J Ind Microbiol Biotechnol (2015) 42:779–786
DOI 10.1007/s10295-015-1598-5

SIMB
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Engineered *Serratia marcescens* for efficient (3*R*)-acetoin and (2*R*,3*R*)-2,3-butanediol production

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Received: 5 November 2014 / Accepted: 31 January 2015 / Published online: 10 February 2015

Abstract (3*R*)-Acetoin and (2*R*,3*R*)-2,3-butanediol are important pharmaceutical intermediates. However, until now, the quantity of natural microorganisms with the ability to produce single configuration of optically pure (3*R*)-acetoin and (2*R*,3*R*)-2,3-butanediol is rare. In this study, a *meso*-2,3-butanediol dehydrogenase encoded by the *slaC* gene from *Serratia marcescens* MG1 was identified for *meso*-2,3-butanediol and (2*S*,3*S*)-2,3-butanediol biosynthesis. Inactivation of the *slaC* gene could significantly decrease *meso*-2,3-butanediol and (2*S*,3*S*)-2,3-butanediol and result in a large quantity of (3*R*)-acetoin accumulation. Furthermore, a (2*R*,3*R*)-2,3-butanediol dehydrogenase encoded by the *bdhA* gene from *Bacillus subtilis* 168 was introduced into the *slaC* mutant strain of *Serratia marcescens* MG1. Excess (2*R*,3*R*)-2,3-butanediol dehydrogenase could accelerate the reaction from (3*R*)-acetoin to (2*R*,3*R*)-2,3-butanediol and lead to (2*R*,3*R*)-2,3-butanediol accumulation. In fed-batch fermentation, the excess (2*R*,3*R*)-2,3-butanediol dehydrogenase expression strain could produce 89.81 g/l (2*R*,3*R*)-2,3-butanediol with a productivity of 1.91 g/l/h at 48 h. These results provided potential applications for (3*R*)-acetoin and (2*R*,3*R*)-2,3-butanediol production.

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Keywords (3*R*)-acetoin · (2*R*,3*R*)-2,3-butanediol · *Meso*-2,3-butanediol dehydrogenase · (2*R*,3*R*)-2,3-butanediol dehydrogenase · *Serratia marcescens*

Introduction

As one of the promising bulk chemicals, 2,3-butanediol (2,3-BD) has attracted increasing attention due to its potential industrial application in butadiene and liquid fuel [2, 6, 26]. 2,3-BD contains two stereo centers and has three stereo isomers: (2*R*,3*R*)-2,3-BD, *meso*-2,3-BD and (2*S*,3*S*)-2,3-BD [21, 25]. The optically active isomers of 2,3-BD can be used as building blocks for asymmetric synthesis of highly valuable chiral compounds [7]. Due to its low freezing point of −60 °C, optically pure 2,3-BD is also used as an antifreeze agent [21]. Acetoin (AC), the precursor of 2,3-BD, exists in two stereoisomeric forms, (3*R*)-AC and (3*S*)-AC, which are widely used to synthesize novel optically active α-hydroxyketone derivatives and liquid crystal composites [17, 18]. In addition, all of the isomers of 2,3-BD and AC are important potential pharmaceutical intermediates [8, 21]. Therefore, production of 2,3-BD and AC with high optical purities is desirable.

AC and 2,3-BD isomers can be produced via the mixed acid fermentation pathway by different native strains such as *Klebsiella pneumoniae* [1], *Klebsiella oxytoca* [4], *Paenibacillus polymyxa* [3], *Bacillus amyloliquefaciens* [20] and *Bacillus licheniformis* [15]. Of these, *Paenibacillus polymyxa* can produce 111 g/l (2*R*,3*R*)-2,3-BD with a purity up to 98 % [3]. At the present, the highest production of 150 g/l and 152 g/l 2,3-BD was, respectively, with *S. marcescens* H30 [24] and *K. pneumoniae* SDM [9] without considering its isomers. So *S. marcescens* and *K. pneumonia* were potential strains to produce optically pure

(2*R*,3*R*)-2,3-BD with high concentration after the purity was taken into consideration.

In previous studies, several heterologous hosts such as *E. coli* [5, 19] *and Synechocystis* [12] were also metabolically engineered to produce optically pure AC or 2,3-BD. α-Acetolactate, diacetyl and AC are the three main intermediate compounds of 2,3-BD fermentation in bacteria [25]. In general, 2,3-BD can be produced by natural microorgansims as a mixture of 2,3-BD isomers, and the stereoisomeric composition of 2,3-BD formed by bacteria differs among strains, which is related to the existence of various 2,3-butanediol dehydrogenases (BDHs) differing in their stereospecificities [14, 25]. Several BDHs with different stereospecificities have been identified and characterized in previous studies. *K. pneumoniae* mainly produced *meso*-2,3-BD with a small amount of (2*S*,3*S*)-2,3-BD from (3*R*)-AC and (3*S*)-AC due to the existence of *meso*-BDH [8, 23]. *Brevibacterium saccharolyticum* could express (2*S*,3*S*)-BDH which led to (2*S*,3*S*)-2,3-BD production from (3*S*)-AC [13, 14], whereas (2*R*,3*R*)-BDH from *Bacillus subtilis* and *Paenibacillus polymyxa* could catalyze (3*R*)-AC and (3*S*)-AC into (2*R*,3*R*)-2,3-BD and *meso*-2,3-BD, respectively [10, 22]. So some gene modifications of native strains are necessary for single configuration production of optically pure AC and 2,3-BD.

Previously, we reported an *S. marcescens* MG1 strain, which mainly produces (3*R*)-AC and *meso*-2,3-BD with a small amount of (2*S*,3*S*)-2,3-BD and (2*R*,3*R*)-2,3-BD from sucrose and demonstrated the potential for industrial-scale (3*R*)-AC and *meso*-2,3-BD production [11]. In this present study, we identified a *meso*-BDH enzyme encoded by the *slaC* gene in *S. marcescens* MG1, which was responsible for *meso*-2,3-BD and (2*S*,3*S*)-2,3-BD formation from (3*R*)-AC and (3*S*)-AC. Further, inactivation of the *slaC* gene from *S. marcescens* MG1 could lead to a large quantity of optically pure (3*R*)-AC accumulation. The expression of (2*R*,3*R*)-BDH from *B. subtilis* 168 in the *slaC* mutant strain could accelerate (3*R*)-AC into (2*R*,3*R*)-2,3-BD and result in a high purity of (2*R*,3*R*)-2,3-BD production. These results would provide further useful hints for single configuration production of AC and 2,3-BD.

Materials and methods

Enzymes, chemicals and primers

Restriction enzymes, T4 DNA ligase and Taq DNA polymerase were from Takara Biotech (Dalian, China). Bacterial DNA kit, plasmid mini kit, cycle-pure kit and gel extraction kit were purchased from Omega Biotech (Norcross, America). Oligonucleotide primers were synthesized in SBSbio (Shanghai, China). Diacetyl, (3*S*/3*R*)-AC, (2*S*,3*S*)-2,3-BD (97.0 %), (2*R*,3*R*)-2,3-BD (97.0 %) and *meso*-2,3-BD (99.0 %) were obtained from Sigma–Aldrich (Shanghai, China).

Bacterial strains, plasmids and growth conditions

The strains, plasmids and primers used in this study are listed in Table 1. Luria–Bertani (LB) broth was used for culturing *E. coli*, *S. marcescens* MG1 and its derivatives. *S. marcescens* MG1 was grown at 30 °C, and *E. coli* was grown at 37 °C. Plasmid pET-28a (+) (Novagen) was used for the *meso*-BDH expression. Antibiotics were added in the following amounts (per ml) if necessary: 50 μg kanamycin or 100 μg ampicillin.

For fermentation experiments, a full loop of *S. marcescens* MG1 strains or its derivatives from the slants were inoculated into 250-ml shake flasks containing 30 ml of fresh seed medium and cultivated for 12 h at 200 rpm and 30 °C. Seed culture (5 %, v/v) was then inoculated into 250-ml shake flasks containing 50 ml of fresh fermentation medium, followed by 30 h of incubation at 30 °C on a rotary shaker (200 rpm). The seed medium was composed of (g/l): glucose 10, peptone 2, yeast extract 1, $(NH_4)_2SO_4$ 6, KH_2PO_4 10, NaCl 0.5 and $MgSO_4$ 0.5 at pH 7.2. The fermentation medium was composed of (g/l): sucrose 90, yeast extract 33.36, sodium citrate 10, sodium acetate 4, $NH_4H_2PO_4$ 1, $MgSO_4$ 0.3 and $MnSO_4$ 0.1 [24].

For fed-batch fermentations, the seed culture was inoculated (5 %, v/v) into the above fermentation medium in a 7-l jar (ALF, Bioengineering, Switzerland) with an initial broth volume of 3 l. The cultivation was carried out at 30 °C with an aeration rate of 1.0 vvm and agitation speed of 500 rpm during the first 15 h of strain growth. After 15 h, the aeration rate and agitation speed were decreased to 0.5 vvm and 300 rpm, respectively. The feeding substrate was pumped into the bioreactor using a computer-coupled peristaltic pump. Sucrose solution (80 %, w/v) was added to 40 g/l into the bioreactor at different time points when the residual sucrose concentration was below 5 g/l. When the pH decreased to 6.0, it was maintained at 6.0 by automatic addition of 4 M KOH using a computer-coupled peristaltic pump.

Construction of the *slaC* recombinant strain

The ORF of the *slaC* gene was amplified by PCR with the genomic DNA of *S. marcescens* MG1 as template using the primer pair slaC-1 and slaC-2, which contain the *Eco*RI and *Hin*dIII restriction sites, respectively. The amplified product was digested and ligated into the vector pET-28a (+) at *Eco*RI and *Hin*dIII sites to generate pET28a-slaC.

Table 1 Bacteria strains, plasmids and primers used in this study

Strains, plasmids and primers	Description	Source
S. marcescens MG1	Wild type, Tc^rAp^r	Laboratory stock
S. marcescens-ΔslaC	*S. marcescens slaC* mutant	This study
S. marcescens-ΔslaC-bdhA	*S. marcescens-ΔslaC* with pUC-pbdhA	This study
E. coli BL21(DE3)	*F−, ompT, hsdSB(rB-mB-), gal, dcm(DE3)*	Laboratory collection
E. coli S17-1 (λ-pir)	*thi pro hsdR hsdM recA rpsL RP4-2* (*Tcr::Mu*) (*Kmr::Tn7*)	Laboratory collection
Bacillus subtilis 168	Wild type	Laboratory collection
pET28a(+)	Km^r lacZ'; expressing vector	Laboratory collection
pET28a-slaC	pET28a(+) vector cloned with the *slaC* gene	This study
pUTKm1	Ap^r Km^r *ori*R6 K *oriT*RP4	Laboratory collection
pUT-slaC	pUTKm1 with a combined fragment from upstream sequence and downstream sequence of the *slaC* gene inserted into the *Kpn*I-*Sca*I sites	This study
pUC19	Ap^r	Laboratory collection
pUC-pbdhA	pUC 19 vector cloned with the *bdhA* gene and the promoter of the *slaC* gene	This study
slaC-1	attgaattcATGCGTTTTGACAATAAAGTCG	This study
slaC-2	attaagcttTTAGACGATCTTCGGTTGGCC	This study
UpslaC-1	ggggtaccCATGCGGCAAGGAGCGCCATC	This study
UpslaC-2	GGCCTGTGCGTTAACGCGAGACCTCCTCCATGTGAAC	This study
DnslaC-1	GTTCACATGGAGGAGGTCTCGCGTTAACGCACAGGCC	This study
DnslaC-2	gtgagtactCAGCCGCATCAGCCGCTAC	This study
pslaC-1	gtaagcttTCGCGGCCGCCTGCGGGC	This study
pslaC-2	CCATCTTGCTGCCTTCATGAGACCTCCTCCATGTG	This study
bdhA-1	CACATGGAGGAGGTCTCATGAAGGCAGCAAGATGG	This study
bdhA-2	gtggatccTTAGTTAGGTCTAACAAG	This study

The recombinant pET28a-slaC vector was transformed into *E. coli* BL21(DE3) for protein expression.

Enzyme preparation and enzymatic reactions

The recombinant *E. coli* BL21(DE3)/pET28a-slaC strain was cultivated at 37 °C in a 250-ml flask containing 30 ml LB medium (pH 7.0) with kanamycin (50 μg/ml). The cells were induced at about 0.6 OD_{600} with 1 mM IPTG and harvested by centrifugation after 5 h. The *meso*-BDH was purified by Ni-affinity chromatography using a His-trap column according to the purification protocol (GE healthcare, USA).

The enzymatic reactions were carried out following a previously described method [25]. Briefly, for the oxidation reactions, a mixture containing 100 mM substrates (*meso*-2,3-BD, (2*S*,3*S*)-2,3-BD and (2*R*,3*R*)-2,3-BD), 4 mM NAD^+, 50 mM of potassium phosphate buffer (pH 8.0) and 10 μl of purified enzyme in a final volume of 1 ml was incubated at 40 °C for 2 h. The reduction reactions was performed in a 1-ml mixture containing 100 mM substrates (diacetyl and (3*S*/3*R*)-AC), 0.2 mM NADH, 50 mM of sodium acetate buffer (pH 5.0) and 10 μl of purified enzyme at 40 °C for 2 h. The products in these reaction systems were analyzed by gas chromatography.

Construction of *S. marcescens-ΔslaC* and *S. marcescens-ΔslaC-bdhA*

Two DNA fragments from upstream sequence (about 800 bp) and downstream sequence (about 1,000 bp) of the *slaC* gene with overlapping ends were amplified from *S. marcescens* MG1 genomic DNA using the primers listed in Table 1. The two fragments were then fused by overlapping PCR, generating an in-frame deletion construct of the *slaC* gene. The overlapping PCR fragment was cloned into the suicide vector pUTkm1 to produce pUT-slaC. The pUT-slaC vector was transformed into *E. coli* S17-1 λ_{pir} for conjugation with *S. marcescens* MG1. The single-crossover strains were selected on LB medium agar plate containing 50 μg/ml kanamycin. Then, a single-corssover colony was grown in LB broth overnight and plated onto LB agar, which was incubated overnight at 30 °C. Colonies were screened through the double-crossover resistance phenotype. The kanamycin-sensitive colonies were verified by PCR using the primers UpslaC-1/DnslaC-2. The *slaC* disruption mutant was designated as *S. marcescens-ΔslaC* and stored in a glycerol suspension at −80 °C.

To develop a constitutive expression vector for expression of (2*R*,3*R*)-BDH in *S. marcescens*, the *slaC* promoter (P_{slaC}) sequence and *bdhA* gene [coding for (2*R*,3*R*)-BDH]

were amplified from the genomic DNA of *S. marcescens* MG1 and *B. subtilis* 168 using the primers of pslaC-1/pslaC-2 and bdhA-1/bdhA-2. Two amplified products were combined by overlapping PCR resulting in the merged fragment, designated as p-bdhA. The p-bdhA fragment was digested and ligated into the vector pUC19 at *Bam*HI and *Hin*dIII sites to produce the recombinant pUC-pbdhA vector. The pUC-pbdhA vector was then transformed into the *slaC* mutant strain by electroporation, generating *S. marcescens-ΔslaC-bdhA* strain.

The stability of pUC-pbdhA plasmid in *S. marcescens-ΔslaC*

The stability of pUC-pbdhA plasmid in *S. marcescens-ΔslaC* was investigated using the following experimental procedure. Appropriately diluted fermentation samples were spread on selective (100 μg/ml ampicillin) and nonselective LB agar plates. The plates were incubated at 37 °C for 24 h. Plasmid stability was estimated as the ratio of number of colonies on the selective agar plates to the number on the nonselective plates.

Analytical methods

The sucrose concentration of the samples was measured by reagent kit (Jiemen Bio-Tech Co. China) of glucose after centrifugation and sucrose hydrolysis.

The biomass concentration was determined from the optical density (OD) at 600 nm using a spectrophotometer (UV-2008 h, Unic) and correlated with dry cell weight (DCW).

AC and 2,3-BD isomers in the samples were analyzed and quantified by GC (Agilent GC9860) equipped with a chiral column (FID-detector, Supelco β-DEX™ 120, 30 m length, 0.25 mm inner diameter). The operation conditions were as follows: N_2 was used as the carrier gas at a flow rate of 1.2 ml/min; the injector temperature and the detector temperature were 215 and 245 °C, respectively; the oven temperature was maintained at 50 °C for 1.5 min, then raised to 180 °C at a rate of 8 °C/min.

Results and discussion

Expression, purification and stereospecific characteristics of *meso*-BDH

The *slaC* gene from *S. marcescens* MG1 was obtained by PCR amplification using the primers (slaC-1/slaC-2) designed according to our previous submitted sequence (Genbank accession number JF519738). The PCR-amplified products were ligated into the expression vector pET-28a (+) and transformed into *E. coli* BL21(DE3) for heterologous expression. As shown in Fig. 1, SDS-PAGE analysis showed that the *meso*-BDH was successfully expressed in *E. coli*. The subunit of *meso*-BDH was approximately 26 kDa which was consistent with the predicted size. The stereospecific characteristics of the *meso*-BDH were studied by catalysis reactions using 2,3-BD, diacetyl and AC as substrates. The results are given in Table 2. In oxidation reactions, *meso*-BDH could convert *meso*-2,3-BD, and (2*S*,3*S*)-2,3-BD to (3*R*)-AC and (3*S*)-AC, respectively, while it could not convert (2*R*,3*R*)-2,3-BD to any form of AC. In reduction reactions, the *meso*-BDH could convert diacetyl to (3*S*)-AC and could not further convert (3*S*)-AC into any form of 2,3-BD at pH 5.0. As reported by Zhang et al. [25], the (3*S*)-AC product from diacetyl could be further transformed into (2*S*,3*S*)-2,3-BD at pH 9.0. When racemic acetoin was used as the substrate, only *meso*-2,3-BD was observed.

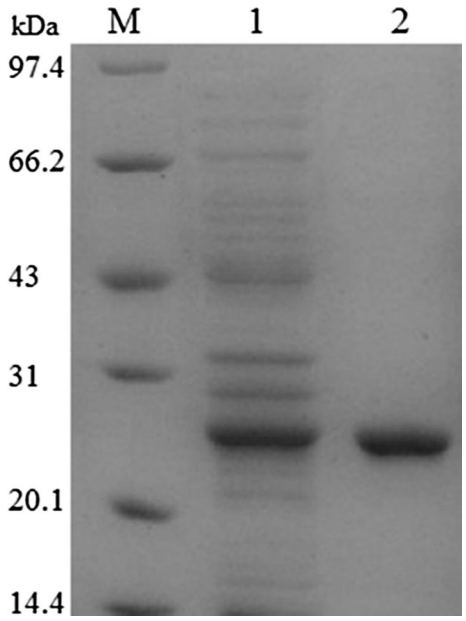

Fig. 1 Expression and purification of the *meso*-BDH protein encoded by the *slaC* gene from *S. marcescens* MG1 by SDS-PAGE. *Lane M* protein marker (97.4, 66.2, 43, 31, 20.1, 14.4 kDa). *Lane 1* soluble protein from the cell lysate. *lane 2* purified *meso*-BDH

Table 2 Stereospecific characteristics of the *meso*-BDH

Reactions	Substrates (pH)	Products
Oxidation reactions	*meso*-2,3-BD	(3*R*)-AC
	(2*S*,3*S*)-2,3-BD	(3*S*)-AC
	(2*R*,3*R*)-2,3-BD	ND[a]
Reduction reactions	Diacetyl	(3*S*)-AC
	(3*S*)-AC	(2*S*,3*S*)-2,3-BD
	(3*S*/3*R*)-AC	*meso*-2,3-BD

[a] Not detected

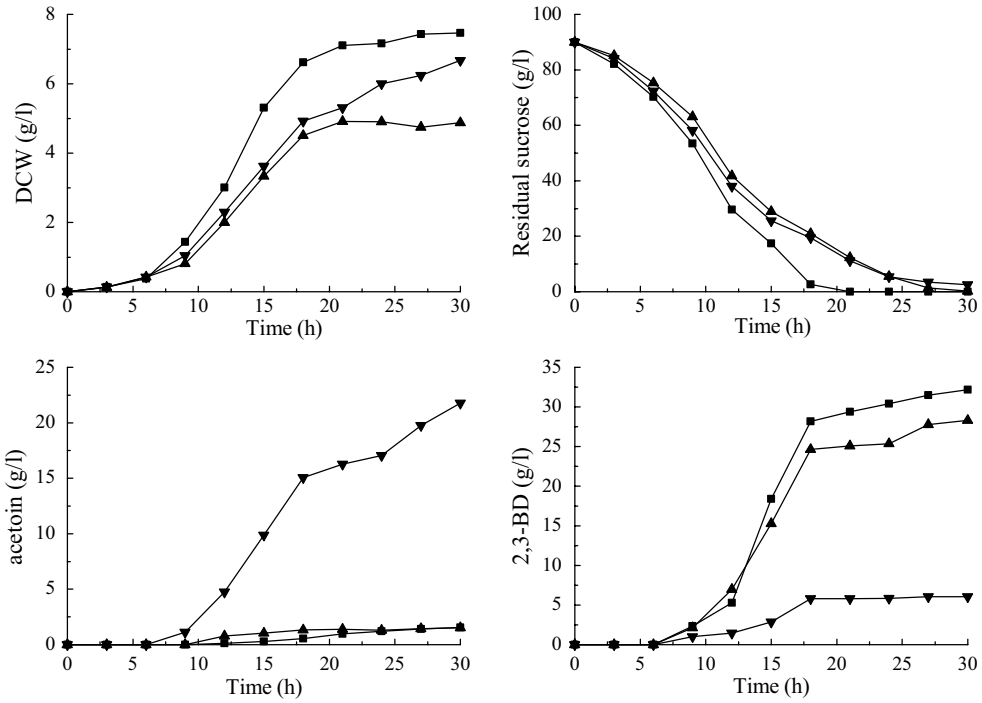

Fig. 2 DCW, residual sucrose and metabolic products of *S. marcescens* MG1 (*square*), *S. marcescens-ΔslaC* (*inverted triangle*) and *S. marcescens-ΔslaC-bdhA* (*upright triangle*)

Considering (2*S*,3*S*)-2,3-BD from 3*S*-acetoin, therefore, *meso*-2,3-BD was formed from 3*R*-acetoin as substrate.

Effects of *slaC* inactivation on cell growth and metabolic profiles

Figure 2 demonstrated the effects of *slaC* inactivation on cell growth and metabolic profiles in batch culture. The maximum cell concentration (6.67 g/l) of the *slaC* mutant strain was lower than that of the wild-type strain MG1 (7.47 g/l), which indicated that the cell growth was somewhat impaired due to *slaC* inactivation. Similar to cell growth, the sucrose consumption rate for *S. marcescens-ΔslaC* appeared significantly lower when compared with that of the wild-type strain MG1. The sucrose was completely exhausted by the wild-type strain MG1 at 21 h, whereas for *S. marcescens-ΔslaC* the residual sucrose of 2.56 g/l remained in the broth after 30 h. This might be because excess NADH produced by *S. marcescens-ΔslaC* led to the decrease of bacterial glycolysis rate. As described in previous studies, 2,3-BD production played a major role in oxidizing NADH and regulated the intracellular redox balance during sugar metabolism [18]. Inactivation of the *slaC* gene in *S. marcescens* MG1 blocked the 2,3-BD biosynthesis pathway and resulted in NADH accumulation, thus inhibiting the glycolysis pathway of *S. marcescens*. For AC and 2,3-BD production, inactivation of the *slaC* gene could result in a large quantity of AC accumulation (21.78 g/l), implying that the *slaC* gene played a major role in 2,3-BD biosynthesis in *S. marcescens* MG1. However, *S. marcescens-ΔslaC* still produced a small amount of 2,3-BD (6.07 g/l), which suggested that another enzyme involved in 2,3-BD formation might exist in *S. marcescens* MG1. According to our previous study [24] and another study [16], glycerol dehydrogenase was proved to be involved in 2,3-BD formation. Therefore, it is possible that glycerol dehydrogenase was responsible for the formation of 2,3-BD in the *ΔslaC* mutant. The production of *meso*-2,3-BD and (2*R*,3*R*)-2,3-BD in the *ΔslaC* mutant also support the hypothesis. As shown in Fig. 3 and Table 3, 21.78 g/l of (3*R*)-AC with 1.45 g/l *meso*-2,3-BD and 4.62 g/l (2*R*,3*R*)-2,3-BD was produced by *S. marcescens-ΔslaC* at 30 h. (2*S*,3*S*)-2,3-BD and (3*S*)-AC could not be detected in the broth of *S. marcescens-ΔslaC*. In the broth of the wild-type strain MG1, 30.58 g/l of *meso*-2,3-BD with a small amount of (2*R*,3*R*)-2,3-BD (0.67 g/l), (2*S*,3*S*)-2,3-BD (0.92 g/l) and (3*R*)-AC (1.39 g/l) could be observed at 30 h. These results indicated that optically pure 3*R*-acetoin production could be achieved by *S. marcescens-ΔslaC*.

Effects of *slaC* inactivation and expressed *bdhA* on cell growth and metabolic profiles

The above results showed that *meso*-BDH encoded by the *slaC* gene from *S. marcescnes* MG1 were responsible for

meso-2,3-BD and (2*S*,3*S*)-2,3-BD biosynthesis and *slaC* inactivation could lead to optically pure (3*R*)-AC accumulation. Here, we attempted to introduce a (2*R*,3*R*)-BDH enzyme encoded by the *bdhA* gene from *B. subtilis* 168 into *S. marcescens-ΔslaC* for (2*R*,3*R*)-2,3-BD production, since the (2*R*,3*R*)-BDH enzyme showed the ability of the conversion from (3*R*)-AC to (2*R*,3*R*)-2,3-BD and diacetyl to (2*R*,3*R*)-2,3-BD via (3*R*)-AC with concomitant oxidation of NADH to NAD^+. The expression vector pUC-pbdhA was constructed and transformed into *S. marcescens-ΔslaC* as described in "Materials and methods". The obtained recombinant strain designated as *S. marcescens-ΔslaC-bdhA* was cultured in the fermentation medium to analyze plasmid stability, expression of the *bdhA* gene and its metabolic profiles. As shown in Fig. 4, the fraction of plasmid-containing cells remained about 93 % at 30 h, suggesting that the vector pUC-pbdhA was stably expressed in *S. marcescens-ΔslaC*. SDS-PAGE analysis showed that (2*R*,3*R*)-BDH was successfully expressed in the *S. marcescens-ΔslaC* (Fig. 4).

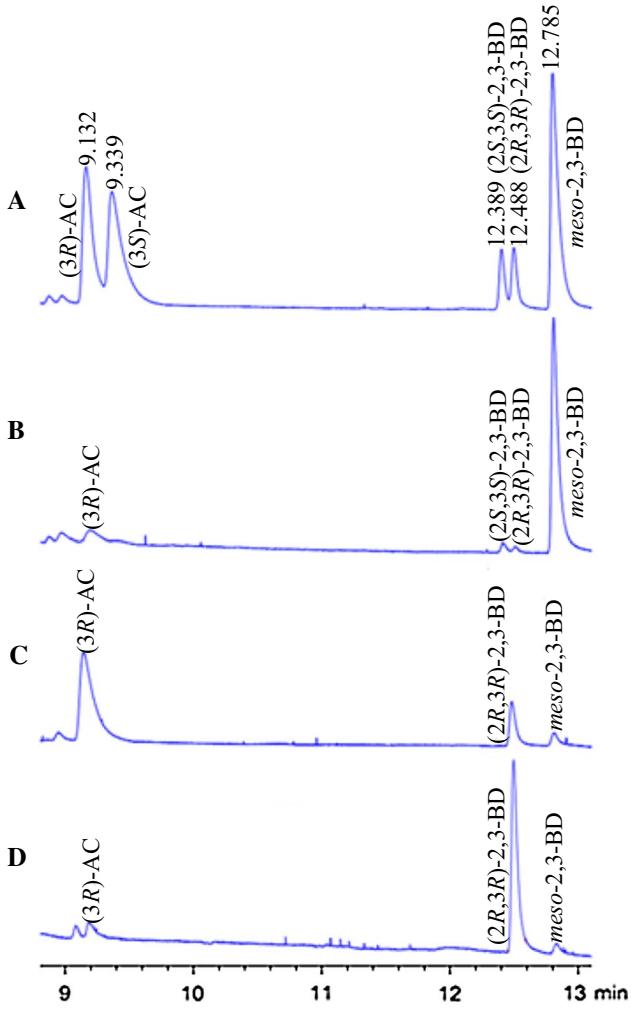

Fig. 3 Chiral-column GC analysis of the products by *S. marcescnes* MG1 and its derivatives. **a** Profile of mixture of standard chemicals. **b** Fermentation products by *S. marcescens* MG1. **c** Fermentation products by *S. marcescens-ΔslaC*. **d** Fermentation products by *S. marcescens-ΔslaC-bdhA*

The effects of *bdhA* gene expression in *S. marcescens-ΔslaC* on cell growth, sucrose consumption, AC and 2,3-BD production were investigated in 250-ml flask containing 50 ml of fresh fermentation medium. As shown in Fig. 2, the maximum cell concentration (4.87 g/l) of *S. marcescens-ΔslaC-bdhA* was obviously lower than that of the wild-type strain MG1 due to *slaC* inactivation and *bdhA* gene expression. Similar to *S. marcescens-ΔslaC*, the sucrose consumption rate of *S. marcescens-ΔslaC-bdhA* was lower than that of the wild-type strain MG1, even though BDH was working to consume NADH for BDO biosynthesis. For AC and 2,3-BD production, excess (2*R*,3*R*)-BDH in *S. marcescens-ΔslaC* could increase 2,3-BD yield and decrease AC accumulation. Ultimately, 28.31 g/l of 2,3-BD with a small amount of 1.53 g/l AC could be produced by *S. marcescens-ΔslaC-bdhA* at 30 h, which indicated that *slaC* inactivation could be relieved by *bdhA* gene expression. Configuration analysis of the products by *S. marcescens-ΔslaC-bdhA* was also determined by GC with a chiral column. As shown in Fig. 3 and Table 3, 27.56 g/l of (2*R*,3*R*)-2,3-BD occupying a weight fraction of 97.4 % could be produced by *S. marcescens-ΔslaC-bdhA* at 30 h, showing that most of (3*R*)-AC was converted into (2*R*,3*R*)-2,3-BD by (2*R*,3*R*)-BDH. Additionally, a small amount of *meso*-2,3-BD (0.75 g/l) was observed in the

Table 3 Comparison of the fermentation of *S. marcescens* MG1 and its derivatives

Strains (g/l)	*S. marcescens* MG1	*S. marcescens-ΔslaC*	*S. marcescens-ΔslaC-bdhA*	
			In flask	Fed-batch
Cell dry weigh	7.43 ± 0.12	6.64 ± 0.11	4.84 ± 0.03	10.85 ± 0.15
Sucrose consumed	90.00 ± 0.37	87.44 ± 1.31	89.72 ± 0.73	255.05 ± 2.16
(3*R*)-AC	1.39 ± 0.06	21.78 ± 0.99	1.53 ± 0.03	1.98 ± 0.07
(3*S*)-AC	ND[a]	ND	ND	ND
meso-2,3-BD	30.58 ± 0.72	1.45 ± 0.01	0.75 ± 0.01	2.11 ± 0.06
(2*S*,3*S*)-2,3-BD	0.92 ± 0.03	ND	ND	ND
(2*R*,3*R*)-2,3-BD	0.67 ± 0.01	4.62 ± 0.07	27.56 ± 0.44	89.81 ± 1.13

[a] Not detected

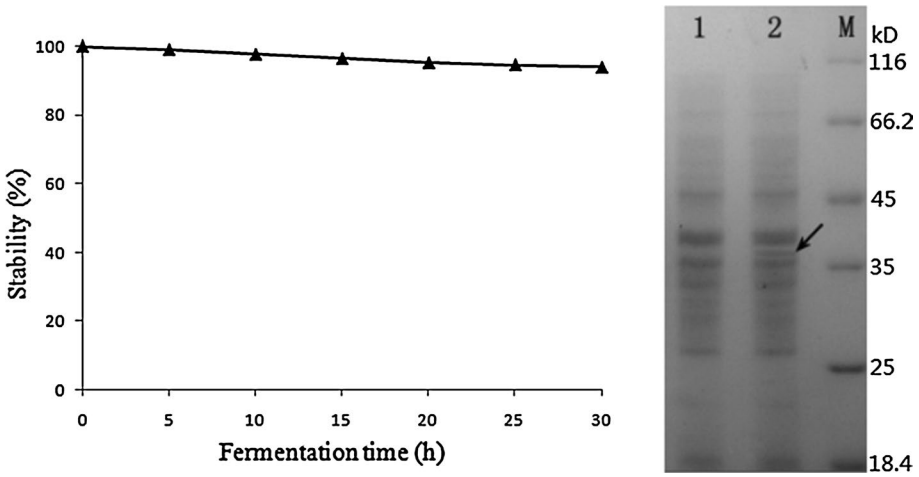

Fig. 4 Stability of pUC19-pbdhA and SDS-PAGE analysis of expressed *R*,*R*-BDH in *S. marcescens-ΔslaC-bdhA*. *Lane M* protein marker (116, 66.2, 45, 35, 25, 18.4 kDa), *lane 1* soluble protein from the cell lysate of *S. marcescens-ΔslaC*, *lane 2* soluble protein from the cell lysate of *S. marcescens-ΔslaC-bdhA*

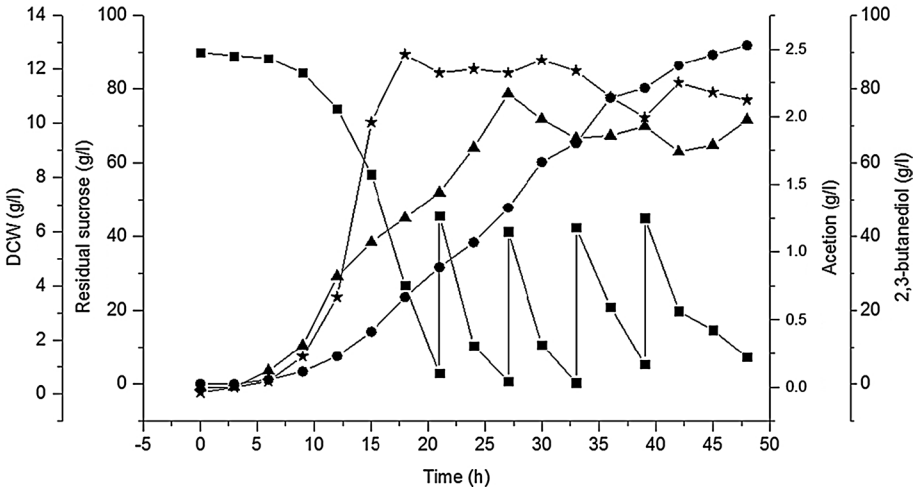

Fig. 5 Time course of 2,3-butanediol production by fed-batch culture of the *S. marcescens-ΔslaC-bdhA* strain. DCW (*filled stars*), residual sucrose (*filled squares*), 2,3-BD (*filled circles*) and AC (*filled upright triangles*)

broth. The reason might be related to another enzyme existing in *S. marcescens* MG1. Further identification of another enzyme would be helpful for optically pure (2*R*,3*R*)-2,3-BD production.

Fed-batch fermentation with *S. marcescens-ΔslaC-bdhA* strain

To improve the final concentration of (2*R*,3*R*)-2,3-BD, the strain *S. marcescens-ΔslaC-bdhA* was used to perform fed-batch culture in a 7-l bioreactor with an initial broth volume of 3.0 l. As shown in Fig. 5 and Table 3, the maximum cell concentration was 12.56 g/l, which was more than twice that of shake flask culture, indicating that the engineered strain could also grow to a higher density in a more suitable environment. When the residual sucrose decreased below 5 g/l, a fed-batch culture was performed to increase the residual sucrose concentration to about 40 g/l. Finally, 255.05 g/l sucrose was consumed and 89.81 g/l of (2*R*,3*R*)-2,3-BD with 2.11 g/l of *meso*-2,3-BD was produced by *S. marcescens-ΔslaC-bdhA* at 48 h. The productivity and yield of (2*R*,3*R*)-2,3-BD were 1.91 g/l/h and 0.35 g (2*R*,3*R*)-2,3-BD/g sucrose, respectively. These results suggested that the *S. marcescens-ΔslaC-bdhA* strain had potential to improve its (2*R*,3*R*)-2,3-BD production and might be a candidate strain for large-scale production of (2*R*,3*R*)-2,3-BD with high optical purity. In addition, the AC concentration was just 1.98 g/l, showing again that the exogenous (2*R*,3*R*)-BDH was very efficient in converting AC to 2,3-BD.

Conclusion

In this study, a *meso*-BDH enzyme encoded by the *slaC* gene from *S. marcescens* MG1 was identified and responsible for *meso*-2,3-BD and (2*S*,3*S*)-2,3-BD formation. Inactivation of the *slaC* gene could lead to a large quantity of optically pure (3*R*)-AC accumulation, while (2*R*,3*R*)-BDH from *B. subtilis* 168 introduced in the *slaC* mutant strain

could result in (2*R*,3*R*)-2,3-BD accumulation which occupied with a weight fraction of 97.4 %. Therefore, the two recombinant strains might be potential alternatives for optically pure (3*R*)-AC and (2*R*,3*R*)-2,3-BD production.

Acknowledgments This work was supported by Shanghai Leading Academic Discipline Project (project B505) and National Special Fund for State Key Laboratory of Bioreactor Engineering (no. 2060204).

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