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

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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.[1–4] 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.[5] 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.[5] 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[6] (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*)-2,3-BDO was also produced using engineered *E. coli* by feeding diacetyl as precusor[7] (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.[8] 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

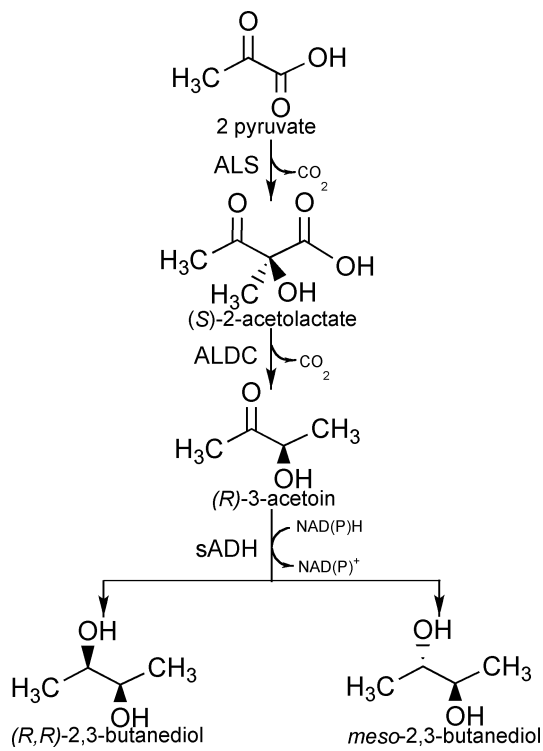

Fig. 1 Schematic illustration of the 2,3-BDO biosynthesis pathway. ALS: acetolactate synthase; ALDC: acetolactate decarboxylase; sADH: secondary alcohol dehydrogenase.

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† 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

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

	Organism	sADH gene	Precursor	Isomer	Titer(g/L)	Enantio purity	Reference
native producer	*B. cereus* YUF-4	Native	glucose	*meso-*	0.3	60%	
				(R,R-)	0.2	40%	13
	K. pneumoniae IAM 1063	Native	glucose	*meso-*	21.2	74%	
				(S,S)-	1.6	6%	10
				(R,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	(*R,R*)-	6.1	99%	This study

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†) 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.[9,10] 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. coli*[11] (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 s^{-1} and 0.91 s^{-1}, respectively) towards acetoin with high affinities (K_m = 0.26 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 mM) although activity is relatively high (K_{cat} = 8.2 s^{-1}). The enzyme encoded by *budC* from *K. pneumoniae* shows the highest activity (K_{cat} = 58s^{-1}) despite having a slightly lower affinity (K_m = 0.85 mM) (Table 2).

Table 2 Kinetic parameters of secondary alcohol dehydrogenases on substrate acetoin

Organism	Gene	Cofactor	K_m(mM)	K_{cat}(s^{-1})	K_{cat}/K_m ($mM^{-1}.s^{-1}$)
B. subtilis	*bdhA*	NADH	0.26 ± 0.02	0.98 ± 0.05	3.8
C. beijerinkii	*adh*	NADPH	8.3 ± 0.3	8.2 ± 0.3	0.99
T. brockii	*adh*	NADPH	0.23 ± 0.02	0.91 ± 0.06	4.0
K. pneumoniae	*budC*	NADH	0.85 ± 0.04	58 ± 3	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^{-1} until 45 °C, and held for 4 min. Then it was increased with a gradient 15 °C min^{-1} 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.[11] The synthetic operons included three structural genes: *alsS* and *alsD* from *B. subtilis*

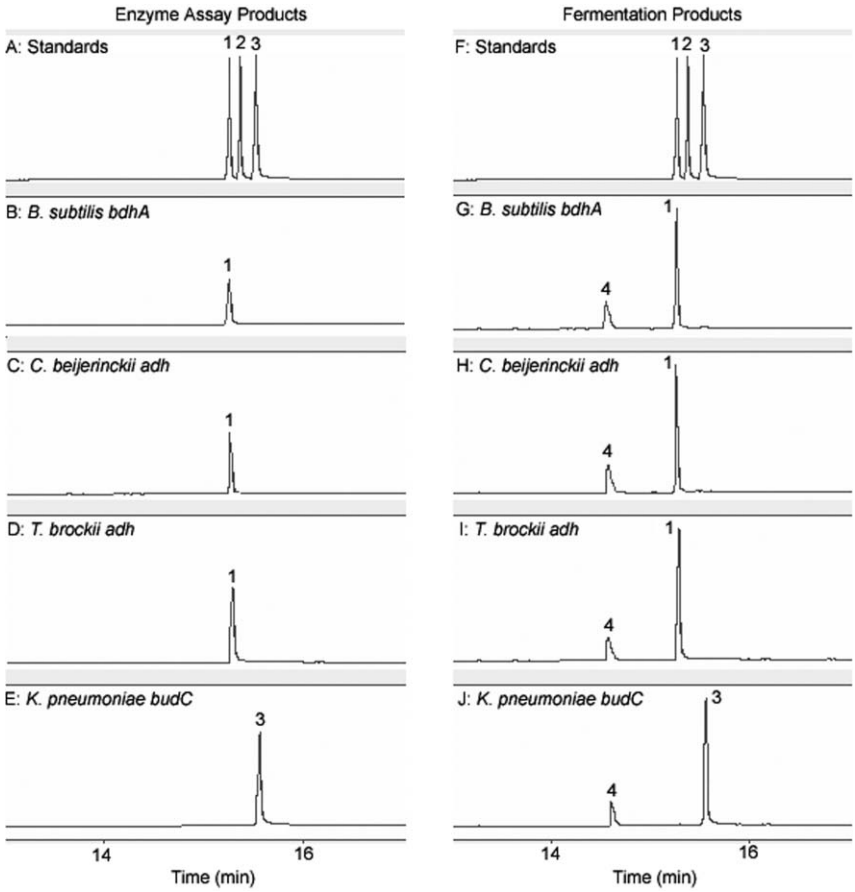

Fig. 2 Identification of 2,3-BDO isomers by GC-FID. A and F: 2,3-BDO isomer standards; B: Assay product of *bdhA* from *B. subtilis*; C: Assay product of *adh* from *C. beijerinckii*; D: Assay product of *adh* from *T. brockii*; E: Assay product of *budC* from *K. pneumoniae* MGH78578; G: Fermentation 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 *T. brockii adh* in the pathway; J: 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 *SalI* and *BamHI*. The generated plasmids carrying four synthetic operons were named pZE12-alsS-alsD-bdhA, pZE12-alsS-alsD-CBADH, pZE12-alsS-alsD-TBADH, and pZE12-alsS-alsD-BudC (the plasmid construct is provided in the ESI†). 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 50μg/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 $MgSO_4$, 0.1 mM $CaCl_2$, 2×10^{-4}% 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*]) [12] 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

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

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

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

sADH gene	Glucose consumption (g/L)	(*R,R*)-2,3-BDO (g/L)	*meso*-2,3-BDO (g/L)	Enantio purity	Yield (g/g glucose)
B. subtilis bdhA	18.9 ± 0.5	5.8 ± 0.3	<0.03	>99%	0.30
C.beijerinckii adh	17.8 ± 0.8	5.1 ± 0.4	<0.03	>99%	0.29
T.brockii adh	19.7 ± 0.7	6.1 ± 0.5	<0.03	>99%	0.31
K. pneumoniae budC	29.6 ± 2.8	<0.06	10 ± 0.8	>99%	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.[12] 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.

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