## Enantioselective synthesis of pure (R,R)-2,3-butanediol in *Escherichia coli* with stereospecific secondary alcohol dehydrogenases<sup>†</sup>

Yajun Yan, Chia-Chi Lee and James C. Liao\*

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We characterized the activity and stereospecificity of four secondary alcohol dehydrogenases (sADHs) towards acetoin reduction and constructed synthetic pathways in *E. coli* to produce enantiomerically pure (R,R)-2,3-butanediol (2,3-BDO) from glucose with a titer of 6.1 g/L (enantio purity >99%), and yield of 0.31 g product/g glucose (62% of theoretical maximum).

Synthesis of high-value pharmaceutical or agricultural compounds often requires enantiomerically pure molecules, such as secondary alcohols, as the important building blocks. However, chiral synthesis or separation remains a costly step in chemical synthesis. As an alternative, using enzymes or whole living cells provides an effective approach to synthesize compounds with high enantiomeric purity.<sup>1-4</sup> In this study, we demonstrated chiral synthesis of (*R*, *R*)-2,3-butanediol (2,3-BDO) from glucose with an engineered pathway in *Escherichia coli*.

2,3-BDO contains two stereo centers and has three stereo isomers including (R,R)-, *meso*- and (S,S)-forms. The optically active isomers can be used as antifreeze agents. 2,3-BDO can also be converted to 1,3-butandiene with established chemical processes in the rubber industry. Moreover, dehydration of 2,3-BDO generates 2-butanone, which is an effective liquid fuel additive.<sup>5</sup> In general, 2,3-BDO can be naturally produced by several microorganisms as a mixture of (R,R)- and *meso*-2,3 BDO. *Bacillus* and *Klebsiella* are two native producers that have potential for industrial applications in 2,3-BDO production. However, the ratio of produced 2,3-BDO stereo isomers can vary dramatically depending on the microorganisms and fermentation conditions (Table 1).

There are several possible explanations for the mixed formation of 2,3-BDO isomers, including aeration conditions (redox balance), non-stereospecific dehydrogenases, multiple pathways, and multiple stereospecific dehydrogenases.<sup>5</sup> Ui *et al.* introduced gene fragments containing genes encoding acetolactate synthase (ALS), acetolactate decarboxylase (ALDC) and a single *meso*dehydrogenase into *E. coli*, and obtained production of enantiomerically pure *meso*-2,3-BDO<sup>6</sup> (Table 1) which indicated that mixed formation of 2,3-BDO is mainly due to the existence of multiple pathways or dehydrogenases. Recently, (*S,S*)-2,3BDO was also produced using engineered *E. coli* by feeding diacetyl as precusor<sup>7</sup> (Table 1). However, no enantiomerically pure (R,R)-2,3-BDO production was achieved with microbial fermentation. In this study, we characterized the activity and stereospecificity of four sADHs towards (*R*)-acetoin reduction and found that three of them produce specifically (R,R)-2,3-BDO, while one produces *meso*-2,3-BDO. Using these enzymes, we further constructed a synthetic pathway in *E. coli* to produce enantiomerically pure (R,R)- or *meso*-2,3-BDO from glucose. To our knowledge, production of pure (R,R)-form from glucose has not been achieved previously (Table 1).

The 2,3-BDO biosynthesis pathway (Fig. 1) involves the decarboxylation of acetolactate by ALDC to form acetoin, which is stereospecific and only leads to the formation of the (*R*)-enantiomer.<sup>8</sup> When the ketone group of acetoin was reduced to a hydroxyl group by sADH, either (*S*)-configuration or (*R*)-configuration is formed depending on the enzyme stereospecificity. This step creates the second stereo-center and yields either *meso*-2,3-BDO or (*R*,*R*)-2,3-BDO. Production of enantiomerically pure



Fig. 1 Schematic illustration of the 2,3-BDO biosynthesis pathway. ALS: acetolactate synthase; ALDC: acetolactate decarboxylase; sADH: secondary alcohol dehydrogenase.

Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, 5531, Boelter Hall, 420 Westwood Plaza, Los Angeles, CA 90095, USA. E-mail: Liaoj@ucla.edu; Fax: 310-206-4107; Tel: 310-205-1656

<sup>†</sup> Electronic supplementary information (ESI) available: *B. subtilis alsS* cDNA, *B. subtilis alsD* cDNA, *B. subtilis bdhA* cDNA, *K. pneumoniae* MGH78578 *budC* cDNA, *C. beijerinckii adh* cDNA and *T. brockii adh* cDNA sequences. See DOI: 10.1039/b913501d

	Organism	sADH gene	Precursor	Isomer	Titer(g/L)	Enantio purity	Reference
native producer	B. cereus	Native	glucose	meso-	0.3	60%	
	YUF-4		8	(R,R-)	0.2	40%	13
	K. pneumoniae	Native	glucose	meso-	21.2	74%	
	IAM 1063		e	(S,S)-	1.6	6%	10
				$(\vec{R},\vec{R})$ -	6.0	20%	
	E. coli	K. pneumoniae IAM 1063 budC	glucose	meso-	17.7	98%	6
non-native producer	E. coli	B. saccharolyticum C-1012 bdh	diacetyl	(S,S)-	2.2	98%	7
	E. coli	B. subtilis bdhA	glucose	(R, R)-	5.8	99%	This study
	E. coli	C. beijerinckii adh	glucose	(R, R)-	5.1	99%	This study
	E. coli	T. brockii adh	glucose	( <i>R</i> , <i>R</i> )-	6.1	99%	This study

Table 1 Previous studies on enantioselective biosynthesis of stereo isomers of 2,3-BDO

2,3-BDO requires the use of a sADH with high stereospecifity. In 2,3-BDO native producers, the presence of multiple sADHs with inconsistent stereospecificity contributes to formation of a mixture of stereo isomers of 2,3-BDO. Therefore, reconstruction of the 2,3-BDO biosynthesis pathway in a non-native host with expression of only single sADH was expected to allow for the synthesis of enantiomerically pure 2,3-BDO.

In order to obtain such a sADH, candidate sADH genes (the sequences provided in the ESI<sup>†</sup>) were amplified from the genomic DNA of 2,3-BDO native producer Bacillus subtilis and Klebsiella pneumoniae MGH78578, since B. subtilis and K. pneumoniae were reported to be able to produce (R,R)-form and meso-form 2,3-BDO, respectively.<sup>9,10</sup> The genes bdhA from B. subtilis and budC from K. pneumoniae were cloned. In addition, two adh genes (Thermoanaerobacter brockii adh and Clostridium beijerinckii adh) were completely synthesized with codon optimized for expression in E. coli11 (Epoch Biolabs, Missouri City, TX). For protein overexpression and purification, the amplified genes were inserted into vector pETDuet-1 (Novagen, Madison, WI) with His-tag at the N-terminal, generating expression plasmids pET-bdhA, pET-budC, pET-CBADH, pET-TBADH. The proteins were expressed in the E. coli strain BL21(DE3) (Invitrogen, Carlsbad, CA) harboring the constructed plasmids. The proteins were purified with Ni-NTA spin columns (Qiagen, Valencia, CA). The protein concentration was measured by Bradford assay (Bio-Rad, Hercules, CA). Dehydrogenase activity was measured by monitoring the absorbance decrease of NADH or NADPH at wavelength 340 nm. To determine the kinetic parameters, the assay reaction was prepared with Tris-HCl buffer (50 mM, pH = 8.0) containing 100 µM of NADH or NADPH and various concentration of acetoin ranging from 10 µM to 20 mM at room temperature. The Km and Kcat values were obtained by non-linear fitting with the Michaelis-Menten equation.

The sADHs from *B. subtilis bdhA* and *T. brockii adh* demonstrated similar activities ( $K_{cat} = 0.98 \text{ s}^{-1}$  and 0.91 s<sup>-1</sup>, respectively) towards acetoin with high affinities ( $K_m = 0.26 \text{ mM}$  and 0.23 mM, respectively), although the latter enzyme was not previously reported to reduce acetoin. On the other hand, the enzyme coded by *C. beijerinckii adh* has a low affinity towards acetoin ( $K_m = 8.3 \text{ mM}$ ) although activity is relatively high ( $K_{cat} = 8.2 \text{ s}^{-1}$ ). The enzyme encoded by *budC* from *K. pneumoniae* shows the highest activity ( $K_{cat} = 58\text{ s}^{-1}$ ) despite having a slightly lower affinity ( $K_m = 0.85 \text{ mM}$ ) (Table 2).

 Table 2
 Kinetic parameters of secondary alcohol dehydrogenases on substrate acetoin

Organism	Gene	Cofactor	$K_m(\mathrm{mM})$	$K_{cat}(\mathbf{s}^{-1})$	$\frac{K_{cat}/K_m}{(\mathrm{m}\mathrm{M}^{-1}.\mathrm{s}^{-1})}$
B. subtilis C. beijerinkii T. brockii K. pneumoniae	bdhA adh adh budC	NADH NADPH NADPH NADH	$\begin{array}{c} 0.26 \pm 0.02 \\ 8.3 \pm 0.3 \\ 0.23 \pm 0.02 \\ 0.85 \pm 0.04 \end{array}$	$\begin{array}{c} 0.98 \pm 0.05 \\ 8.2 \pm 0.3 \\ 0.91 \pm 0.06 \\ 58 \pm 3 \end{array}$	3.8 0.99 4.0 68

In order to determine the stereospecificity of these sADHs, enantiomerically pure (R)-acetoin was used for the enzyme assay. (R)-Acetoin was prepared by culturing E. coli that overexpressed B. subtilis alsS (encoding ALS) and alsD (encoding ALDC) in M9 glucose medium containing 4% glucose. The assay condition was as described above with (R)-acetoin as the substrate at a concentration of 5 mM. After 5 min, the reaction mixture was analyzed with GC-FID (Hewlett Packard) equipped with a HP-chiral 20β column (30 m, 0.32-mm internal diameter, 0.25-μm film thickness; Agilent Technologies). For the analysis, the GC oven temperature was initially set at 40 °C for 2 min, increased with a gradient of 5 °C min<sup>-1</sup> until 45 °C, and held for 4 min. Then it was increased with a gradient 15 °C min<sup>-1</sup> until 230 °C and held for 4 min. Helium was used as the carrier gas. The temperature of the injector and detector were set at 225 °C. The stereo isomers were identified by the standard (R,R)-2,3-BDO, meso-2,3-BDO and (S,S)-2,3-BDO purchased from Sigma-Aldrich. As the results show in Fig. 2(A-E), all four tested sADHs demonstrated strict stereospecificity on (R)-acetoin reduction. Among them, the gene products of B. subtilis bdhA, C. beijerinckii adh and T. brockii adh exclusively catalyzed the formation of (R,R)-2,3-BDO, while the gene product of K. pneumoniae MGH78578 budC exclusively catalyzed the production of meso-2,3-BDO. To our knowledge, the stereospecificity of these enzymes have not been characterized previously for the synthesis of 2,3-BDO isomers, and the gene product of bdhA is the first sADH characterized from B. subtilis that is responsible for 2,3-BDO formation.

With the characterized sADHs, we further engineered *E. coli* as a whole-cell biocatalyst to produce enantiomerically pure 2,3-BDO from glucose. Vector pZE12-Luc was utilized to carry each of the synthetic operons consisting of the 2,3-BDO biosynthesis pathway with different sADHs.<sup>11</sup> The synthetic operons included three structural genes: *alsS* and *alsD* from *B. subtilis* 



**Fig. 2** Identification of 2,3-BDO isomers by GC-FID. A and F: 2,3-BDO isomer standards; B: Assay product of *bdhA* from *B. subtilis*; C: Assay product of *adh* from *C. beijerinckii*; D: Assay product of *adh* from *T. brockii*; E: Assay product of *budC* from *K. pneumoniae* MGH78578; G: Fermentation product with *E. coli* expressing *B. subtilis bdhA* in the pathway; H: Fermentation product with *E. coli* expressing *C. beijerinckii adh* in the pathway; I: Fermentation product with *E. coli* expressing *K. pneumoniae* MGH78578 budC in the pathway. Peak 1: (*R*,*R*)-2,3-BDO; peak 2: (*S*,*S*)-2,3-BDO; peak 3: *meso*-2,3-BDO; peak 4: an unknown component from culture medium.

encoding ALS and ALDC, respectively, and the four sADHs characterized above, each in a separate plasmid. The operons were regulated by a pLlacO1 promoter with separated RBS located upstream of each structure gene to facilitate the translation. To construct these plasmids, the genes alsS and alsD were first amplified from B. subtilis genomic DNA. These two genes were integrated together by splicing overlap extension PCR with the introduction of RBS sequence (AGGAGATATACC) in front of alsD. Various sADH genes were inserted downstream of alsD between Sall and BamHI. The generated plasmids carrying four synthetic operons were named pZE12-alsS-alsD-bdhA, pZE12alsS-alsD-CBADH, pZE12-alsS-alsD-TBADH, and pZE12-alsSalsD-BudC (the plasmid construct is provided in the ESI<sup>†</sup>). The constructed plasmids were then transformed into E. coli strains respectively. The transformants were tested for the production of enantiomeric 2,3-BDO through fermentation.

To test the production of 2,3-BDO, 1 ml of seed culture was prepared in LB medium containing  $50\mu g/L$  of ampicillin and grown overnight at 37 °C inside a shaker set at 250 rpm. After overnight incubation, the culture was inoculated (1% vol/vol) into 10 ml of M9 medium containing 4% glucose, 0.5% yeast extract, 1 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 2 × 10<sup>-4</sup>% thiamine, and 50 µg/L ampicillin with a pH value at around 7.0. After growing

at 37 °C for 3 hours, IPTG was added into the culture to a final concentration of 0.2 mM to induce protein expression. Then the fermentation was carried out at 30 °C with rigorous shaking (250 rpm). The samples were taken after 48 hours and analyzed with GC-FID. The analytic method was as described above. The stereo isomers were identified and quantified by the standard (R,R)-2,3-BDO, *meso*-2,3-BDO and (S,S)-2,3-BDO purchased from Sigma-Aldrich. Glucose consumption was measured using a glucose analyzer (YSI Inc., OH).

The wild type *E. coli* JCL16 (BW25113/F'[*traD36, proAB+, lacIq ZDM15*]) <sup>12</sup> carrying the operon with *budC* was able to produce 4.5 g/L *meso-2,3-BDO* with only a trace amount of the (*R,R*)- form from 17.1 g/L of glucose consumed. Since the theoretical maximum yield of 2,3-BDO from glucose is 0.5 (g product/g glucose consumed), we accomplished 53% of the theoretical maximum. When *budC* from *K. pneumoniae* MGH78578 was replaced by *bhdA* from *B. subtilis,* only (*R,R*)-2,3-BDO was detected as the exclusive product (Fig. 2(F–J)). The titer was around 2.2 g/L with the yield of 0.16 g product/g glucose (32% of theoretical maximum). When the operons containing either *T. brockii adh* or *C. beijerinckii adh* were expressed in wild type *E. coli* JCL16, (*R,R*)-2,3-BDO was produced exclusively with titers of 1.5 g/L and 2.8 g/L, respectively, with the yields of 0.12 g

sADH gene	Glucose consumption (g/L)	( <i>R</i> , <i>R</i> )-2,3-BDO (g/L)	meso-2,3-BDO (g/L)	Enantio purity	Yield (g/g glucose)	
B. subtilis bdhA	$13.8 \pm 0.6$	$2.2 \pm 0.2$	<0.01	>99%	0.16	
C.beijerinckii adh	$12.8 \pm 0.4$	$1.5 \pm 0.1$	< 0.01	>99%	0.12	
T. brockii adh	$15.2 \pm 0.7$	$2.8 \pm 0.3$	< 0.01	>99%	0.19	
K. pneumoniae budC	17.1 ±1.1	< 0.04	$4.5 \pm 0.4$	>99%	0.27	

Table 3 Enantiomeric 2,3-BDO production in wild type E. coli JCL16 with different sADHs

Table 4 Enantiomeric 2,3-BDO production in engineered E. coli JCL260 with different sADHs

sADH gene	Glucose consumption (g/L)	( <i>R</i> , <i>R</i> )-2,3-BDO (g/L)	meso-2,3-BDO (g/L)	Enantio purity	Yield (g/g glucose)
B. subtilis bdhA C.beijerinckii adh T.brockii adh K. pneumoniae budC	$\begin{array}{c} 18.9 \pm 0.5 \\ 17.8 \pm 0.8 \\ 19.7 \pm 0.7 \\ 29.6 \pm 2.8 \end{array}$	$5.8 \pm 0.3 \\ 5.1 \pm 0.4 \\ 6.1 \pm 0.5 \\ < 0.06$	< 0.03 < 0.03 < 0.03 $10 \pm 0.8$	>99% >99% >99% >99%	0.30 0.29 0.31 0.34

product/g glucose (24% of theoretical maximum) and 0.19 g product/g glucose (38% of theoretical maximum), respectively (Table 3).

In order to conserve the carbon source and reducing power (either NADH or NADPH) for 2,3-BDO synthesis, the previously created E. coli strain JCL260 with knockouts, adhE, ldhA, frdBC, fnr, pta and pflB, was employed.<sup>12</sup> With this host, the 2,3-BDO production was dramatically increased without changing the product chirality (Table 4). When budC-contained operon was expressed in JCL260, meso-2,3-BDO was produced at 10 g/L (122% increase) with a yield of 0.34 g product/g glucose (68% of theoretical maximum). Similarly, (R,R)-2,3-BDO was produced with expression of B. subtilis bdhA, C. beijerinckii adh or T. brockii adh-contained operons at titers of 5.8 g/L, 5.1 g/L and 6.1 g/L, respectively, which were 160%, 240% and 120% increases of titers in JCL16, with yields of 0.30 g product/g glucose (60% of theoretical maximum), 0.29 g product/g glucose (58% of theoretical maximum), and 0.31 g product/g glucose (62% of theoretical maximum), respectively.

In conclusion, we described for the first time the enantioselective synthesis of pure (R, R)-2,3-BDO isomer in *E. coli* from glucose by employing stereospecific sADHs which were characterized to produce the *R*-configuration specifically. By using engineered whole cell biocatalysts expressing sADHs with strict stereospecificity instead of native producers, we were able to increase the enantiomeric selectivity in (R, R)-2,3-BDO biosynthesis. Switching the *R*-specific sADHs to the *S*-specific sADH in the pathway led to the synthesis of enantiomerically pure *meso*-2,3-BDO. Both (R, R)- and *meso*-2,3BDO were synthesized from glucose with

enantiomeric purity over 99%. Due to the strict stereospecificity and promiscuous activity of the characterized sADHs, they will have broad applications in chiral synthesis. Furthermore, we also demonstrated the advantage of using *E. coli* as a whole cell biocatalyst with expression of certain biosynthesis pathway by avoiding the costly reducing cofactor recycling procedures.

## Notes and references

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