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Engineering of cofactor regeneration enhances (2*S*,3*S*)-2,3-butanediol production from diacetyl

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(2*S*,3*S*)-2,3-Butanediol ((2*S*,3*S*)-2,3-BD) is a potentially valuable liquid fuel and an excellent building block in asymmetric synthesis. In this study, cofactor engineering was applied to improve the efficiency of (2*S*,3*S*)-2,3-BD production and simplify the product purification. Two NADH regeneration enzymes, glucose dehydrogenase and formate dehydrogenase (FDH), were introduced into *Escherichia coli* with 2,3-BD dehydrogenase, respectively. Introduction of FDH resulted in higher (2*S*,3*S*)-2,3-BD concentration, productivity and yield from diacetyl, and large increase in the intracellular NADH concentration. In fed-batch bioconversion, the final titer, productivity and yield of (2*S*,3*S*)-2,3-BD on diacetyl reached 31.7 g/L, 2.3 g/(L·h) and 89.8%, the highest level of (2*S*,3*S*)-2,3-BD production thus far. Moreover, cosubstrate formate was almost totally converted to carbon dioxide and no organic acids were produced. The biocatalytic process presented should be a promising route for biotechnological production of NADH-dependent microbial metabolites.

2,3-Butanediol (2,3-BD) is a promising bulk chemical with extensive industry applications[1–3]. The heating value of 2,3-BD (27,200 J/g) is comparable with that of other liquid fuels e.g. methanol (22,100 J/g) and ethanol (29,100 J/g), making 2,3-BD a potentially valuable fuel or a fuel additive[4]. Dehydration of 2,3-BD yields methyl ethyl ketone that is a high quality aviation fuel[3,5]. There are three isomeric forms of 2,3-BD: (2*R*,3*R*)- and (2*S*,3*S*)- forms that are optically active, as well as an optically inactive *meso*-form[5]. Optically pure 2,3-BD can act as an excellent building block in asymmetric synthesis of chiral compounds that contain 2 vicinal stereocenters[6]. Thus, it is desirable to develop a practical technique for the production of optically pure 2,3-BD[7]. Since native microorganisms generally produce a mixture of two isomers (*meso*- and (2*S*,3*S*)- or *meso*- and (2*R*,3*R*)-), metabolic engineering and synthetic biology have been applied to improve the productivity and purity of optically pure 2,3-BD[5,8–11]. Researchers reported previously that (2*S*,3*S*)-2,3-BD could be produced from diacetyl (DA) by engineered *Escherichia coli* strains expressing 2,3-butanediol dehydrogenase (2,3-BDH)[9,11]. Glucose was used for the internal cofactor regeneration. However, two problems need to be solved for economical industrial production: (1) the yield of (2*S*,3*S*)-2,3-BD was not satisfactory due to the accumulation of intermediate product acetoin (AC); (2) organic acids excreted during the glucose metabolism would make the purification difficult.

The transformation between 2,3-BD and AC was reversible and coupled with the NADH/NAD^+ conversion. Through manipulating the level of intracellular cofactors, production of 2,3-BD or AC can be improved[12,13]. The accumulation of AC can be ascribed to the unbalance between NADH consumption and regeneration. Goldberg et al. also reported that the internal cofactor regeneration is usually not fast or efficient enough due to the high heterogeneous enzyme concentration in recombinant biocatalysts[14]. In addition, glucose was metabolized to organic acids such as acetic acid and lactic acid when it was used for the internal cofactor regeneration. Organic acids will cause high energy consumption and loss of the aim product in downstream purification processes[15]. It has been reported that regeneration of the cofactor makes the production process economically and industrially feasible by driving the reaction to completion, preventing the accumulation of by-products and simplifying the reaction work-up[16]. Therefore, a potential strategy to solve the aforementioned two problems is to introduce efficient cofactor regeneration system instead of merely using the internal one.

Glucose dehydrogenase (GDH) and formate dehydrogenase (FDH) are widely-used for cofactor regeneration[17–22]. GDH oxidizes the cosubstrate glucose with the regeneration of NAD(P)H. It was rather inexpensive, highly active and stable. Because of the hydrolysis of the coproduct gluconolactone to gluconic acid, the reaction is

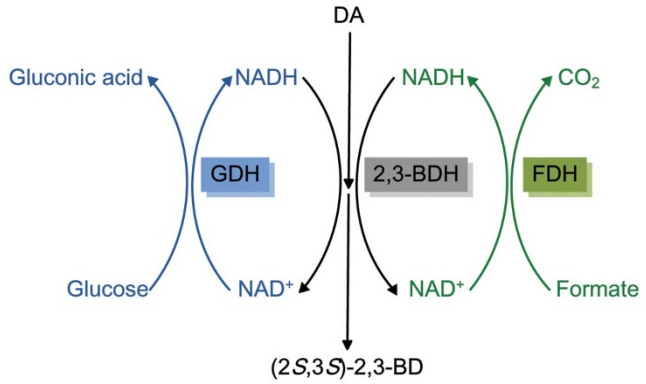

Figure 1 | Biocatalytic process for (2*S*,3*S*)-2,3-BD production with NADH regeneration by GDH or FDH.

nearly irreversible, providing a strong driving force for NADH regeneration[18]. In the case of FDH, NADH is formed when formate is converted to carbon dioxide by FDH. The advantages of FDH involve the use of formate as a cheap and innocuous substrate and ready removal of the coproduct carbon dioxide, thereby a favorable thermodynamic equilibrium[18,23].

In the present study, FDH or GDH was co-expressed with 2,3-BDH in *E. coli*, respectively, as shown in Figure 1. The performance of the two resulting recombinant strains, *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*) and *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*) was evaluated in terms of enzymatic activities, (2*S*,3*S*)-2,3-BD production and intracellular NADH/NAD$^+$ level. Our intention is to develop an efficient biocatalytic process for the production of (2*S*,3*S*)-2,3-BD by cofactor engineering.

Results

Cloning and expression of *bdh*, *gdh* and *fdh*. To investigate co-expression of 2,3-BDH with GDH or FDH, *gdh* gene from *Bacillus subtilis* 168 and *fdh* gene from *Candida boidinii* NCYC 1513 were cloned and different engineered *E. coli* strains were constructed. The resulting recombinant strains, *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*) and *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*) were characterized for their enzymatic activities and the results were shown in Table 1.

Crude extract of induced *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*) showed a 2,3-BDH activity of 39.1 U/mg and a GDH activity of 17.6 U/mg. Meanwhile, a 2,3-BDH activity of 38.9 U/mg and an FDH activity of 0.3 U/mg were detected in crude extract of induced *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*). SDS-PAGE analyses of the recombinant proteins were shown in Figure 2. 2,3-BDH and GDH have similar molecular weights, 26.8 kDa for 2,3-BDH and 28.1 kDa for GDH, respectively. These two enzymes were poorly resolved by SDS-PAGE analysis. However, the results of enzyme activity assay and SDS-PAGE indicated that *gdh* and *fdh* were successfully co-expressed with *bdh*, respectively.

Bioconversion with different cofactor regeneration systems. In order to evaluate the potential of the recombinants with different cofactor regeneration systems for the (2*S*,3*S*)-2,3-BD production from DA, batch bioconversion was carried out under the same conditions as presented by Li et al.[11]. Whole cells of recombinants were used as biocatalysts because they were convenient to prepare and suitable for the reactions that require cofactor regenerations[14,24]. Glucose and formate were added as the cosubstrates for GDH and FDH, respectively, to produce reducing equivalent.

As shown in Table 2, 16.8 g/L of (2*S*,3*S*)-2,3-BD (purity > 99%) was obtained after 6 h with a productivity of 2.8 g/(L·h) by *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*). The conversion yield of (2*S*,3*S*)-2,3-BD on DA was 85.4%. Gluconic acid, acetic acid and lactic acid were the main byproducts. Using *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*) as biocatalyst resulted in higher (2*S*,3*S*)-2,3-BD concentration, productivity and yield. After 5 h of reaction, 17.8 g/L of (2*S*,3*S*)-2,3-BD (purity > 99.0%) was accumulated with a productivity of 3.6 g/(L·h) and a conversion yield of 91.8% by *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*). The pH changes during the batch bioconversion were also monitored. Concerning *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*), pH was decreased, for the formation of acetic acid, lactic acid and gluconic acid from glucose. Therefore, 10 M NaOH was added periodically to control pH at 7.0. With respect to *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*), the consumption of formate resulted in the increase of pH, and HCl was added to maintain pH.

Effects of NADH regeneration system on intracellular NADH and NAD$^+$. Heterogeneous expression of GDH or FDH in *E. coli* was expected to regenerate cofactors and increase the overall intracellular NADH pool, thereby improving the flux of NADH-dependent pathways[22]. The results of batch bioconversions showed that *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*) achieved a higher concentration, productivity and yield of (2*S*,3*S*)-2,3-BD than the other two biocatalysts, possibly indicating more NADH was produced by FDH. To explain the phenomenon, intracellular concentrations of NADH and NAD$^+$ were determined.

In all three recombinants, total concentrations of NADH and NAD$^+$ remained constant. Compared with expression of 2,3-BDH alone, co-expression of GDH with 2,3-BDH didn't change the level of NADH remarkably (Figure 3 a and b). As we expect, *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*) showed a relatively high level of NADH and a low one of NAD$^+$. The ratio of NADH to NAD$^+$ was also higher and increased continuously. During the bioconversion, DA was converted to (2*S*,3*S*)-2,3-BD with NADH consumption. The increased NADH level and ratio of NADH to NAD$^+$ suggested that NADH was indeed regenerated in the engineered strain. After 5 h, the intracellular NADH concentration of *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*) increased by 0.43 (0.74 to 1.17) μmol/g DCW, which was slightly lower than those of the other two recombinants (0.45 (0.33 to 0.78) for *E. coli* BL21 (DE3) (pETDuet-*bdh*) and 0.49 (0.35 to 0.84) for *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*)).

Fed-batch bioconversion by biocatalyst containing 2,3-BDH and FDH. Efficient fed-batch strategies could enhance the concentrations of the aim products[5,25,26]. To obtain a higher concentration of (2*S*,3*S*)-2,3-BD, fed-batch bioconversion was carried out using *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*), with an initial DA concentration of 20.0 g/L and adding 5.0 g/L of DA at 2, 4, and 6 h. Formate was used as the driving force for NADH regeneration and added at 0, 4 and 6 h. As shown in Figure 4a, after 14 h of reaction, 31.7 g/L of (2*S*,3*S*)-2,3-BD (purity > 99.0%) was produced from

Table 1 | Activities of 2,3-BDH, GDH and FDH

Strain	2,3-BDH activity (U/mg)	GDH activity (U/mg)	FDH activity (U/mg)	Reference
E. coli BL21 (DE3) (pETDuet-*bdh*)	87.9 ± 3.90	ND	ND	[11]
E. coli BL21 (DE3) (pETDuet-*bdhgdh*)	39.1 ± 0.76	17.6 ± 0.51	ND	This work
E. coli BL21 (DE3) (pETDuet-*bdhfdh*)	38.9 ± 1.42	ND	0.3 ± 0.02	This work

ND: not detected.

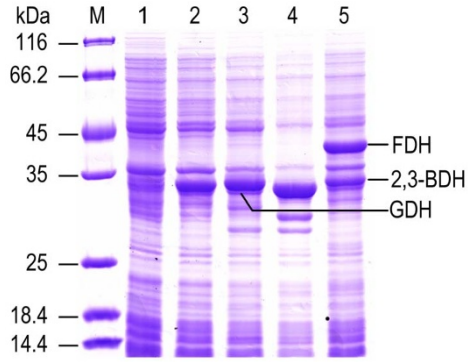

Figure 2 | Validation of the expression of 2,3-BDH, GDH and FDH in *E. coli* through SDS-PAGE. M, maker; lane 1, *E. coli* BL21 (DE3); lane 2, *E. coli* BL21 (DE3) (pETDuet-*bdh*); lane 3, *E. coli* BL21 (DE3) (pETDuet-*gdh*); lane 4, *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*); lane 5, *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*).

35.3 g/L of DA with a productivity of 2.3 g/(L·h). Only 2.6 g/L of AC was accumulated, leading to a high yield of (2*S*,3*S*)-2,3-BD on DA (89.8%). Formate was almost used up at the end of the reaction (Figure 4b), and no organic acids were detected.

Discussion

As a predominant redox product of catabolism, NADH plays an important role in over 700 biochemical reactions, a number of which are synthetically practical enzymatic reactions[16,27]. Given the high cost of NADH, its stoichiometric use is not economically feasible. Instead, *in situ* regeneration of the cofactor is necessary[16,23]. (2*S*,3*S*)-2,3-BD is an important precursor of asymmetric synthesis and potentially valuable liquid fuel[1–3]. Engineered biocatalysts were constructed to achieve (2*S*,3*S*)-2,3-BD production[9–11]. However, the processes usually suffer from low yield and difficult purification. Here, the efficiency of (2*S*,3*S*)-2,3-BD production was improved and the downstream process was simplified by cofactor engineering. The *gdh* gene encoding GDH and *fdh* gene encoding FDH were co-expressed with *bdh* encoding 2,3-BDH in *E. coli*, respectively. The two resulting recombinants, *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*) and *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*), shared a similar 2,3-BDH activity, which was nearly half of that of *E. coli* BL21 (DE3) (pETDuet-*bdh*)[11]. The expression levels of 2,3-BDH were quantified using the Glyko BandScan 5.0 software (Glyko, USA). When 2,3-BDH was expressed individually and co-expressed with FDH, the amounts of the recombinant 2,3-BDH accounted for about 25.8% and 15.8% of the total protein in the crude extracts, respectively. It revealed that the 2,3-BDH expression level in co-expression system was about 61.2% of individual recombinant protein. The 2,3-BDH expression level in *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*) was not quantified since 2,3-BDH and GDH were overlapped in the gel image. This suggests that the expression levels of the co-expression systems were relatively lower than those of the individual recombinant proteins. Similar results were obtained in other engineered *E. coli* strains when two genes were co-expressed[20,28].

Batch bioconversion was carried out to evaluate the potential of (2*S*,3*S*)-2,3-BD production from DA by the recombinants. Compared with the results achieved previously by *E. coli* BL21 (DE3) (pETDuet-*bdh*), co-expressing 2,3-BDH with GDH slightly improved the production of (2*S*,3*S*)-2,3-BD[11]. Analysis of by-products indicated that only 5.2 g/L of gluconic acid was produced from glucose by GDH to regenerate NADH (Table 2). Rest of the consumed glucose was metabolized to acetic acid and lactic acid during glucolysis. While co-expressing 2,3-BDH with FDH benefited the production of (2*S*,3*S*)-2,3-BD markedly by improving the concentration, productivity and conversion yield. Only 1.1 g/L of AC was accumulated, which contributed to the high conversion yield of (2*S*, 3*S*)-2,3-BD on DA (91.8%). Moreover, no organic acids were detected in the bioconversion broth of *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*). In the recovery process of alcohols such as 2,3-BD and 1, 3-propanediol, vacuum evaporation is usually employed to concentrate the broth. The existence of organic acids will make the operating temperature rise and the on-off concentration ratio decrease, resulting in high energy consumption. Besides, parts of aim product will be lost during the concentration process[15]. Therefore, preventing the synthesis of organic acids is of great significance.

To investigate the effect of cofactor regeneration systems on intracellular NADH/NAD^+ level, concentrations of NADH and NAD^+ were determined. Introduction of FDH resulted in higher intracellular NADH/NAD^+ level, indicating a high efficiency of NADH regeneration. The relatively lower increased amount of NADH concentration might be due to two reasons. Firstly, a high NADH level was achieved by *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*), which might drive the production of (2*S*,3*S*)-2,3-BD and consumption of NADH. As shown in Table 2, the highest titer of (2*S*,3*S*)-2,3-BD was produced by *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*). Therefore, the amount of increased NADH level of *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*) might be lower. Secondly, there are systems in microbial cells for maintaining the NADH/NAD^+ ratio, for the homeostasis of NADH/NAD^+ is essential to sustain the metabolism[27]. As a result, the intracellular NADH concentration might be difficult to improve when it reach a high level.

As mentioned above, a high GDH activity (17.6 U/mg) was detected in crude extract of *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*), but only part of glucose was metabolized to gluconic acid by GDH in the batch bioconversion (Table 2). The low efficiency of GDH in whole-cell biocatalysis might be due to permeation resistance of glucose. Glucose is mainly transported into cells by the active transporter of the phosphotransferase system (PTS) with phosphorylation, producing intracellular glucose-6-phosphate. Meanwhile, glucose can also enter in a non-phosphorylated form through permeation[29]. In crude extract, glucose acted as the substrate of GDH

Table 2 | The products of batch bioconversion with different cofactor regeneration systems

Strain	(2*S*,3*S*)-2,3-BD (g/L)	AC (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Gluconic acid (g/L)	Productivity of (2*S*,3*S*)-2,3-BD (g/(L·h))	Yield of (2*S*,3*S*)-2,3-BD (%)
E. coli BL21 (DE3) (pETDuet-*bdh*)	16.1 ± 0.30	2.4 ± 0.08	7.3 ± 0.21	11.6 ± 0.21	ND	2.7 ± 0.05	82.5 ± 0.62
E. coli BL21 (DE3) (pETDuet-*bdhgdh*)	16.8 ± 0.49	1.9 ± 0.26	6.6 ± 0.21	6.2 ± 0.13	5.2 ± 0.20	2.8 ± 0.08	85.4 ± 1.59
E. coli BL21 (DE3) (pETDuet-*bdhfdh*)	17.8 ± 0.59	1.1 ± 0.28	ND	ND	ND	3.6 ± 0.12	91.8 ± 1.18

ND: not detected.
Yield of (2*S*,3*S*)-2,3-BD: the (2*S*,3*S*)-2,3-BD produced divided by the DA consumed and multiplied by 100.

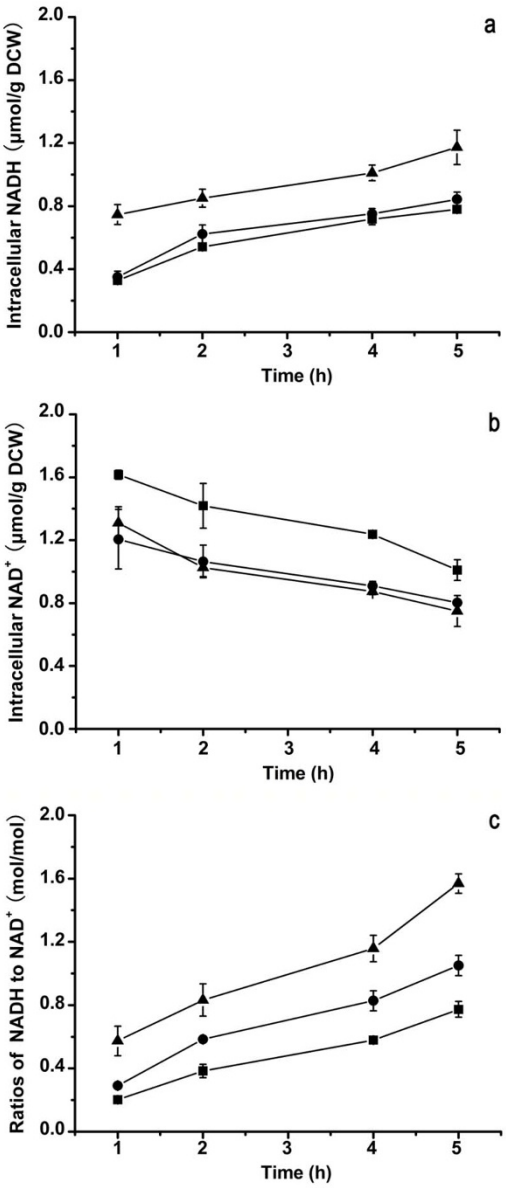

Figure 3 | Effects of different NADH regeneration systems on levels of intracellular NADH, NAD$^+$ and ratios of NADH to NAD$^+$. (■) *E. coli* BL21 (DE3) (pETDuet-*bdh*), (●) *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*), (▲) *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*). Error bars indicate s.d. (n = 3).

without permeation across the membrane, leading to a high GDH activity. While in the whole-cell biocatalysis, permeation resistance limited the amount of intracellular glucose. As a result, only part of NADH was produced by GDH, which did not improve the level of intracellular NADH significantly.

To improve the concentration of (2*S*,3*S*)-2,3-BD, fed-batch bioconversion was carried out using *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*). In comparison with the results achieved by *E. coli* BL21 (DE3) (pETDuet-*bdh*) previously[11], co-expressing FDH with 2,3-BDH improved the final (2*S*,3*S*)-2,3-BD titer from 26.8 g/L to 31.7 g/L, and the productivity from 1.9 g/(L·h) to 2.3 g/(L·h). It is worth noting that the conversion yield was increased from 66.7% to 89.8%. In addition, the absence of glucose avoided the production of organic acids, which can make the product extraction easier. It has been reported that the cost of substrates accounted for more than about 30% of total production-cost of 2,3-BD[30]. Therefore, the cost analyses of substrates were performed to evaluate the fed-batch bioconversions of (2*S*,3*S*)-2,3-BD by *E. coli* BL21 (DE3) (pETDuet-*bdh*) and *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*), without consideration of production process (see Supplementary Data 1 online). The formate cost of *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*) was 0.5 US$/kg (2*S*,3*S*)-2,3-BD, which was decreased by over 60% than the glucose cost (1.3 US$/kg (2*S*,3*S*)-2,3-BD) of *E. coli* BL21 (DE3) (pETDuet-*bdh*). Due to the high yield of the recombinant with FDH, the DA cost was 24.5 US$/kg (2*S*,3*S*)-2,3-BD, which was decreased by over 25% than recombinant without FDH (33.0 US$/kg (2*S*,3*S*)-2,3-BD). Considering the easier purification, introduction of FDH would futher decrease the cost of (2*S*,3*S*)-2,3-BD production.

In summary, the introduction of NADH regeneration system may improve the efficiency of (2*S*,3*S*)-2,3-BD production and simplify the downstream process. Co-expression of FDH and 2,3-BDH can increase the availability of intracellular NADH and resulted in higher titer, productivity and yield than those of previous studies. Moreover, no organic acids were produced, which may make the product extraction easier. The method developed in this work suggests a useful approach to improve the production efficiency of similar biotechnological processes.

Methods

Enzymes and chemicals. The FastPfu DNA polymerase was acquired from TransGen Biotech (China). The restriction enzymes and T_4 DNA ligase were purchased from New England Bio-Labs (Beverly, MA). *meso*-2,3-BD (98.0%), (2*R*,3*R*)-2,3-BD (98.0%) and (2*S*,3*S*)-2,3-BD (99.0%) were obtained from ACROS (The Kingdom of Belgium). Isopropyl-β-D-1-thiogalactopyranoside (IPTG), Dithiothreitol (DTT), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Merck (Germany). Ampicillin, NADH and NAD$^+$ were purchased from Amresco (USA). All other chemicals were of analytical grade and commercially available.

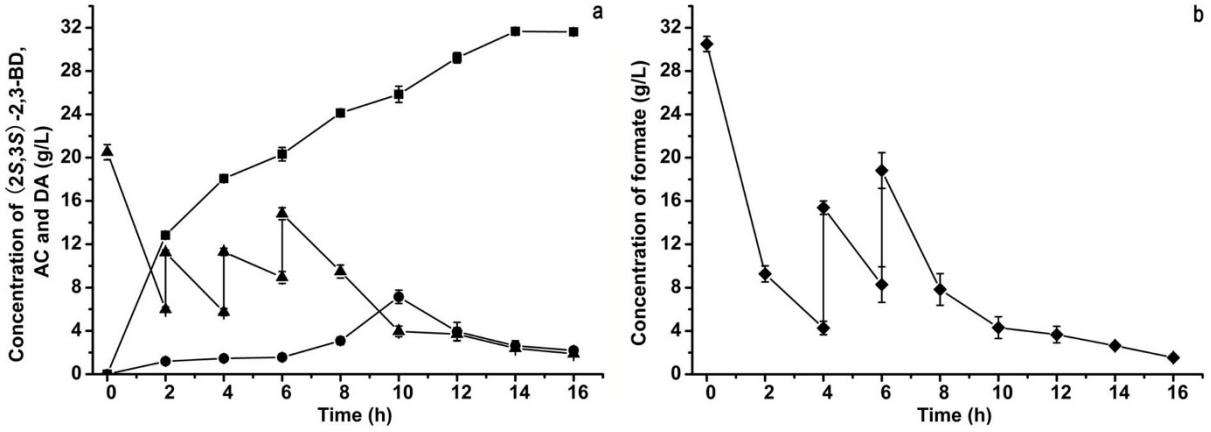

Figure 4 | Time course of fed-batch bioconversion by *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*). (■) (2*S*,3*S*)-2,3-BD, (●) AC, (▲) DA, and (◆) formate. Error bars indicate s.d. (n = 3).

Table 3 | Strains, plasmids, and primers used in this study

Strain, plasmid or primer	Genotype, property or sequence	Source or reference
Strain		
B. subtilis 168	Wild type	ATCC 23857
C. boidinii	Wild type	NCYC 1513
E. coli DH5α	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Novagen
E. coli BL21 (DE3)	*F⁻ ompT hsdSB (rB⁻mB⁻) gal (λ c I 857 ind1 Sam7 nin5 lacUV5 T7gene1) dcm* (DE3)	Novagen
Plasmid		
pEASY-Blunt	Cloning vector; Ap^r	TransGen
pETDuet-1	Overexpression vector; Ap^r	Novagen
pETDuet-*bdh*	*bdh* in pETDuet-1	[11]
pETDuet-*gdh*	*gdh* in pETDuet-1	This work
pETDuet-*bdhgdh*	*bdh* and *gdh* in pETDuet-1	This work
pETDuet-*bdhfdh*	*bdh* and *fdh* in pETDuet-1	This work
Primer		
pg1	5′-CCC*AGATCT*AATGTATCCGGATTTAAAAGG-3′	This work
pg2	5′-*CTCGAG*TTAACCGCGGCCTGCCT-3′	This work
pf1	5′-*CATATG*AAGATCGTTTTAGTCTTATATGATGCTGGTA-3′	This work
pf2	5′-*CTCGAG*TTATTTCTTATCGTGTTTACCGTAAGCTTTG-3′	This work

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 3. *E. coli* DH5α and BL21 (DE3) were used as cloning and expression host, respectively. The pEASY-Blunt cloning vector (TransGen Biotech, China) was used for gene cloning, and pETDuet-1 with two T7 promoters was used for gene expression. Luria-Bertani (LB) medium was used for *E. coli* and *B. subtilis* 168 cultivations. *C. boidinii* NCYC 1513 was cultured in YPD medium (2.0% glucose, 2.0% peptone and 1.0% yeast extract). Ampicillin was used at a concentration of 100 μg/mL.

Cloning and expression of *bdh*, *gdh* and *fdh*. *B. subtilis* 168 and *C. boidinii* NCYC 1513 genomic DNAs were extracted with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The *gdh* gene was amplified by PCR using forward primer pg1 with a *Bgl*II restriction site insertion and reverse primer pg2 with an *Xho*I restriction site insertion. The PCR product was firstly ligated to the pEASY-Blunt vector, and the resulting plasmid was designated pEASY-Blunt-*gdh*. The pEASY-Blunt-*gdh* was then sequenced (Sangon, Shanghai, China) to verify that no mutations were introduced by PCR. Next, to construct the recombinant plasmid pETDuet-*bdhgdh* under the control of the T7 promoter, pEASY-Blunt-*gdh* was digested with *Bgl*II and *Xho*I, and the gel-purified *gdh* fragment was ligated to the pETDuet-*bdh* vector digested with the same restriction enzymes. The resulting plasmid was designated pETDuet-*bdhgdh*. The *gdh* gene fragment was also ligated to the pETDuet-1 vector with the same restriction sites to obtain the pETDuet-*gdh*. Using the same process that described above, the *fdh* gene fragment was obtained from the genome of *C. boidinii* NCYC 1513 using primers pf1 (with the *Nde*I restriction site) and pf2 (with the *Xho*I restriction site), and the pETDuet-*bdhfdh* was constructed.

Biocatalyst preparation and bioconversion conditions. The recombinant strains were grown in LB medium containing 100 μg/mL of ampicillin at 37°C on a rotary shaker (180 rpm). The cultures were induced with 1 mM IPTG at an $OD_{620\ nm}$ of 0.6. 16°C was used for induction to avoid the formation of inactive inclusion bodies for about 10 h. The cells were harvested by centrifugation at 6,000 × *g* for 5 min at 4°C, and then washed twice with 0.85% NaCl. The cell pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and maintained at 4°C for further study.

The bioconversion conditions were as same as that presented by Li et al.[11]. 10 mL of mixture was reacted at 30°C and 200 rpm in 100 mL flasks. The cell concentration in the reaction was 6.0 g dry cell weight (DCW)/L. pH was controlled at 7.0 by adding HCl or 10 M NaOH. (2*S*,3*S*)-2,3-BD production using *E. coli* BL21 (DE3) (pETDuet-*bdhgdh*) as the whole-cell biocatalyst was carried out using 20.0 g/L of DA as substrate and 40.0 g/L of glucose as the source of reducing equivalent (molar ratio of 1 : 1 (DA: glucose)) in 200 mM Tris–HCl buffer (pH 7.0). For (2*S*,3*S*)-2,3-BD production catalyzed by *E. coli* BL21 (DE3) (pETDuet-*bdhfdh*), 20.0 g/L of DA and 32.0 g/L of formate were required (molar ratio of 1 : 2 (DA: formate)).

Assays for 2,3-BDH, GDH and FDH activities. The harvested cells were resuspended in 20% glycerol, 0.1 mM PMSF, and 1 mM DTT in 50 mM Tris-HCl buffer (pH 7.4) and disrupted by sonication in an ice bath. The homogenate was centrifuged at 18,000 × *g* for 30 min, and the supernatant (crude extract) was recovered. The expressed enzyme was determined by SDS-PAGE. Enzyme activities were assayed spectrophotometrically by measuring the change in absorbance at 340 nm corresponding to the oxidation of NADH or the reduction of NAD^+ (ε_{340} = 6220/M cm) at 30°C. One unit of 2,3-BDH activity was defined as the amount of enzyme that consumed 1 μmol of NADH per min. The reaction solution contained 5 mM of DA and 0.2 mM of NADH in 50 mM Tris-HCl buffer (pH 7.4). One unit of GDH and FDH activity was defined as the amount of enzyme that produced 1 μmol of NADH per min. The reaction solution contained 5 mM of glucose for GDH or formate for FDH and 0.2 mM of NAD^+ in 50 mM Tris-HCl buffer (pH 7.4).

Determination of NADH and NAD^+ concentrations. The intracellular concentrations of NADH and NAD^+ were determined using the EnzyChrom NAD^+/NADH Assay kit (E2ND-100) from BioAssay Systems (Hayward, CA, USA) according to the manufacturers' instructions. About 10^6 cells were required for each sample. The assay kit is based on a lactate dehydrogenase cycling reaction, in which the formed NADH reduces a formazan (MTT) reagent. The intensity of the reduced product color, measured at 565 nm, is proportionate to the NAD^+/NADH concentration in the sample.

Analytical methods. The software Glyko BandScan 5.0 (Glyko, USA) was used for analyzing gel images of SDS-PAGE and quantifying the amount of cellular proteins. The concentrations of 2,3-BD and AC were determined by GC using the method described previously[10]. The purity of (2*S*,3*S*)-2,3-BD was defined as $\frac{[S]}{[S]+[M]+[R]} \times 100\%$, where [S], [M] and [R] represent the concentrations of (2*S*,3*S*)-2,3-BD, *meso*-2,3-BD and (2*R*,3*R*)-2,3-BD, respectively. Glucose was measured enzymatically by a bio-analyzer (SBA-40C, Shandong Academy of Sciences, China) after diluted to the appropriate concentration. The concentration of DA was measured using the colorimetric method[31]. Organic acids were determined by HPLC using the method described previously[11]. Gluconic acid was poorly resolved from glucose in this HPLC method and quantified by the gluconokinase/6-phosphogluconate dehydrogenase assay kit (product number 10 428 191 035; R-Biopharm AG, Darmstadt, Germany) after appropriate dilution of the sample.

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Author contributions

Y.W., C.M. and P.X. designed experiments. Y.W. and L.L. performed experiments. C.M. and C.G. and P.X. contributed reagents and materials. Y.W., L.L., F.T. and C.G. analyzed data. Y.W., P.X. and F.T. wrote the manuscript. All authors have read and approved the final manuscript.

Additional information

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