J Ind Microbiol Biotechnol (2015) 42:1419–1425
DOI 10.1007/s10295-015-1648-z

SHORT COMMUNICATION

Enhanced production of (*R*,*R*)-2,3-butanediol by metabolically engineered *Klebsiella oxytoca*

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Received: 29 April 2015 / Accepted: 22 June 2015 / Published online: 15 August 2015

Abstract Microbial fermentation produces a racemic mixture of 2,3-butanediol ((*R*,*R*)-BD, (*S*,*S*)-BD, and *meso*-BD), and the compositions and physiochemical properties vary from microorganism to microorganism. Although the *meso* form is much more difficult to transport and store because of its higher freezing point than those of the optically active forms, most microorganisms capable of producing 2,3-BD mainly yield *meso*-2,3-BD. Thus, we developed a metabolically engineered (*R*,*R*)-2,3-BD overproducing strain using a *Klebsiella oxytoca ΔldhA ΔpflB* strain, which shows an outstanding 2,3-BD production performance with more than 90 % of the *meso* form. A *budC* gene encoding 2,3-BD dehydrogenase in the *K. oxytoca ΔldhA ΔpflB* strain was replaced with an exogenous gene encoding (*R*,*R*)-2,3-BD dehydrogenase from *Paenibacillus polymyxa* (*K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain), and then its expression level was further amplified with using a pBBR1MCS plasmid. The fed-batch fermentation of the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain with intermittent glucose feeding allowed the production of 106.7 g/L of (*R*,*R*)-2,3-BD [*meso*-2,3-BD, 9.3 g/L], with a yield of 0.40 g/g and a productivity of 3.1 g/L/h, which should be useful for the industrial application of 2,3-BD.

Keywords *meso*-2,3-butanediol · (*R*,*R*)-2,3-butanediol · *Klebsiella oxytoca ΔldhA ΔpflB*

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Introduction

2,3-Butanediol (2,3-BD), one of the products of fermentation, is receiving increasing attention as a promising platform compound due to its extensive industrial applications. The dehydration of 2,3-BD yields 1,3-butadiene, a substance used extensively in synthetic rubber production [2, 8]. The other dehydration product of 2,3-BD, methyl ethyl ketone, serves as an effective fuel additive as well as an industrial solvent for resins and lacquers [2, 8, 28, 32]. 2,3-BD can also be dehydrogenated readily to acetoin and diacetyl, both used as flavoring agents for food [1]. Despite its great potential for industrial applications, the use of 2,3-BD itself is very limited by the isomer type. 2,3-BD exists in three stereoisomers, the *meso*-, (*R*,*R*)-, and (*S*,*S*)-forms, and each stereoisomer has different physiochemical properties [4, 5, 17]. For example, the freezing point of (*R*,*R*)-2,3-BD is lower than −30 °C but the freezing point of *meso*-2,3-BD is higher than 10 °C in around 50 % 2,3-BD content [4, 5, 17]. This makes the transportation and storage of *meso*-2,3-BD difficult for its industrial applications. *meso*- and (*R*,*R*)-2,3-BD have similar applicability potential in chemical industry sectors, such as 1,3-butadiene, methyl ethyl ketone, printing inks, spandex, and softening agents [2, 8, 32]. However, their applications are strongly limited to some special sectors by their physiochemical properties. For example, only (*R*,*R*)-2,3-BD has an applicability in agricultural industry, which is to activate plants' own defense systems against diseases, drought, and the elements [25, 26]. In addition, (*R*,*R*)-2,3-BD can be used as an antifreeze agent due to its low freezing point [7]. On the other hand, the physiochemical activity of (*R*,*R*)-2,3-BD might restrict its application to cosmetics and personal cares.

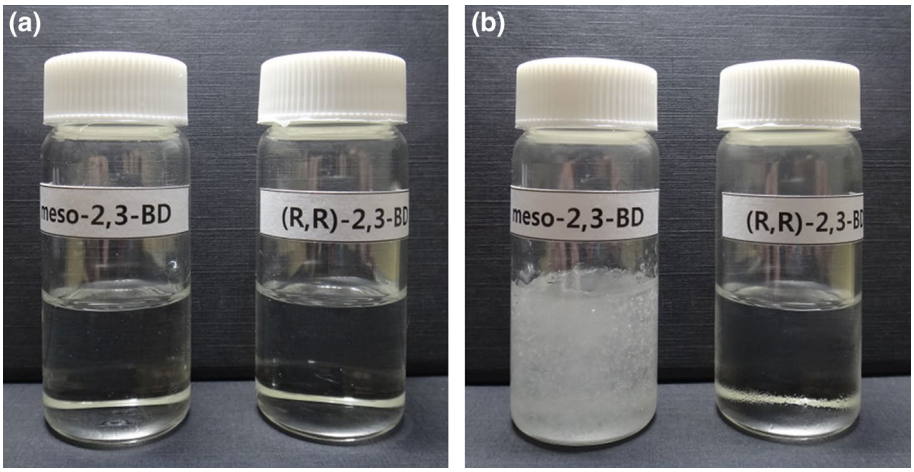

Fig. 1 The property of freezing for the (*R*,*R*)-2,3-BD-rich and *meso*-2,3-BD-rich solution of high purity (>95 %) at **a** 26 °C and **b** 5 °C. In the normal temperature on cold weather, the *meso*-2,3-BD-rich solution can be more often frozen than (*R*,*R*)-2,3-BD-rich solution. This property of *meso*-2,3-BD can bring about the difficulties for transportation and storage in industrial processes

Many bacterial species have an ability to synthesize 2,3-BD as a fermentation product via homologous pathways with three enzymes: acetolactate synthase, acetolactate decarboxylase, and 2,3-BD dehydrogenase [2, 3, 8, 10, 12, 13, 24, 28, 29, 31, 32]. However, the production ratio of 2,3-BD stereoisomers varies considerably depending on the bacterial species. *Klebsiella oxytoca*, *K. pneumoniae*, *Enterobacter aerogenes*, and *Serratia marcescens* produce mainly *meso*-2,3-BD, while *Bacillus subtilis* yields *meso*- and (*R*,*R*)-2,3-BD concurrently [2, 33]. In *Panebacillus polymyxa*, (*R*,*R*)-2,3-BD is produced as a major product making up a 98 % of the total weight of 2,3-BD [20, 31]. On the other hand, *Brevibacterium saccharolyticum* has (*S*,*S*)-2,3-BD dehydrogenase, which exclusively converts acetoin to (*S*,*S*)-2,3-BD [31].

We selected *K. oxytoca* strain as host strain for the overproduction of (*R*,*R*)-2,3-BD. To the best of our knowledge, the highest titer of 2,3-BD achieved by *P. polymyxa* is 111 g/L after 54 h fed-batch fermentation in a medium containing 60 g/L yeast extract [9]. However, *P. polymyxa* could produce only about 70 g/L (*R*,*R*)-2,3-BD in a medium containing 5 g/L yeast extract. Although *P. polymyxa* can produce (*R*,*R*)-2,3-BD with a high enantioselective purity, the overall productivity and cell density should be increased in a cheap medium containing corn steep liquor or whey for its industrial application. We improved *K. oxytoca* strain by genetic manipulation for (*R*,*R*)-2,3-BD production because the overall 2,3-BD productivity and cell growth were not much different in the medium containing yeast extract and corn steep liquor.

Klebsiella oxytoca has been considered a convincing candidate for the production of 2,3-BD because of its exceptional performance [2, 3, 8, 10–14, 24, 28, 32]. In order to enhance the production of 2,3-BD but reduce the formation of byproducts in *K. oxytoca*, many studies seeking strain improvement and fermentation optimization have been executed [10, 11, 13, 16, 23]. Recently, we developed a metabolically engineered *K. oxytoca* strain, in which *ldhA* and *pflB* genes were deleted (*K. oxytoca ΔldhA ΔpflB*), based on its in silico simulation using a genome-scale metabolic model of *K. oxytoca*, KoxGSC1457 [22, 23]. In the *K. oxytoca ΔldhA ΔpflB* strain, the overall 2,3-BD yield on glucose increased remarkably (0.45 g/g, 90 % of theoretical maximum yield). The final titer and productivity of 2,3-BD were achieved by the optimization of the fed-batch fermentation strategy up to 113 g/L and 2.1 g/L/h, respectively, in which more than 90 % of the total weight of 2,3-BD was *meso*-2,3-BD [23].

As expected, *meso*-2,3-BD-rich solution of high purity (>95 %) was shown to be more frequently frozen at normal cold weather temperatures than (*R*,*R*)-2,3-BD-rich solution (Fig. 1). Therefore, in this study, we introduced (*R*,*R*)-2,3-BD dehydrogenase from *P. polymyxa* into the *K. oxytoca ΔldhA ΔpflB* strain in order to widen the potential applications of 2,3-BD. The overexpression of (*R*,*R*)-2,3-BD dehydrogenase was conducted further to enhance (*R*,*R*)-2,3-BD production. The final engineered strain, *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH), could produce up to 106.7 g/L of (*R*,*R*)-2,3-BD together with 9.3 g/L of *meso*-2,3-BD in the fed-batch fermentation. The yield and productivity of (*R*,*R*)-2,3-BD were comparable to those of *meso*-2,3-BD obtained from the *K. oxytoca ΔldhA ΔpflB* strain.

Materials and methods

Bacterial strain and plasmid

The *K. oxytoca* KCTC12133BP strain (Korean Collection for Type Cultures, Daejeon, Korea) was used in this study. For overexpression of (*R*,*R*)-2,3-BD dehydrogenase, pBBR1MCS plasmid was used [19]. The pBBR1MCS plasmid contains a multiple cloning site,

mob gene, lacZα fragment, and chloramphenicol resistance gene.

Construction of recombinants for (*R*,*R*)-2,3-BD production

In-frame gene deletions and the replacement of an endogenous gene with an exogenous gene were carried out based on the *sacB* homologous recombination system using overlapped polymerase chain reaction (PCR) products. The overlapped products of the two fragments, in which each ~500 bp upstream and downstream regions of the target gene were amplified and overlapped by PCR, were transformed into the competent cells of the *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 with genome-specific primers.

The pBBR-PBDH plasmid (pBBR1MCS containing the gene encoding (*R*,*R*)-2,3-BD dehydrogenase from *P. polymyxa*) was also transformed into the competent cells of the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain by electroporation. Then, the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain was selected in LB chloramphenicol (25 mg/L) plates at 37 °C.

Culture medium

Culture medium used in batch and fed-batch fermentations contained (per liter) yeast extract (Becton–Dickinson, Le Pont de Claix, France), 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $(NH_4)_2SO_4$, 6.6 g; K_2HPO_4, 8.7 g; KH_2PO_4, 6.8 g; trace metal solution, 10 mL. The trace metal solution contained (per liter) $FeSO_4 \cdot 7H_2O$, 5 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; $MnSO_4 \cdot H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; HCl, 10 mL.

Culture conditions

For inoculum preparation, the suspended cells from single colonies on LB agar (Difco Laboratories, Detroit, MI) plates were precultured in 20 mL test tubes containing 5 mL culture medium (10 g/L D-glucose) for 5 h. 1 mL of the preculture was then 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 (OD_{600}). The tube and flask cultivations were conducted in a rotary shaker at 150 rpm and at 37 °C (JEIO Tech. Co. SI-900R). 300 mL of the seed culture was then transferred to a 5 L bioreactor. Batch fermentations were performed in a 5 L BIOFLO®& CELLIGEN®310 bioreactor (New Brunswick. Scientific Co., Edison, NJ) containing 3 L culture medium (90 g/L D-glucose). The bioreactor was continuously aerated through a 0.2-μm membrane filter at a flow rate of 1 vvm (air volume/working volume/minute). The temperature was maintained at 37 °C. The pH was controlled at 6.5 ± 0.1 by the automatic feeding of NH_4OH (28 % vol/vol). Foaming was controlled by the addition of Antifoam 289 (Sigma, St. Louis, MO). As needed, chloramphenicol was added at 25 mg/L in the medium. 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 the 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 then switched to 350 rpm [23]. Samples were periodically taken for the measurement of OD_{600} as well as for the determination of metabolite concentrations. After centrifugation at 13,200×*g* for 5 min, the resulting supernatant was used to measure the concentrations of glucose and metabolites.

Analytical procedures

(*R*,*R*)-2,3-BD, *meso*-2,3-BD, and (*S*,*S*)-2,3-BD were determined by a gas chromatograph with flame ionization detector (GC-FID; HP 6890 series, Hewlett Packard, Palo Alto, CA, USA) equipped with a HP-chiral 20ß column (30 m, 0.32-mm internal diameter, 0.25-μm film thickness; Agilent Technologies, Waldbronn, Germany). The oven temperature was initially set at 40 °C for 5 min. It was increased with a gradient of 15 °C/min until it reached 160 °C, at which it was kept for the final 2 min. The temperature of the injector and detector was set at 230 °C. Argon was used as the carrier gas and run through the column at a flow rate of 2 mL/min. The sample injection volume was 0.2 μL.

The concentrations of D-glucose and metabolites, including *meso*-2,3-BD, (*R*,*R*)-2,3-BD, formic acid, ethanol, acetic acid, lactic acid, succinic acid, and acetoin, were also determined by a high-performance liquid chromatography (HPLC) equipped with UV/VIS and RI detectors (Agilent 1260 series, Agilent Technologies). An Aminiex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Hercules, CA) was isocratically eluted with 0.01 N H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min and at 80 °C. The OD_{600} was measured using UV–Vis spectrophotometry (DR5000, Hach Company, CO) to monitor cell growth. Cell concentration, defined as dry cell weight (DCW) per liter of culture broth, was calculated from the pre-determined standard curve relating OD_{600} to DCW (1 OD_{600} = 0.3877 ± 0136 g DCW/L) [16].

Results and discussion

Strain development for the production of (*R*,*R*)-2,3-BD based on *K. oxytoca ΔldhA ΔpflB*

We used the *K. oxytoca ΔldhA ΔpflB* strain as a base strain for the development of a novel strain capable of (*R*,*R*)-2,3-BD production. In previous work, the metabolic fluxes toward 2,3-BD were maximized, while the metabolic fluxes of byproduct production were minimized in the *K. oxytoca ΔldhA ΔpflB* strain [23]. A 2,3-BD dehydrogenase encoded by the *budC* gene in *K. oxytoca* catalyzes the formation of *meso*-2,3-BD from acetoin. However, *P. polymyxa* yields mostly (*R*,*R*)-2,3-BD (>98 % of total 2,3-BD) [31]. Consequently, the native *budC* gene in the *K. oxytoca ΔldhA ΔpflB* strain was deleted firstly by homologous recombination. Then, the gene encoding (*R*,*R*)-2,3-BD dehydrogenase from *P. polymyxa* was introduced into the chromosomal site of the deleted *budC* gene.

The batch and fed-batch fermentations of the constructed *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain were carried out to investigate its ability for (*R*,*R*)-2,3-BD production. The GC analysis showed that the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain could produce 2,3-BD with over 90 % (*R*,*R*)-2,3-BD (Fig. 2). However, the final titer, yield, and productivity of total 2,3-BD (88 g/L, 0.32 g/g, and 1.6 g/L/h) were significantly lower than those of the *K. oxytoca ΔldhA ΔpflB* strain (113 g/L, 0.45 g/g, and 2.1 g/L/h) under the same fermentation conditions (Fig. 3, [23]). It is most likely that the expression level of the introduced (*R*,*R*)-2,3-BD dehydrogenase was not high enough to completely metabolize the reinforced metabolic fluxes from pyruvate toward 2,3-BD synthesis via metabolic engineering in the *K. oxytoca ΔldhA ΔpflB* strain. Based on the observation that *K. oxytoca ΔldhA ΔpflB* strain and the engineered final strain produced similar amount of total 2,3-BD (113 and 116 g/L), the properties of 2,3-BD dehydrogenase like cofactor specificity and kinetics seem not much different from *K. oxytoca* and *P. polymyxa*. This can be supported by the observation that acetoin, the direct precursor of 2,3-BD, accumulated more in the fed-batch fermentation of the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain (15.5 g/L) than that of the *K. oxytoca ΔldhA ΔpflB* strain (4.7 g/L, [23]).

Gene overexpression for the enhancement of (*R*,*R*)-2,3-BD production

In order to solve the metabolic interruption, we attempted to implement overexpression of (*R*,*R*)-2,3-BD dehydrogenase using a pBBR1MCS plasmid [19] in the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain. The gene encoding (*R*,*R*)-2,3-BD dehydrogenase from *P. polymyxa* was cloned into the multiple cloning site in the pBBR1MCS plasmid, and then the cloned plasmid was transferred into the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain by electroporation. Thus, the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain was constructed.

Fed-batch fermentation using the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain was then performed to evaluate the performance of (*R*,*R*)-2,3-BD production. The final titer, yield, and productivity of total 2,3-BD were reached up to 116 g/L of total 2,3-BD [(*R*,*R*)-2,3-BD, 106.7 g/L; *meso*-2,3-BD, 9.3 g/L], 0.40 g/g, and 3.1 g/L/h, respectively (Fig. 3). The results were 31.8 % (concentration), 25 % (yield), and 93.8 % (productivity) higher than those obtained from the fed-batch fermentation of the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain. The formation of acetoin also decreased from 15.5 g/L in the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain to 8.9 g/L in the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain. These results strongly support the assumption that the expression level of (*R*,*R*)-2,3-BD dehydrogenase was not enough to completely metabolize acetoin accumulated by reinforced metabolic fluxes from pyruvate by means of the deletion of the *ldhA* and *pflB* genes. This caused the interruption of the metabolic fluxes originated from the consumption of glucose and glycolysis. Consequently, the cell growth rate and 2,3-BD production rate of *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain were also decreased (Fig. 3). The interrupted metabolic fluxes were solved by improving the expression level of (*R*,*R*)-2,3-BD dehydrogenase. The *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain metabolized acetoin to 2,3-BD more intensively without the significant metabolic bottleneck seen in the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strain. With the efficient consumption of glucose, the cell growth and 2,3-BD production rates were increased in the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain.

The *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain excreted 8.9 g/L of acetoin into culture medium (Fig. 3). The reaction of 2,3-BD dehydrogenase is a reversible reaction, which could be mainly controlled by NADH/NAD ratio because 1 mol NADH per reaction is required to convert 1 mol acetoin to 1 mol 2,3-BD. In general, the metabolism involved in the supply of NADH (e.g., TCA cycle) slows down during the stationary phase of fermentation which leads to the limited availability of NADH [18, 21]. Thus, it is a reasonable to assume that the accumulation of acetoin resulted from the NADH-limited reaction of acetoin to 2,3-BD [6].

The *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain still formed a significant level of

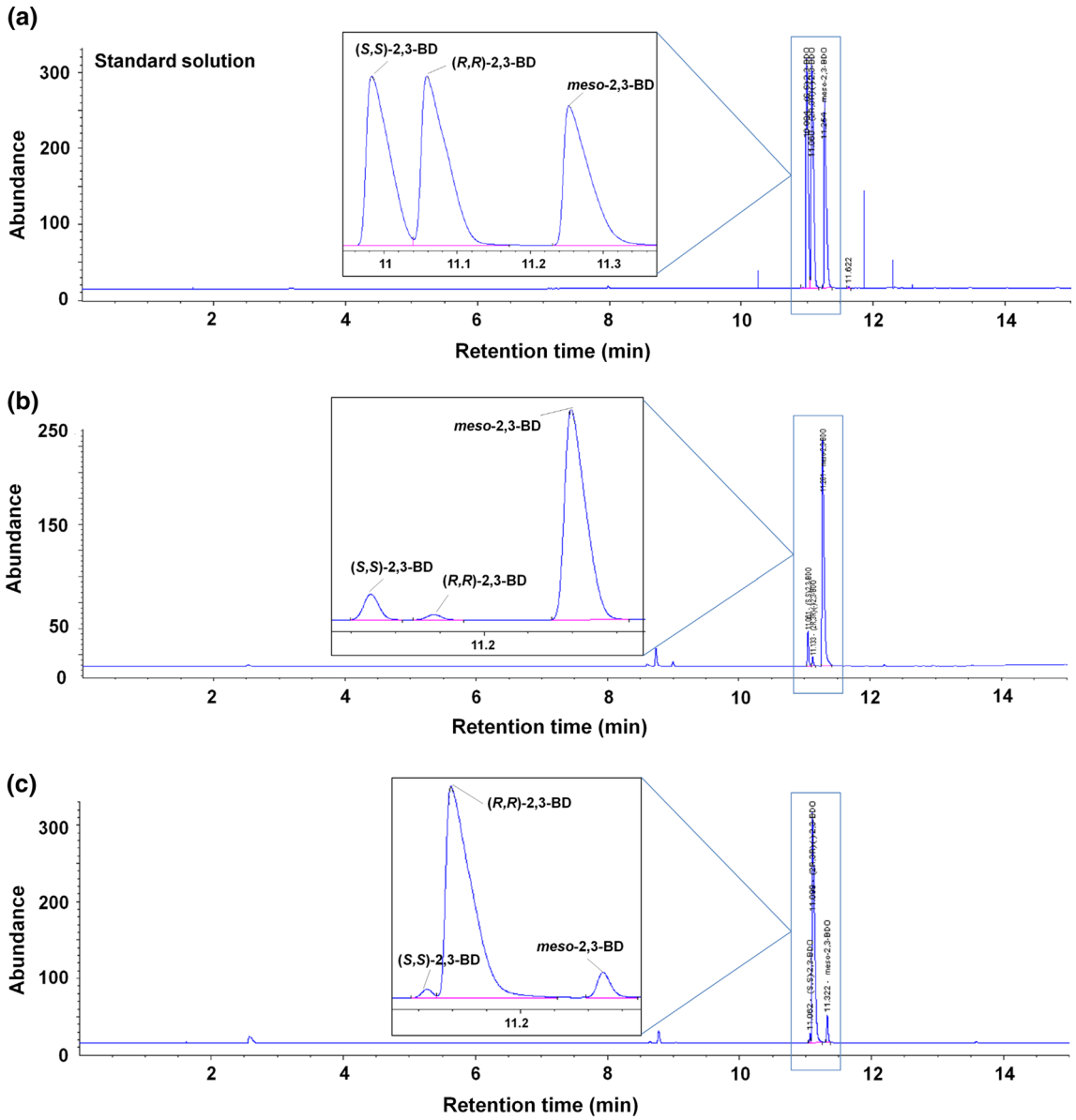

Fig. 2 GC analysis results of **a** standard solution and fermentation samples from **b** *K. oxytoca ΔldhA ΔpflB* and **c** *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strains. (*R*,*R*)-2,3-BD, *meso*-2,3-BD, and (*S*,*S*)-2,3-BD were determined by a gas chromatograph with flame ionization detector (GC-FID; HP 6890 series, Hewlett Packard, Palo Alto, CA, USA) equipped with a HP-chiral 20ß column (30 m, 0.32-mm internal diameter, 0.25-μm film thickness; Agilent Technologies, Waldbronn, Germany). The GC analysis showed that the *K. oxytoca ΔldhA ΔpflB* and *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH strains could produce 2,3-BD with over 90 % *meso*-2,3-BD and over 90 % (*R*,*R*)-2,3-BD, respectively

meso-2,3-BD (9.3 g/L). Although the reaction of 2,3-BD dehydrogenase encoded by the *budC* gene has been known as a major route to produce *meso*-2,3-BD in *K. oxytoca*, several other pathways for the formation of *meso*-2,3-BD were suggested. The pathways include spontaneous reaction (acetoin to diacetyl), non-specific reactions by other dehydrogenases, and cyclic pathways [12, 15, 27]. Yang et al. recently reported that the *budC*-deleted *K. oxytoca* strain produced large amount of acetoin, but this strain still formed a high level of 2,3-BD via the alternative pathways [30].

From the total quantity of 2,3-BD produced, the *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strain yields over 92 % (*R*,*R*)-2,3-BD, thus helping to overcome difficulties in industrial applications whereby transportation and storage of 2,3-BD results in high freezing of *meso*-2,3-BD in cold weather. Furthermore, the high concentration, high yield, and high productivity of the (*R*,*R*)-2,3-BD

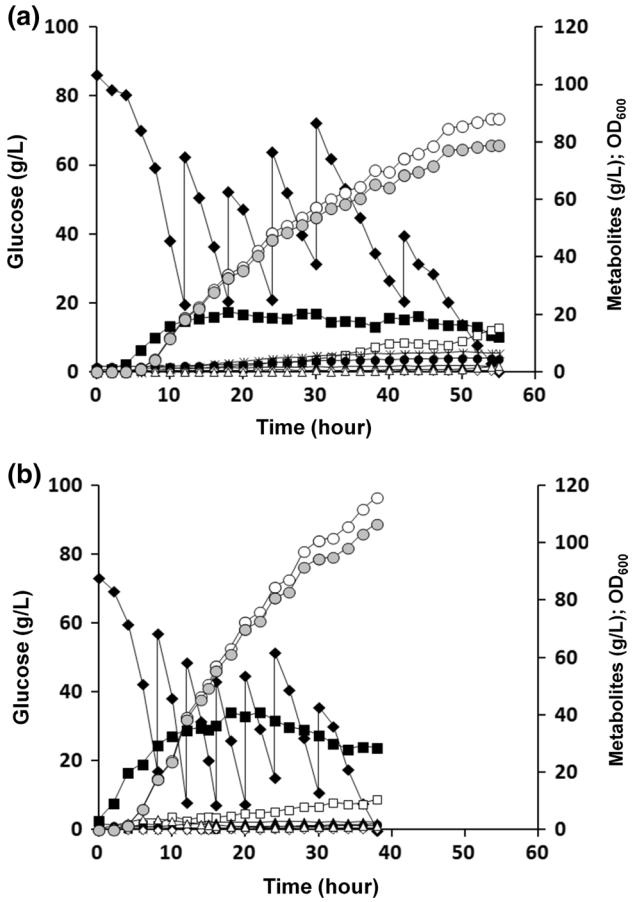

Fig. 3 Fed-batch fermentation by **a** *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH and **b** *K. oxytoca ΔldhA ΔpflB ΔbudC*::PBDH (pBBR-PBDH) strains. Fed-batch fermentation was performed with a working volume of 3 L at 37 °C. The aeration rate was maintained at 1 vvm, and pH was controlled at 6.5 by the automatic feeding of NH_4OH. For the fed-batch fermentation, the agitation speed was maintained at 450 rpm until the concentration of acetoin was reached at about 10 g/L, and then switched to 350 rpm. The *symbols* in fermentation profiles indicate OD_{600} (*filled square*) and the concentration of glucose (*filled diamond*), total 2,3-BD (*open circle*), (*R*,*R*)-2,3-BD (*filled gray circle*), acetoin (*open square*), ethanol (*filled triangle*), succinic acid (*asterisk*), lactic acid (*filled circle*), formic acid (*open diamond*), and acetic acid (*open triangle*)

production system may be widely applied in the agricultural and gardening sectors as an eco-friendly alternative.

Acknowledgments This work was supported by the Industrial Strategic Technology Development Program (No. 10050407) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea).

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