor experiments were performed at least three times independently, and the representative

results are shown in the figures. Fed-batch fermentations were performed under the same conditions as batch fermentations, except for the feeding of glucose into the fermentor to maintain the concentration at 10–60 g/L. For the fed-batch fermentation, the agitation speed was maintained at 450 rpm until the concentration of acetoin reached about 10 g/L and was then switched to 350 rpm. Samples were periodically taken to measure OD₆₀₀ to determine metabolite concentrations. After centrifugation at 13,200×g for 5 min, the resulting supernatant was used to determine the concentrations of glucose and metabolites.

Analytical procedures

Concentrations of D-glucose and metabolites, including 2,3-BD, formic acid, ethanol, acetic acid, lactic acid, succinic acid, and acetoin, were determined by a high-performance liquid chromatography (HPLC) equipped with ultraviolet-visible (UV-VIS) and reactive-index (RI) detectors (Agilent 1260 series, Agilent Technologies, Waldbronn, Germany). An Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA) was isocratically eluted with 0.01 N H₂SO₄ at 80 °C, and a flow rate of 0.6 ml/min was maintained. The OD₆₀₀ was measured using a UV-VIS spectrophotometry (DR5000, Hach Company, CO, USA) to monitor cell growth. Cell concentration, as DCW per liter of culture broth, was calculated from the predetermined standard curve relating OD₆₀₀ to DCW (1 OD₆₀₀ = 0.3877 ± 0.136 g DCW/L) [24].

Results and discussion

In silico prediction of gene knockout candidates for enhanced 2,3-BD production

K. oxytoca wild-type strain produced 2,3-BD along with several byproducts, including formic acid, acetic acid, ethanol, lactic acid, succinic acid, and acetoin during batch fermentation under microaerobic conditions [17, 21, 30] (Figs. 1, 2). For enhanced 2,3-BD production, the formation of byproducts should be reduced, after which the remaining metabolic fluxes from carbon sources should be redirected toward the synthesis of 2,3-BD. In our previous study [38], knockout of the *ldhA* gene, encoding lactate dehydrogenase, was targeted by in silico simulation, with top priority to eliminate the formation of lactic acid because *K. oxytoca* wild-type strain formed lactic acid as a main byproduct (32.2 g/L, 71 % of total byproducts) (Figs. 1, 2). The best candidate for gene knockout was found based on criteria of the maximization of 2,3-BD production rate and minimization of byproduct formation rate with viable-cell growth rate (>50 % cell growth rate of

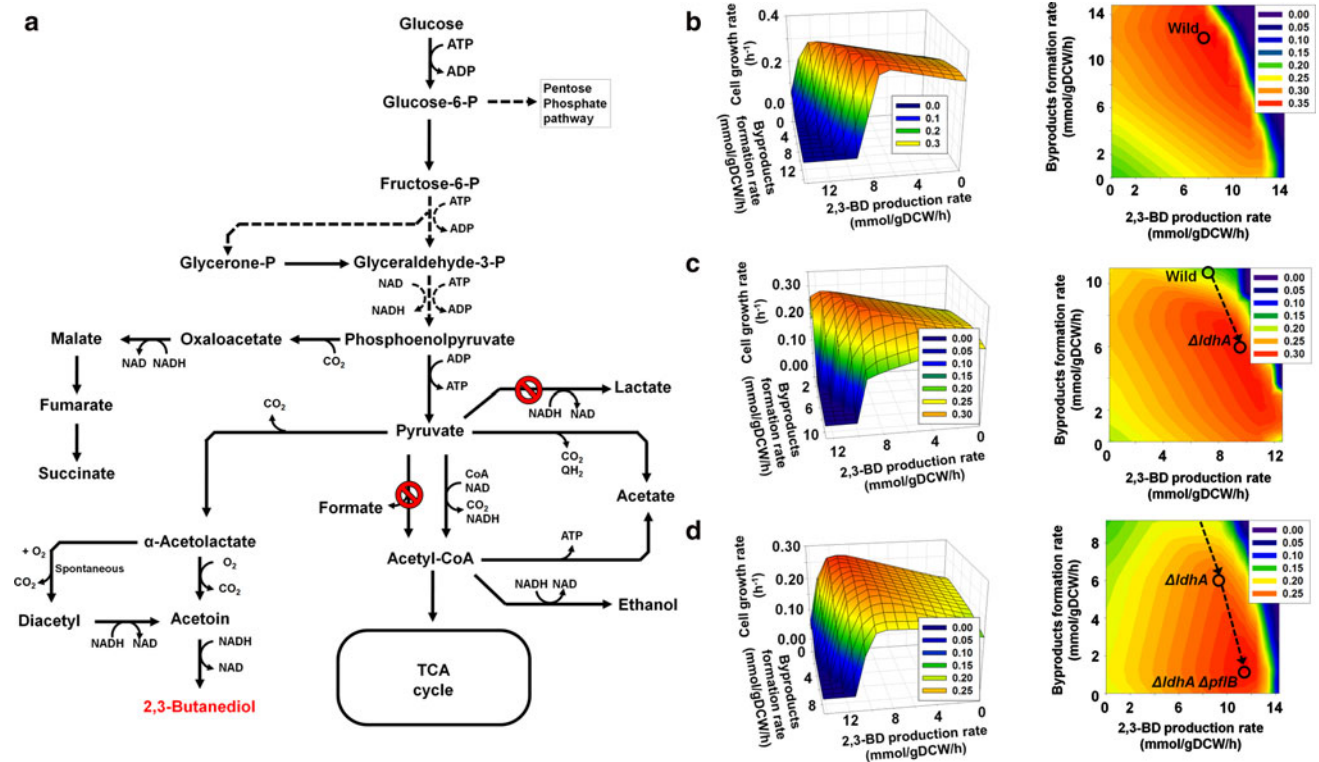


Fig. 1 In silico investigation for flux solution spaces of *Klebsiella oxytoca* wild-type and mutant strains. **a** Metabolic pathway related with 2,3-butanediol (2,3-BD) production. Three-dimensional mesh plot graph as a continuous surface for in silico flux solution spaces of *K. oxytoca*, **b** wild-type, **c** $\Delta ldhA$, and **d** $\Delta ldhA \Delta pflB$ strains. The maximal point of in silico cell growth rate is generally

base strain). Knockout of the *ldhA* gene did not disrupt the redox balance of nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide, reduced (NAD⁺/NADH). The inactivation of lactate-dehydrogenase-oxidizing NADH seemed to be compensated with 2,3-BD-dehydrogenase-producing 2,3-BD with the oxidation of NADH as well. As a result, the constructed *K. oxytoca* $\Delta ldhA$ strain successfully decreased the formation of lactic acid to 1.9 g/L (6 % of lactic acid produced by the wild-type strain) and significantly increased 2,3-BD. However, the *K. oxytoca* $\Delta ldhA$ strain still produced large amounts of byproducts, especially formic acid and ethanol. The Pareto diagram in Fig. 2c visually identifies the main byproducts, formic acid and ethanol, in *K. oxytoca* $\Delta ldhA$ strain. In silico analysis based on the KoxGSC1457 model also suggests that it left room for further strain improvement (Fig. 1). Although the optimal status of in silico flux solution space of the *ldhA* knockout strain was shifted to the increase of 2,3-BD production compared with that of the wild-type strain, byproduct formation rates of the *ldhA* knockout strain were still >5 mmol/g DCW/h (Fig. 1c).

In order to further improve the *K. oxytoca* $\Delta ldhA$ strain, an in silico single-gene knockout simulation was

acceptable to predict the actual state of a cell, which is denoted by a *small circle*, because the first priority of an actual cell is survival [32, 35, 41]. The *dotted arrow* indicates the transition of maximal cell growth rate by gene manipulation. *Colors* in the contour graph indicate the values of cell growth rate. Byproduct contains ethanol and several organic acids: lactic, acetic, succinic, and formic

performed on the criteria of the maximization of 2,3-BD production but minimization of byproduct formation rates. As a result, the *pflB* gene encoding pyruvate formate lyase was given as a knockout candidate of top priority for 2,3-BD overproduction. The metabolic characteristics of *K. oxytoca* $\Delta ldhA \Delta pflB$ strain were investigated by in silico flux solution space (Fig. 1d). The additional knockout of the *pflB* gene dramatically decreased the formation rate of byproducts, particularly those of formic acid and ethanol, <2 mmol/g DCW/h, and redirected the remaining metabolic fluxes into 2,3-BD synthesis. Inactivation of pyruvate formate lyase—which converts pyruvic acid into acetyl coenzyme A (acetyl-CoA) and formic acid—by *pflB* gene knockout, reduced effectively the formation of both formic acid and ethanol. The reduction of ethanol can be explained by the fact that the inactivation of pyruvate formate lyase decrease the formation of acetyl-CoA, which is the precursor for the formation of ethanol. In addition, the knockouts of *ldhA* and *pflB* genes significantly increased the pyruvic acid (an important precursor for 2,3-BD synthesis) pool, which was redirected into 2,3-BD synthesis without the disruption of redox balance. These predictions and observations were validated by the construction of *K.*

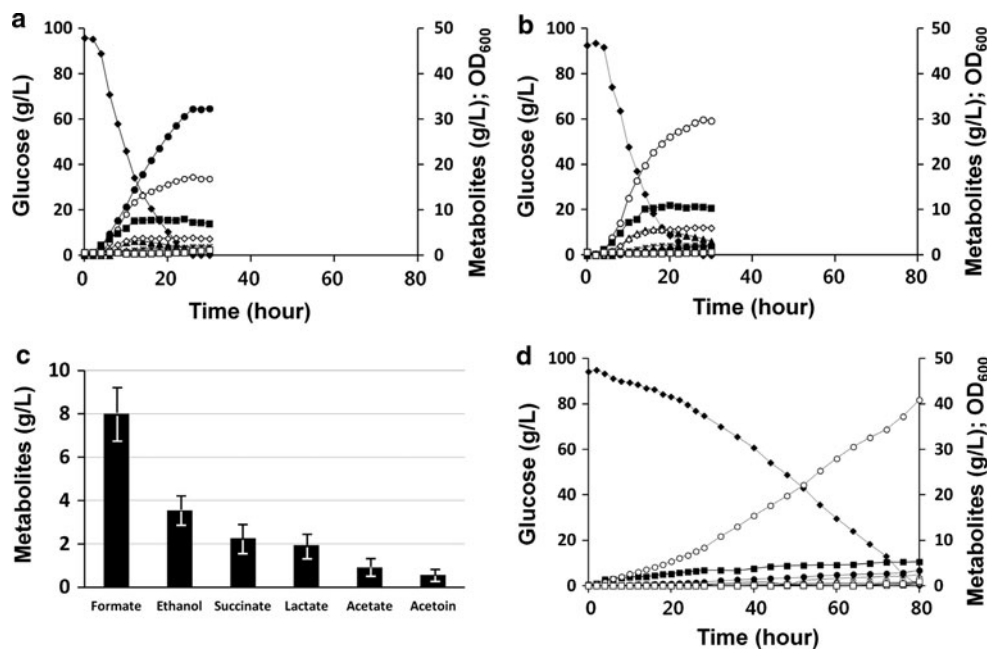


Fig. 2 Batch fermentation results. **a** *Klebsiella oxytoca* wild-type strain. **b** *K. oxytoca AldhA* strain. **c** Byproducts produced by *K. oxytoca AldhA* strain. **d** *K. oxytoca AldhA ΔpflB* strain. Batch fermentations were performed with a working volume of 3 L at 37 °C. Aeration rate and agitation speed were maintained at 1 vvm and 150 rpm, respectively; pH was controlled at 6.5 by the automatic

feeding of NH₄OH. Symbols in fermentation profiles indicates the concentration of glucose (filled diamonds), 2,3-butanediol (2,3-BD) (open circles), optical density at 600 nm (OD₆₀₀ (filled squares), acetoin (open squares), ethanol (filled triangles), succinic acid (asterisks), lactic acid (filled circles), formic acid (open diamonds), and acetic acid (open triangles)

Table 1 Batch fermentation results of *Klebsiella oxytoca* wild-type and mutant strains

Strains	Wild type	<i>AldhA</i>	<i>AldhA ΔpflB</i>
Glucose consumed (g/L)	95.7 ± 1.12	92.6 ± 1.53	94.3 ± 1.46
2,3-BD (g/L)	17.31 ± 2.15	29.91 ± 2.23	40.89 ± 2.04
Acetoin (g/L)	0.97 ± 0.34	0.57 ± 0.21	0.97 ± 0.26
Ethanol (g/L)	1.61 ± 0.56	3.54 ± 0.67	0.97 ± 0.24
Succinic acid (g/L)	1.92 ± 0.52	2.27 ± 0.65	2.50 ± 0.58
Lactic acid (g/L)	32.20 ± 1.89	1.93 ± 0.54	3.38 ± 0.87
Formic acid (g/L)	3.88 ± 1.45	8.01 ± 1.51	0.80 ± 0.16
Acetic acid (g/L)	1.27 ± 0.32	0.92 ± 0.35	0.59 ± 0.23
2,3-BD (2,3-BD) yield (g/g glucose)	0.18 ± 0.03	0.32 ± 0.02	0.44 ± 0.02
2,3-BD productivity (g/L/h)	0.58 ± 0.03	1.07 ± 0.04	0.51 ± 0.02

Batch fermentations were performed in a 5-L BIOFLO & CELLIGEN 310 bioreactor containing 3 L culture medium at 37 °C. Aeration rate and agitation speed were maintained at 1 vvm and 150 rpm, respectively; pH was controlled at 6.5 by the automatic feeding of NH₄OH

oxytoca AldhA ΔpflB strain and its application to fermentations.

High-yield production of 2,3-BD by *K. oxytoca AldhA ΔpflB* strain

According to the in silico predictions, *K. oxytoca AldhA ΔpflB* strain was constructed by a marker-free and homologous recombination knockout method. Batch fermentation of *K. oxytoca AldhA ΔpflB* strain was performed on the culture medium containing 90 g/L of D-glucose at 150 rpm and 37 °C. Fermentation results were compared

with those of the wild-type and *ldhA* knockout strains (Fig. 2). *K. oxytoca AldhA ΔpflB* strain was able to produce about 40.9 g/L of 2,3-BD, which is more than 2.3 times and 1.3 times higher than those produced by the wild-type (17.3 g/L of 2,3-BD) and *ldhA* knockout (29.9 g/L of 2,3-BD) strains, respectively (Fig. 2; Table 1). As the in silico predictions, the formation of ethanol and formic acid (<1 g/L) was decreased in *K. oxytoca AldhA ΔpflB* strain, whereas the *ldhA* knockout strain formed >3 g/L of ethanol and 6 g/L of formic acid. In particular, the 2,3-BD production yield from glucose by *K. oxytoca AldhA ΔpflB* strain was much higher (0.44 g/g, about 90 % of

theoretical maximum yield) than that of the wild-type (0.18 g/g) and *ldhA* knockout (0.32 g/g) strains (Table 1; Fig. 2). It can be speculated that the substantially increased pool of pyruvic acid, 2,3-BD precursor, by the inactivation of pyruvate formate lyase, enhanced the synthesis of 2,3-BD. In addition, we compared the experiments with in silico predictions (Fig. 3). Given the fact that multiple equivalent solutions of intracellular metabolic fluxes can be computed for the same state [28, 36, 40], we should investigate the ranges of in silico metabolic fluxes by flux variability analysis. Flux patterns predicted by in silico simulation for the wild-type and mutant strains showed good agreement with experimentally measured metabolic fluxes. As represented in Fig. 3, the knockouts of *ldhA* and *pf1B* genes dramatically decreased byproduct formation rate and increased 2,3-BD production rate in comparison with wild-type and *ldhA* knockout strains.

However, overall 2,3-BD productivity (0.51 g/L/h) of *K. oxytoca* Δ *ldhA* Δ *pf1B* strain was lower than that of the wild-type (0.58 g/L/h) and *ldhA* knockout (1.07 g/L/h) strains (Fig. 2; Table 1). This is mainly due to the severe inhibition of cell growth resulting from the inactivation of pyruvate formate lyase, which considerably decreased overall cell glucose uptake and 2,3-BD production rates (Fig. 2d). As mentioned earlier, the conversion of pyruvic acid into formic acid and acetyl-CoA is catalyzed by pyruvate formate lyase. In bacteria, acetyl-CoA is essential to energy generation, as it is the substrate that forms citric acid to initiate the tricarboxylic acid (TCA) cycle. It also contributes to biomass synthesis by serving as a precursor to fatty and amino acids. Another way to convert pyruvic acid into acetyl-CoA is conduction by the pyruvate dehydrogenase complex (PDC)—a complex of pyruvate dehydrogenase, dihydrolipoyl, and dihydrolipoyl dehydrogenase—in the presence of oxygen with reduction of NAD⁺. However, PDC activity seemed to be insufficient to replace the reaction of pyruvate formate lyase under the oxygen-limiting condition (150 rpm and 1 vvm) [5, 8]. Therefore, we examined the effect of agitation speed on 2,3-BD fermentation in order to improve overall 2,3-BD productivity of the *K. oxytoca* Δ *ldhA* Δ *pf1B* strain (Table 2).

Strategy to improve 2,3-BD productivity by changing agitation speeds

In a previous study, we examined the response of the 2,3-BD production rate of *K. oxytoca* Δ *ldhA* strain to variations in agitation speed during fermentation based on in silico genome-scale metabolic model (KoxGSC1457) [38]. The prediction showed that the optimal agitation speed existed on 2,3-BD production. The 2,3-BD productivity was proportional to agitation speed to 450 rpm during fermentation. However, further increase of the agitation speed

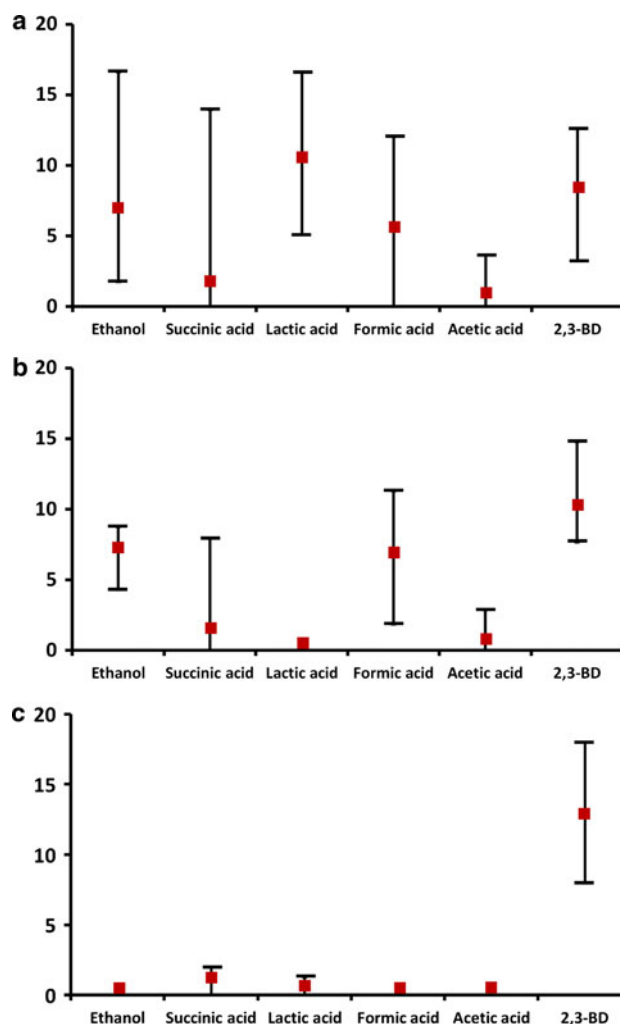


Fig. 3 Comparison between experiments and in silico predictions. **a** *Klebsiella oxytoca* wild-type strain. **b** *K. oxytoca* Δ *ldhA* strain. **c** *K. oxytoca* Δ *ldhA* Δ *pf1B* strain. Bars indicate ranges of in silico metabolic fluxes computed by flux variability analysis. Squares indicate the experimentally measured metabolic fluxes based on fermentation results

gradually decreased 2,3-BD productivity [17, 38]. In order to improve overall 2,3-BD productivity of *K. oxytoca* Δ *ldhA* Δ *pf1B* strain, the effects of agitation speed on 2,3-BD production and cell growth were examined from 150 to 450 rpm, keeping the aeration rate at 1 vvm.

As shown in Fig. 4, the specific cell growth rate (μ) and final cell density (OD₆₀₀) at 450 rpm were increased by more than 10- and 3.5-fold compared with at 150 rpm, respectively. At higher rpm, glucose consumption rate also increased considerably, and the fermentation period was shortened from 90 to 11 h. As a result, the final 2,3-BD productivity drastically increased from 0.43 to 2.7 g/L/h by raising the agitation speed to 450 rpm; no further increase in 2,3-BD production was observed at agitation speeds >450 rpm (data not shown). It seems that PDC recovered

Table 2 Summary of batch fermentations at different agitation speeds by *Klebsiella oxytoca AldhA ApfIB*

Agitation speed (rpm)	150	250	350	450
Glucose consumed (g/L)	87.4 ± 2.52	84.7 ± 3.31	87.3 ± 2.15	86.2 ± 2.78
2,3-Butanediol (2,3-BD) (g/L)	38.55 ± 1.82	33.67 ± 1.53	31.39 ± 1.68	30.79 ± 1.46
Acetoin (g/L)	0.15 ± 0.05	1.18 ± 0.17	1.79 ± 0.54	2.82 ± 0.61
Ethanol (g/L)	0.53 ± 0.06	0.55 ± 0.06	0.68 ± 0.05	1.47 ± 0.08
Succinic acid (g/L)	1.77 ± 0.68	2.30 ± 0.57	2.45 ± 0.46	2.99 ± 0.55
Lactic acid (g/L)	3.24 ± 1.61	1.92 ± 0.72	1.15 ± 0.48	0.32 ± 0.21
Formic acid (g/L)	0.97 ± 0.03	0.47 ± 0.12	0.22 ± 0.52	0.25 ± 0.04
Acetic acid (g/L)	0.15 ± 0.08	0.14 ± 0.06	0.39 ± 0.11	0.08 ± 0.04
2,3-BD yield (g/g glucose)	0.44 ± 0.02	0.40 ± 0.01	0.37 ± 0.02	0.35 ± 0.02
2,3-BD productivity (g/L/h)	0.43 ± 0.02	0.77 ± 0.02	1.40 ± 0.12	2.70 ± 0.21

Batch fermentations were performed in a 5-L BIOFLO & CELLIGEN 310 bioreactor containing 3 L of culture medium at 37 °C. The aeration rate was maintained at 1 vvm; pH was controlled at 6.5 by the automatic feeding of NH₄OH

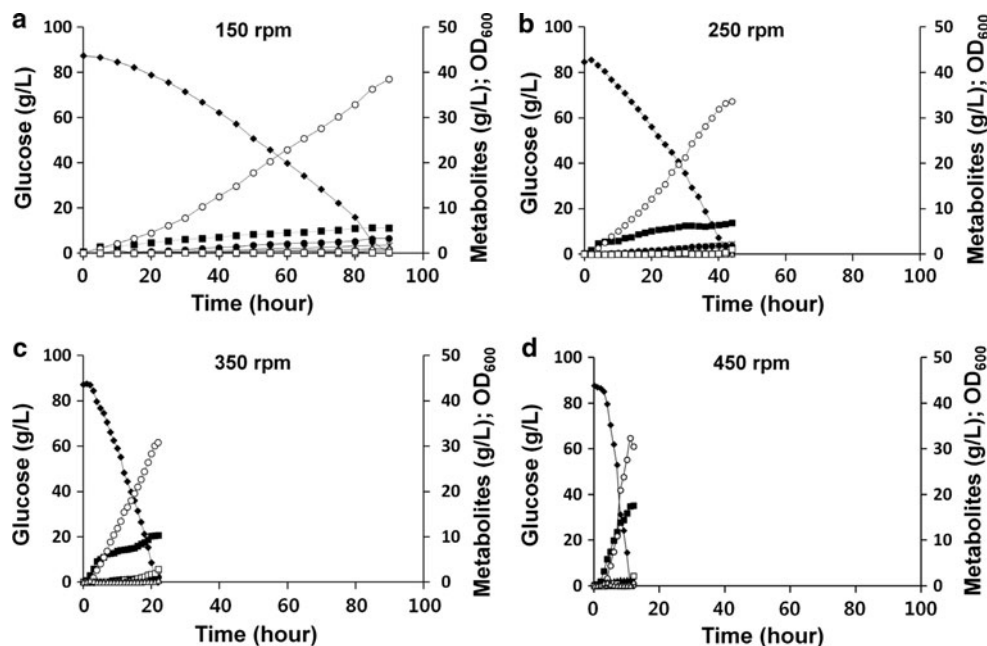


Fig. 4 Batch fermentations of *Klebsiella oxytoca AldhA ApfIB* strain at different agitation speeds. Fermentation results of **a** 150 rpm, **b** 250 rpm, **c** 350 rpm, and **d** 450 rpm. Batch fermentations were performed with a working volume of 3 L at 37 °C. Aeration rate was maintained at 1 vvm, and pH was controlled at 6.5 by the automatic feeding of NH₄OH. Symbols in fermentation profiles indicate glucose

concentration (filled diamonds), 2,3-butanediol (2,3-BD) (open circles), optical density at 600 nm (OD₆₀₀) (filled squares), acetoin (open squares), ethanol (filled triangles), succinic acid (asterisks), lactic acid (filled circles), formic acid (open diamonds), and acetic acid (open triangles)

its enzyme activity to yield enough acetyl-CoA to support cell growth by energy generation and biomass synthesis under more aerobic conditions of 450 rpm compared with that of 150 rpm. Accordingly, the glucose uptake rate improved with increased aeration by activating the TCA cycle and the metabolism of amino acids and fatty acids, resulting in an increase of overall 2,3-BD productivity. However, activation of the TCA cycle suggests that more carbon dioxide is released as carbon waste. Indeed, the final 2,3-BD titer decreased from 38.6 to 30.8 g/L with increasing agitation speed to 450 rpm; therefore, 2,3-BD

yield reduced from 0.44 to 0.35 g/g. Consequently, further optimization of the fed-batch fermentation process should be carried out to maximize 2,3-BD titer, yield, and productivity.

High yield and productivity strategy for 2,3-BD production by fed-batch fermentation

We found that the agitation speed during fermentation was a significant factor for 2,3-BD production in the *K. oxytoca AldhA ApfIB* strain (Fig. 4). Increasing the agitation speed

avored biomass formation, which resulted in the increase of 2,3-BD productivity. Whereas decreasing the agitation speed could increase 2,3-BD yield, it decreased 2,3-BD productivity. Therefore, it was indispensable to establish a strategy of controlling the agitation speed during fermentation for the efficient production of 2,3-BD to high concentration, high yield, and high productivity. Consequently, we adopted the agitation-speed-control strategy of a fed-batch fermentation, consisting of cell growth and 2,3-BD production phases. In the first (cell growth) phase, agitation speed was maintained at 450 rpm for fast cell growth. In the second (2,3-BD production) phase, agitation speed was decreased to 350 rpm, which was beneficial for establishing the 2,3-BD conversion rate and overall yield.

Prior to fed-batch fermentation by controlling the agitation speed, fed-batch fermentation of *K. oxytoca* Δ ldhA Δ pf1B strain with constant agitation speed of 450 rpm was performed to determine the optimal point at which to switch the agitation speed. As a result, the maximum concentration of 2,3-BD reached 57.7 g/L, with a yield of 0.29 g/g and productivity of 1.65 g/L/h (Fig. 5). Fed-batch fermentation was finished before the remaining glucose consumption because the capability of 2,3-BD production was severely depressed after 21 h. As described in Fig. 5, the slope of the trend line for 2,3-BD concentration was maintained until 21 h, which indicated that cellular viability was actively preserved until that time, when the concentration of acetoin reached about 10 g/L. Acetoin, which was mostly produced in the presence of excess

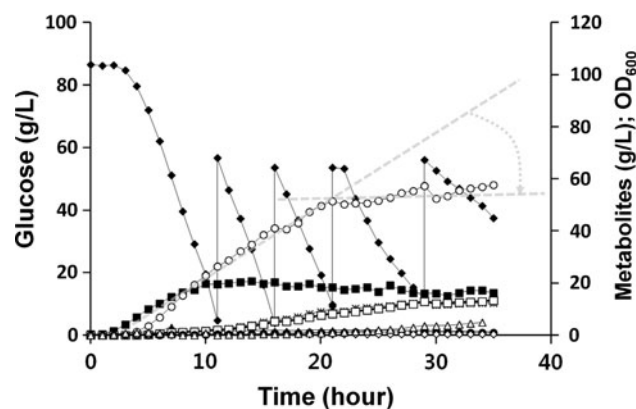


Fig. 5 Fed-batch fermentation with constant agitation speed of 450 rpm by *Klebsiella oxytoca* Δ ldhA Δ pf1B strain. Fed-batch fermentation was performed with a working volume of 3 L at 37 °C. The aeration rate was maintained at 1 vvm; pH was controlled at 6.5 by the automatic feeding of NH_4OH . Dotted line represents the trend of 2,3-butanediol (2,3-BD) production. Symbols in fermentation profiles indicate glucose concentration (filled diamonds), 2,3-BD (open circles), optical density at 600 nm (OD_{600}) (filled squares), acetoin (open squares), ethanol (filled triangles), succinic acid (asterisks), lactic acid (filled circles), formic acid (open diamonds), and acetic acid (open triangles)

oxygen, is known as a toxic metabolite for cellular viability [31]. Accordingly, it is assumed that the disruption of capability for 2,3-BD production was caused by acetoin toxicity. The constant agitation speed of 450 rpm might supply excessive oxygen to inactive cells disrupted by acetoin toxicity. As discussed earlier, the excessive oxygen supply could activate respiration, releasing carbon dioxide as a carbon waste, which is not beneficial for efficient 2,3-BD production. Consequently, a fed-batch fermentation comprising a cell growth and a 2,3-BD production phase was carried out by switching the agitation speed to maintain the concentration of acetoin at <10 g/L and to achieve a high yield and productivity of 2,3-BD (Fig. 6).

In fed-batch fermentation, agitation speed was maintained at 450 rpm until acetoin concentration reached ~ 10 g/L, after which the agitation speed was switched to 350 rpm to decrease acetoin formation and to maintain the capability for 2,3-BD production. It was observed that the maximum concentration of 2,3-BD reached 113 g/L with a yield and productivity of 0.45 g/g and 2.1 g/L/h, respectively, in the fed-batch fermentation (Fig. 6). Results were 95.2 % (concentration), 55.2 % (yield), and 27.3 % (productivity) higher than those of the fed-batch fermentation with a constant agitation speed of 450 rpm. In particular, the final 2,3-BD yield from glucose achieved up to 90 % of the theoretical maximum yield (0.5 g/g glucose). Controlling the agitation speed during fed-batch fermentation

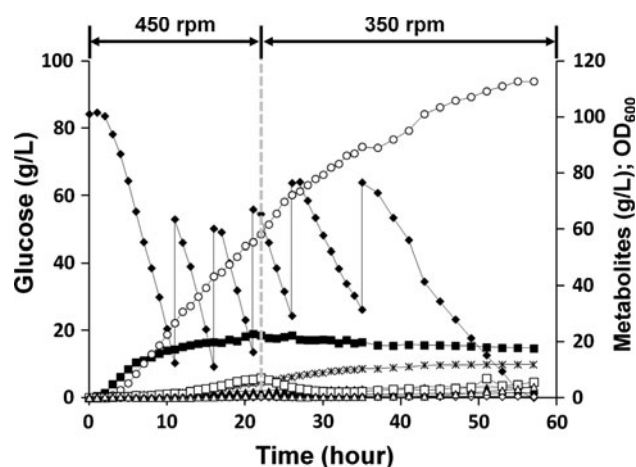


Fig. 6 Fed-batch fermentation by controlling agitation speed (450 \rightarrow 350 rpm) by *Klebsiella oxytoca* Δ ldhA Δ pf1B strain. Fed-batch fermentation was performed with a working volume of 3 L at 37 °C. Aeration rate was maintained at 1 vvm, and pH was controlled at 6.5 by the automatic feeding of NH_4OH . Agitation speed was maintained at 450 rpm until acetoin concentration was reached at about 10 g/L and then switched to 350 rpm. Symbols in fermentation profiles indicate glucose concentration (filled diamonds), 2,3-butanediol (2,3-BD) (open circles), optical density at 600 nm (OD_{600}) (filled squares), acetoin (open squares), ethanol (filled triangles), succinic acid (asterisks), lactic acid (filled circles), formic acid (open diamonds), and acetic acid (open triangles)

could not only significantly improve the final concentration and yield of 2,3-BD but also increase the productivity of 2,3-BD efficiently.

Conclusion

In order to improve the efficiency of *K. oxytoca* for the production of 2,3-BD and to improve its potential for industrial applications, a *K. oxytoca* Δ *ldhA* Δ *pflB* strain was developed based on in silico aided metabolic engineering. In silico analyses predicted that inactivation of *ldhA* encoding lactate dehydrogenase and of *pflB* encoding pyruvate formate lyase effectively reduced byproduct formation and enhanced 2,3-BD production. The mutant was successfully evaluated by a batch fermentation with a high yield but low 2,3-BD productivity compared with wild-type and *ldhA* knockout strains. In order to improve 2,3-BD productivity, the effect of agitation speeds from 150 to 450 rpm was investigated. Then, the fed-batch fermentation with constant agitation speed of 450 rpm was performed, first to examine the optimal time for converting the agitation speed. Subsequently, fed-batch fermentation by controlling the agitation speed was performed by switching the agitation speed from 450 to 350 rpm. Finally, the high concentration, yield, and productivity of 2,3-BD were achieved by fed-batch fermentation up to 113 g/L, 0.45 g/g, and 2.1 g/L/h, respectively. This is an example showing the effectiveness of system metabolic engineering for strain improvement and the importance of agitation speed during fermentation for 2,3-BD production. These results demonstrate one of the best concentration, yield, and productivity of 2,3-BD by fermentation of glucose with *K. oxytoca*, compared with values (e.g., 130 g/L, 0.48 g/g, and 1.64 g/L/h; Ji et al. [21]) previously reported [4, 9, 17, 21, 39]. The strategies of in silico aided metabolic engineering and fermentation process optimization could be applied to overcome the difficulties and hurdles of industrial application of *K. oxytoca*. Likewise, it is expected that the strategies employed in this study will apply to the development of industrial processes on high concentration, high yield, and high productivity of the other products.

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References

- Asadollahi MA, Maury J, Patil KR, Schalk M, Clark A, Nielsen J (2009) Enhancing sesquiterpene production in *Saccharomyces cerevisiae* through in silico driven metabolic engineering. *Metab Eng* 11:328–334
- Becker J, Wittmann C (2012) Systems and synthetic metabolic engineering for amino acid production—the heartbeat of industrial strain development. *Curr Opin Biotechnol* 23:718–726
- Becker J, Zelder O, Hafner S, Schroder H, Wittmann C (2011) From zero to hero—design-based systems metabolic engineering of *Corynebacterium glutamicum* for L-lysine production. *Metab Eng* 13:159–168
- Cao N, Xia Y, Gong CS, Tsao GT (1997) Production of 2,3-butanediol from pretreated corn cob by *Klebsiella oxytoca* in the presence of fungal cellulase. *Appl Biochem Biotechnol* 63–65:129–139
- Cassey B, Guest JR, Attwood MM (1998) Environmental control of pyruvate dehydrogenase complex expression in *Escherichia coli*. *FEMS Microbiol Lett* 159:325–329
- Celinska E, Grajek W (2009) Biotechnological production of 2,3-butanediol—current state and prospects. *Biotechnol Adv* 27:715–725
- Champluvier B, Decallonne J, Rouxhet PG (1989) Influence of sugar source (lactose, glucose, galactose) on 2,3-butanediol production by *Klebsiella oxytoca* NRRL-B199. *Arch Microbiol* 152:411–414
- Chen Z, Liu HJ, Zhang J-A, Liu DH (2009) Cell physiology and metabolic flux response of *Klebsiella pneumoniae* to aerobic conditions. *Process Biochem* 44:862–868
- Cho JH, Rathnasingh C, Song H, Chung BW, Lee HJ, Seung D (2012) Fermentation and evaluation of *Klebsiella pneumoniae* and *K. oxytoca* on the production of 2,3-butanediol. *Bioprocess Biosyst Eng* 35:1081–1088
- Choi HS, Lee SY, Kim TY, Woo HM (2010) In silico identification of gene amplification targets for improvement of lycopene production. *Appl Environ Microbiol* 76:3097–3105
- Edwards JS, Ramakrishna R, Schilling CH, Palsson BO (1999) Metabolic flux balance analysis. In: Lee SY, Papoutsakis ET (eds) *Metabolic engineering*. Marcel Dekker Inc., New York, pp 13–57
- Furusawa C, Horinouchi T, Hirasawa T, Shimizu H (2013) Systems metabolic engineering: the creation of microbial cell factories by rational metabolic design and evolution. *Adv Biochem Eng Biotechnol* 131:1–23
- Garg SK, Jain A (1995) Fermentative production of 2,3-butanediol: a review. *Bioresour Technol* 51:103–109
- Gombert AK, Nielsen J (2000) Mathematical modelling of metabolism. *Curr Opin Biotechnol* 11:180–186
- Hatti-Kaul R, Tornvall U, Gustafsson L, Borjesson P (2007) Industrial biotechnology for the production of bio-based chemicals—a cradle-to-grave perspective. *Trends Biotechnol* 25:119–124
- Jansen NB, Flickinger MC, Tsao GT (1984) Production of 2,3-butanediol from D-xylose by *Klebsiella oxytoca* ATCC 8724. *Biotechnol Bioeng* 26:362–369
- Ji XJ, Huang H, Du J, Zhu JG, Ren LJ, Hu N, Li S (2009) Enhanced 2,3-butanediol production by *Klebsiella oxytoca* using a two-stage agitation speed control strategy. *Bioresour Technol* 100:3410–3414
- Ji XJ, Huang H, Du J, Zhu JG, Ren LJ, Li S, Nie ZK (2009) Development of an industrial medium for economical 2,3-butanediol production through co-fermentation of glucose and xylose by *Klebsiella oxytoca*. *Bioresour Technol* 100:5214–5218
- Ji XJ, Huang H, Li S, Du J, Lian M (2008) Enhanced 2,3-butanediol production by altering the mixed acid fermentation pathway in *Klebsiella oxytoca*. *Biotechnol Lett* 30:731–734
- Ji XJ, Huang H, Ouyang PK (2011) Microbial 2,3-butanediol production: a state-of-the-art review. *Biotechnol Adv* 29:351–364
- Ji XJ, Huang H, Zhu JG, Ren LJ, Nie ZK, Du J, Li S (2010) Engineering *Klebsiella oxytoca* for efficient 2,3-butanediol

- production through insertional inactivation of acetaldehyde dehydrogenase gene. *Appl Microbiol Biotechnol* 85:1751–1758
22. Ji XJ, Nie ZK, Huang H, Ren LJ, Peng C, Ouyang PK (2011) Elimination of carbon catabolite repression in *Klebsiella oxytoca* for efficient 2,3-butanediol production from glucose-xylose mixtures. *Appl Microbiol Biotechnol* 89:1119–1125
 23. Jung YK, Kim TY, Park SJ, Lee SY (2010) Metabolic engineering of *Escherichia coli* for the production of polylactic acid and its copolymers. *Biotechnol Bioeng* 105:161–171
 24. Kim D-K, Rathnasingh C, Song H, Lee HJ, Seung D, Chang YK (2013) Metabolic engineering of a novel *Klebsiella oxytoca* strain for enhanced 2,3-butanediol production. *J Biosci Bioeng* 116:186–192
 25. Lee JW, Kim TY, Jang YS, Choi S, Lee SY (2011) Systems metabolic engineering for chemicals and materials. *Trends Biotechnol* 29:370–378
 26. Lee JW, Na D, Park JM, Lee J, Choi S, Lee SY (2012) Systems metabolic engineering of microorganisms for natural and non-natural chemicals. *Nat Chem Biol* 8:536–546
 27. Lee KH, Park JH, Kim TY, Kim HU, Lee SY (2007) Systems metabolic engineering of *Escherichia coli* for L-threonine production. *Mol Syst Biol* 3:149
 28. Mahadevan R, Schilling CH (2003) The effects of alternate optimal solutions in constraint-based genome-scale metabolic models. *Metab Eng* 5:264–276
 29. Ng CY, Jung MY, Lee J, Oh MK (2012) Production of 2,3-butanediol in *Saccharomyces cerevisiae* by in silico aided metabolic engineering. *Microb Cell Fact* 11:68
 30. Nie ZK, Ji XJ, Huang H, Du J, Li ZY, Qu L, Zhang Q, Ouyang PK (2011) An effective and simplified fed-batch strategy for improved 2,3-butanediol production by *Klebsiella oxytoca*. *Appl Biochem Biotechnol* 163:946–953
 31. Oliver JW, Machado IM, Yoneda H, Atsumi S (2013) Cyanobacterial conversion of carbon dioxide to 2,3-butanediol. *Proc Natl Acad Sci USA* 110:1249–1254
 32. Orth JD, Conrad TM, Na J, Lerman JA, Nam H, Feist AM, Palsson BO (2011) A comprehensive genome-scale reconstruction of *Escherichia coli* metabolism—2011. *Mol Syst Biol* 7:535
 33. Park JH, Lee KH, Kim TY, Lee SY (2007) Metabolic engineering of *Escherichia coli* for the production of L-valine based on transcriptome analysis and in silico gene knockout simulation. *Proc Natl Acad Sci USA* 104:7797–7802
 34. Park JH, Lee SY (2008) Towards systems metabolic engineering of microorganisms for amino acid production. *Curr Opin Biotechnol* 19:454–460
 35. Park JM, Kim TY, Lee SY (2009) Constraints-based genome-scale metabolic simulation for systems metabolic engineering. *Biotechnol Adv* 27:979–988
 36. Park JM, Kim TY, Lee SY (2010) Prediction of metabolic fluxes by incorporating genomic context and flux-converging pattern analyses. *Proc Natl Acad Sci USA* 107:14931–14936
 37. Park JM, Park HM, Kim WJ, Kim HU, Kim TY, Lee SY (2012) Flux variability scanning based on enforced objective flux for identifying gene amplification targets. *BMC Syst Biol* 6:106
 38. Park JM, Song H, Lee HJ, Seung D (2013) Genome-scale reconstruction and in silico analysis of *Klebsiella oxytoca* for 2,3-butanediol production. *Microb Cell Fact* 12:20
 39. Qureshi N, Cheryan M (1989) Production of 2,3-butanediol by *Klebsiella oxytoca*. *Appl Microbiol Biotechnol* 30:440–443
 40. Reed JL, Palsson BO (2004) Genome-scale in silico models of *E. coli* have multiple equivalent phenotypic states: assessment of correlated reaction subsets that comprise network states. *Genome Res* 14:1797–1805
 41. Schuetz R, Kuepfer L, Sauer U (2007) Systematic evaluation of objective functions for predicting intracellular fluxes in *Escherichia coli*. *Mol Syst Biol* 3:119
 42. Shin JH, Kim HU, Kim DI, Lee SY (2012) Production of bulk chemicals via novel metabolic pathways in microorganisms. *Biotechnol Adv*. doi:10.1016/j.biotechadv.2012.12.008
 43. Stephanopoulos G, Aristidou AA, Nielsen JH (1998) *Metabolic engineering: principles and methodologies*. Academic Press, San Diego
 44. Varma A, Palsson BO (1994) *Metabolic flux balancing: basic concepts, scientific and practical use*. *Biotechnology* 12:994–998
 45. Volch M, Jansen NB, Ladisch MR, Tsao GT, Narayan R, Rodwell VW (1985) 2,3-butanediol. In: Murray M-Y (ed) *Comprehensive Biotechnology*, vol 3, 1st edn. Pergamon Press, New York, pp 932–947
 46. Weusthuis RA, Lamot I, van der Oost J, Sanders JP (2011) Microbial production of bulk chemicals: development of anaerobic processes. *Trends Biotechnol* 29:153–158
 47. Yang G, Tian J, Li J (2007) Fermentation of 1,3-propanediol by a lactate deficient mutant of *Klebsiella oxytoca* under microaerobic conditions. *Appl Microbiol Biotechnol* 73:1017–1024
 48. Zeng AP, Sabra W (2011) Microbial production of diols as platform chemicals: recent progresses. *Curr Opin Biotechnol* 22:749–757