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Characterization of acetoin production in a *budC* gene disrupted mutant of *Serratia marcescens* G12

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Abstract The 2,3-butanediol (2,3-BD) dehydrogenase gene *budC* of *Serratia marcescens* G12 was disrupted to construct the acetoin (AC) producing strain G12M. In shake-flask cultures, AC production was enhanced by increased concentrations of glucose or sodium acetate in G12M. In fed-batch fermentation, G12M produced 47.5 g/L AC along with 9.8 g/L 2,3-BD. The expression of the key enzymes for AC synthesis was further investigated. Alpha-acetolactate synthase gene *budB* decreased its expression significantly in G12M compared with G12. This probably explained the moderate AC production in G12M cultures. Additionally, overexpression of *budB* gene and α-acetolactate decarboxylase gene *budA* was conducted in G12M and no significant increase of AC was observed. The results suggested that intracellular AC accumulation might inhibit the expression of *budB* and *budA* gene and induce *budC* gene expression in G12M. Our analyses offered the bases for further genetic manipulations in improving AC production in microbial fermentations.

Keywords Acetoin · *Serratia marcescens* · *budC* mutant · Expression

Introduction

Acetoin (AC) widely exists in fruits, harvests, meats, and dairy products. It is used in the production of flavor agents, drug carriers, and chemical materials. Currently, AC is mainly produced by chemical synthetic methods. However, microbial production of AC may offer an attractive alternative to fossil-based feedstock production [3].

Many microorganisms synthesize AC and 2,3-butanediol (2,3-BD) during the mixed acids fermentation process, mainly including *Saccharomyces cerevisiae*, *Klebsiella oxytoca*, *K. pneumonia*, *Alcaligenes eutrophus*, *Lactococcus lactis*, *Bacillus subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *Serratia marcescens* [3].

Acetoin is an intermediate metabolite of the 2,3-BD biosynthesis pathway. To produce AC purposely, various strategies were employed to minimize the level of the end product 2,3-BD in fermentations. With a two-stage agitation speed control strategy, strain *B. amyloliquefaciens* FMME044 produced 51.2 g/L AC [24]. A similar agitation speed control method was also applied in *S. marcescens* H32 fermentations, 44.9 g/L AC was produced with a relatively high productivity (1.73 g/L/h) [18]. A high amount (56.7 g/L) of pure (*3S*)-AC was obtained in the fermentations using resting cells of *K. pneumonia* CICC 10011 and *B. subtilis* 168 [6]. Genetic manipulation has been employed to introduce the water-forming NADH oxidase gene *nox* from *Lactobacillus brevis* into *S. marcescens* H32 and the resulting strain was able to synthesize 75.2 g/L AC in the fed-batch cultures [17]. *B. subtilis* mutants producing various amounts of AC in fermentation cultures were also obtained in the multiple mutagenesis induced by UV irradiation and or chemical treatments, the strains were identified as 2,3-BD dehydrogenase blocked mutants [7, 23].

Serratia marcescens G12 was characterized in our lab and it produced a relatively high amount of 2,3-BD (87.8 g/L) in the fed-batch fermentation [15]. In this work, 2,3-BD dehydrogenase gene (*budC*) of strain G12 was knocked out by using a suicide vector. The resulting strain

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G12M was characterized for AC productions in fermentations. The expression level of the key enzymes in the 2,3-BD biosynthetic pathway was also investigated to elucidate the underlying mechanism for AC synthesis. Furthermore, overexpression of key enzymes was conducted in G12M.

Materials and methods

Bacterial strains, plasmids, media, and growth conditions

The strains and plasmids used in this study are listed in Table 1. *S. marcescens* G12 was first isolated in our lab from raw bovine milk samples and deposited in the China General Microbiological Culture Collection Center with the collection number CGMCC No. 6770. The previous study had shown that G12 synthesized high levels of the diol (2,3-BD + AC) in fermentation trials [15].

LB medium was routinely used in cultivations of *Escherichia coli* and *S. marcescens* strains. The required antibiotics might be used as following concentrations: for *E. coli* strains, 100 μg/mL ampicillin, 5 μg/mL gentamicin, 25 μg/mL spectinomycin, and 12.5 μg/mL streptomycin; and for *S. marcescens* strains, 100 μg/mL gentamicin, 200 μg/mL spectinomycin, 100 μg/mL streptomycin, and 30 μg/mL tetracycline. Seed medium was composed of glucose 40 g/L, yeast extract 10 g/L, $(NH_4)_2SO_4$ 1 g/L, and K_2HPO_4 1 g/L. Fermentation medium was composed of glucose 130 g/L, yeast extract 5 g/L, peptone 12 g/L, $MnSO_4$ 0.025 g/L, and K_2HPO_4 1 g/L. *E. coli* cultures were incubated at 37 °C and *S. marcescens* cultures were incubated at 35 °C.

Primary seeding cultures were prepared by inoculating *S. marcescens* strains in 5 mL seed medium and cultivated for 12 h. Secondary seeding cultures were prepared by transferring primary seeding cultures into fresh seed medium with an inoculum size of 5 % and cultivated for 10 h. All cultures were incubated on a rotary shaker at a speed of 160 rpm.

Construction of *budC* gene knockout mutant of *S. marcescens* G12

Based on the genome sequence of *S. marcescens* Db11 which is available on the website http://www.sanger.ac.uk/resources/downloads/bacteria/serratia-marcescens.html, primers were designed to amplify the genes encoding the key enzymes of the 2,3-BD synthesis pathway. BudA-F1 (5′ CGCATTGCCAGTCATTGCCTGTTT 3′) and BudA-R1 (5′ CGGGTCTTTTGAACGCGTGGAACC 3′) were used to amplify α-acetolactate decarboxylase gene *budA*. BudB-F1 (5′ AATTCCGCGCATAAAAACGGGCA 3′) and BudB-R1 (5′ GTGGCCGGCTATCACGAACACTTC 3′) were used to amplify α-acetolactate synthase gene *budB*. BudC-F1 (5′ AAACCGCACGTTTTGACTGGCTCTG 3′) and BudC-R1 (5′ CCGTTCTTCGGTGGACGCAATCT 3′) were used to amplify 2,3-BD dehydrogenase gene *budC*. Amplicons were subjected to sequencing directly and gene sequences were analyzed for the similarities by BLAST searching.

Amplified *budC* gene fragments were cloned in vector pGEM-T Easy according to the manual provided by the

Table 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source
E. coli		
XL-1	*E. coli* clone strain	Stratagene
S17-1	RP4-2 Tc::Mu Km::Tn*7* Tp^r Sm^r Pro Res^- Mod^+	Stratagene
S. marcescens		
G12	Isolated from raw bovine milk, producing diol.	This study
G12M	*budC* gene disrupted by Ω fragment in strain G12, $Sp^{200}Sm^{200}$	This study
Plasmids		
pGEM-T Easy	Cloning vector, Ap^r	Promega
pUCP19	Broad-host-range shuttle vector, Ap^r	[14]
pEX18Gm	Gene replacement vector, Gm^r $oriT^+$ $sacB^+$	[4]
pDN19lacΩ	Promoterless lacZ fusion vector, Sp^r Sm^r Tc^r	[19]
pGS01	*budC* gene fragment cloned in pGEM-T Easy vector, Ap^r	This study
pGS02	*budC* gene with Ω fragment cloned in pGEM-T Easy, $Sp^rSm^rAp^r$	This study
pGS03	*budC* gene with Ω fragment cloned in pEX18Gm, $Sp^rSm^rGm^r$	This study
pGS04	*budB* gene promoter fragment cloned in pDN19lacΩ, Sp^r Sm^r Tc^r	This study
pGS05	*budC* gene promoter fragment cloned in pDN19lacΩ, Sp^r Sm^r Tc^r	This study
pGS06	*budA* gene cloned in pUP19, Ap^r	This study
pGS07	*budB* gene cloned in pUP19, Ap^r	This study
pGS08	*budC* gene cloned in pUP19, Ap^r	This study

manufacturer, resulting plasmid pGS01. Ω fragment, harboring spectinomycin and streptomycin resistance determinants, was cut off from pDN19lacΩ by restriction enzyme *Hin*dIII [19]. After filling in the cohesive ends, the fragment was inserted into the single *Eco*RV restriction site in the middle of *budC* gene on plasmid pGS01, resulting plasmid pGS02. The fragment containing *budC* gene with Ω insertion was further cloned into the suicide vector pEX18Gm by restriction site *Eco*RI [4], resulting plasmid pGS03 (Table 1). Plasmid pGS03 was transferred into *E. coli* S17-1 by electroporation. Then plasmid pGS03 were further transferred into strain *S. marcescens* G12 via conjugation. The single-crossover mutants were screened on L-agar plates containing $Sp^{25}Sm^{12.5}Gm^{5}Tc^{30}$. The double-crossover mutants were selected on L-agar plates containing 8 % sucrose and $Sp^{200}Sm^{100}Tc^{30}$. Strain G12 was resistant to tetracycline and this resistance was used for selection of G12 and its derivatives.

Construction of transcription fusion reporter genes

Gene *budB* and *budA* were controlled under the same promoter of an operon and gene *budC* was located in a separate position on the bacterial chromosome. The possible promoter regions of *budB* and *budC* genes were predicted with the tool provided by http://www.fruitfly.org/seq_tools/promoter.html [13]. Primers were designed to amplify promoter regions for construction transcription fusion reporter genes. BudB-F2 (5′ CGCGGATCCCGGAATACC GAAAACGTGTTTGAC 3′) and BudB-R2 (5′ CCGG AATTCGCGAAATCTATCAAATCTCGCTGAT 3′) were used to amplify the promoter region of *budB* gene. BudC-F2 (5′ CCGGAATTCTTAAGGTGCATAAGAAACATGCGG 3′) and BudC-R2 (5′ CGCGGATCCCAAGGCAAATTTG-GAACAGAACAC 3′) were used to amplify the promoter region of *budC* gene. The underlined sequences represent restriction sites *Eco*RI and *Bam*HI, respectively. The amplified fragments were digested with both restriction enzymes *Eco*RI and *Bam*HI, and then cloned in plasmid pDN19lacΩ digested with the same restriction enzymes. The constructed plasmids were pGS04 and pGS05 (Table 1).

Construction of overexpression vectors

To construct overexpression vectors, *budA*, *budB*, and *budC* genes were amplified, subsequently cloned into plasmid pGEM-T Easy, and then subcloned in *Eco*RI restriction site of plasmid pUCP19 [14] (Table 1). BudA-F2 (5′ CGCGGATCCCGAGATTTGATAGATTTCGCCCTC 3′) and BudA-R1 were used to amplify whole *budA* gene. BudB-F2 and BudB-R1 were used to amplify whole *budB* gene. BudC-F2 and BudC-R1 were used to amplify whole *budC* gene. The amplified genes were further cloned into the broad-host-range vector pUCP19, resulting in plasmid pGS06 with *budA* gene, pGS07 with *budB* gene, and pGS08 with *budC* gene (Table 1). Since *budA* gene did not have its own promoter, transcription was driven by the *Plac* promoter of the vector. Both *budB* and *budC* genes were driven by their own promoter. The recombinant plasmids were transferred into *S. marcescens* strains by electroporation.

Batch and fed-batch fermentations

Shake-flask fermentations were carried out in 250-mL standard flasks with 30 mL fermentation medium on a rotary shaker. All experiments were tested in triplicate.

After 72 h incubation, samples were collected for analyses. The fed-batch fermentation was conducted in a 5-L bioreactor with a broth volume of 3 L (60 %, v/v). The aeration rate was 2.5 vvm and the agitation speed was controlled at 400 rpm. The residual glucose concentration was maintained at 10–20 g/L in fed-batch fermentations. The value of pH was controlled at 7.0 in the first stage of fermentation. Whenever bacterial cultures entered stationary phase, pH was switched to 6.0 and the agitation speed was decreased to 300 rpm.

Analytical methods

The β-galactosidase activity assay was carried out as described previously [22]. The glucose concentration was measured by the biosensor SBA-40C [11]. Gas chromatography system Agilent 7890A coupled with column HP–INNOWAX was used to measure the concentrations of 2,3-BD and AC. The analysis was performed according to the manual from the manufacturer. The optical density of fermentation culture was measured at the wavelength of 600 nm.

Accession numbers

Nucleotide sequences of *budA*, *budB*, and *budC* genes were deposited in GenBank with the accession numbers KF547936, KF547937, and KF547938, respectively.

Results

Sequence analysis of *budA*, *budB*, and *budC* genes of strain G12

To obtain the detailed sequence information for subsequent genetic manipulations, PCR products of *budA*, *budB*, and *budC* genes were sequenced directly and the sequences

Table 2 Confirmation of the *budC* gene disrupted mutant

Strain	AC (g/L)	2,3-BD (g/L)
E. coli S17-1	0	0
S. marcescens G12	6.8	29.4
S. marcescens G12M	30.0	2.7
S. marcescens G12M/pUCP19	31.3	4.1
S. marcescens G12M/pGS08[a]	2.1	39.5

[a] The plasmid pGS08 contains the whole *budC* gene with its own promoter on the vector pUCP19

were deposited in GenBank with the accession numbers KF547936, KF547937, and KF547938, respectively. The sequences were further analyzed by alignment using Clustal Omega software [16]. Between strain G12 and Db11, the amino acid sequences of BudA were identical, one semi-conserved substitution of S338N was observed in the amino acid sequence of BudB, and one conserved substitution of I101V was observed in the amino acid sequence of BudC. The results showed that three genes were highly conserved between the two strains.

Construction of *budC* gene knockout mutant

2,3-BD dehydrogenase catalyzed the transformation reaction from AC to 2,3-BD. Knockout of its encoding gene *budC* would generate the strain mainly producing AC. The *budC* gene disrupted mutant was constructed as described in "Materials and methods". The double-crossover mutant G12M was confirmed with PCR (data not shown). The AC production of strain G12M was further analyzed in the shake-flask fermentations. G12M produced 30.0 g/L AC and 2.7 g/L 2,3-BD, while the parent strain G12 produced 6.8 g/L AC and 29.4 g/L 2,3-BD. Complementation of *budC* restored the production level of 2,3-BD to 39.5 g/L in strain G12M/pGS08 (Table 2). The results demonstrated that *budC* gene was successfully disrupted by Ω fragment in strain G12M.

The effect of glucose concentrations on AC synthesis

To select suitable glucose concentration for AC synthesis by G12M, a variety of glucose concentrations were tested in shake-flask fermentations, and strain G12 was used as control. When initial glucose concentration was 70 g/L, strain G12 synthesized 18.1 g/L AC and 7.9 g/L 2,3-BD (Fig. 1a); while mutant G12M produced 23.2 g/L AC and 3.6 g/L 2,3-BD (Fig. 1b). When initial glucose concentration reached 140 g/L, strain G12 synthesized 1.9 g/L AC and 39.9 g/L 2,3-BD (Fig. 1a); while mutant G12M produced 37.8 g/L AC and 5.9 g/L 2,3-BD (Fig. 1b). With the increased initial glucose concentrations, the final pH of

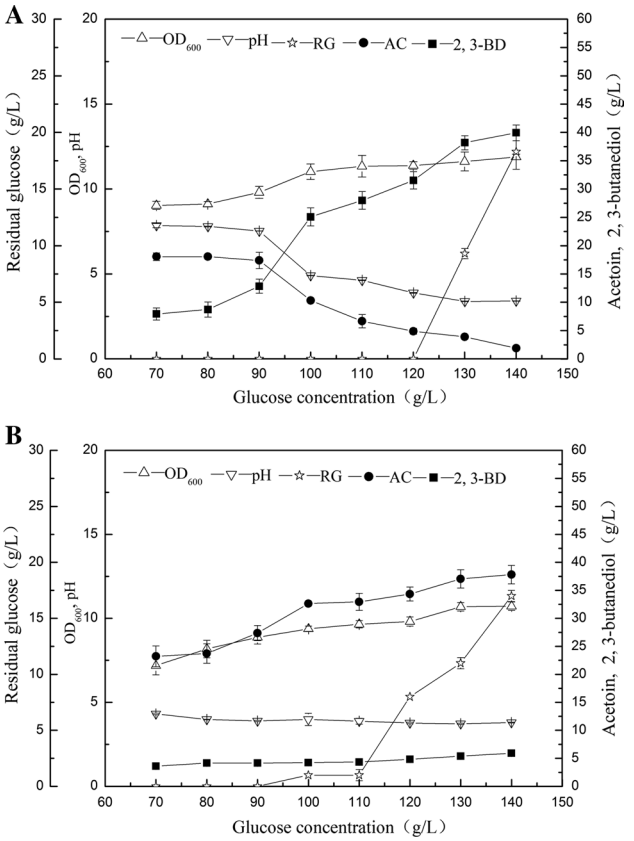

Fig. 1 Effects of different initial glucose concentrations on AC production in the shake-flask fermentations. **a** fermentation of strain *S. marcescens* G12, **b** fermentation of strain *S. marcescens* G12M. *AC* acetoin, *2,3-BD* 2,3-butanediol, *RG* residual glucose, *pH* acidity, OD_{600} the optical density measured at the wavelength of 600 nm

fermentation broth dropped down gradually from 7.9 to 3.4 with the significantly decreased AC production by strain G12 (Fig. 1a). Different from strain G12, pH values in all fermentations by G12M ranged narrowly from 4.3 to 3.7 (Fig. 1b), indicating that strain G12M might produce more acids than its parent strain G12. The results showed that increased glucose concentrations enhanced 2,3-BD production in the parent strain G12 and AC production in *budC* gene mutant strain G12M, respectively.

The effect of sodium acetate on AC synthesis

Various amounts of sodium acetate were added to fermentation medium with 130 g/L glucose to investigate its effect on AC production. In the group with 5 g/L sodium acetate, strain G12 produced 17.0 g/L AC and 29.8 g/L 2,3-BD (Fig. 2a); while G12M produced 43.5 g/L AC, higher than 36.7 g/L of the control group without sodium acetate (Fig. 2b). In cultures of strain G12, fermentations with 3 g/L or more sodium acetate had pH values >8.5 (Fig. 2a); while in cultures of mutant G12M, with the increase of sodium

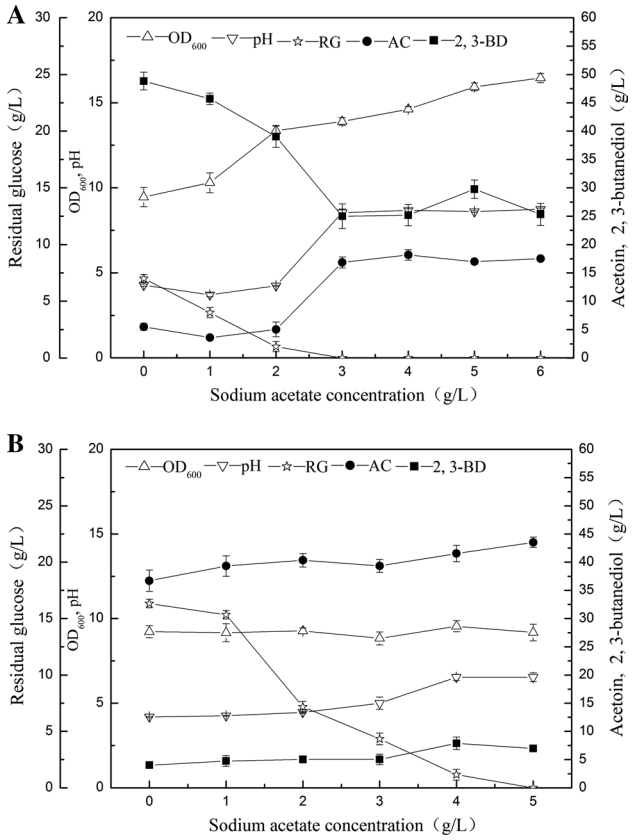

Fig. 2 Effects of different sodium acetate concentrations on AC production in the shake-flask fermentations. **a** Fermentation of strain *S. marcescens* G12, **b** fermentation of strain *S. marcescens* G12M. *AC* acetoin, *2,3-BD* 2,3-butanediol, *RG* residual glucose, *pH* acidity, OD_{600} the optical density measured at the wavelength of 600 nm

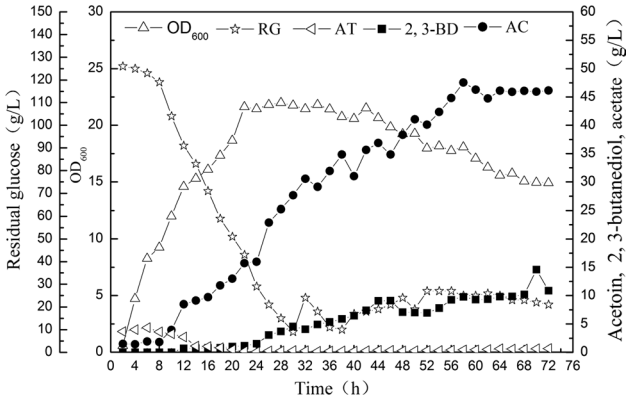

Fig. 3 AC production of *S. marcescens* G12M in the fed-batch fermentation. *AC* acetoin, *2,3-BD* 2,3-butanediol, *RG* residual glucose, *AT* acetate, *pH* acidity. OD_{600} the optical density measured at the wavelength of 600 nm

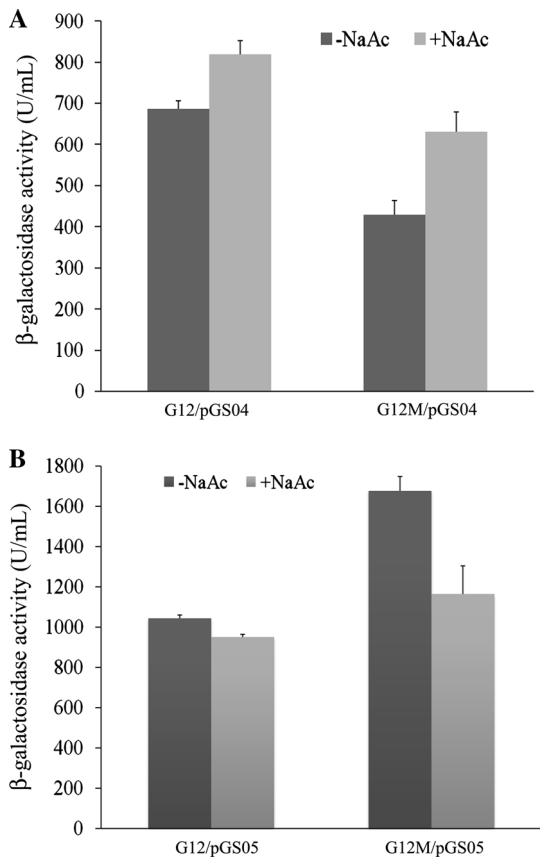

Fig. 4 Expression assays. **a** The expression analysis of the reporter gene *budB-lacZ*, **b** the expression analysis of the reporter gene *budC-lacZ*. G12: the parent strain *S. marcescens* G12. *G12M* the *budC* gene disrupted mutant *S. marcescens* G12M, *pGS04* the plasmid carrying the reporter gene *budB-lacZ*, *pGS05* the plasmid carrying the reporter gene *budC-lacZ*, *NaAc* sodium acetate

acetate concentration, the fermentation broth acidity varied from 4.3 to 6.5 as AC production increased (Fig. 2b).

AC production in the fed-batch fermentation of G12M

Based on the above analyses, the fed-batch fermentation was carried out for AC production by strain G12M. The medium contained 130 g/L glucose and 5.0 g/L sodium acetate. The biomass reached the maximum amount with the OD_{600} value of 21.6 at 22 h. The fermentation broth acidity was switched from pH 7.0 to 6.0 at 24 h to favor AC production. After 58 h fermentation, AC production reached at 47.5 g/L with 9.8 g/L 2,3-BD. The highest productivity for AC was relatively low, about 0.8 g/L h. Compared with the yield in the shake-flask fermentations, G12M produced 9.2 % more AC in the fed-batch fermentation (Fig. 3).

Expression levels of *budB* and *budC* genes

In *budC* gene knockout strain, AC production was not as high as expected. To further elucidate the regulation mechanism underlying the diol production. Expression levels of *budB* and *budC* genes were investigated in both G12 and G12M strains after 24 h cultivation. The expression of

budB gene was significantly lower in strain G12M than in strain G12, 37.5 % in the absence of sodium acetate and 22.9 % in the presence of sodium acetate; while sodium acetate increased *budB* gene expression by 19.1 % in strain G12 and 46.7 % in strain G12M, respectively (Fig. 4a). However, sodium acetate had different effects on *budC* gene expression profiles. In the presence of sodium acetate, the expression level of *budC* gene was decreased slightly in strain G12 (8.9 %) and significantly in strain G12M (30.5 %), respectively (Fig. 4b). The results suggested that low expression of *budB* gene might be responsible for the low production of AC in G12M.

Overexpression of *budA*, *budB*, and *budC* genes to enhance AC production

The genes *budA*, *budB*, or *budC* were overexpressed in the parent and *budC* gene mutant strains, respectively. In strain G12, compared with the control carrying vector pUCP19 only, no significant increase of AC production was observed in the strains carrying *budA*, *budB*, or *budC* genes; while for 2,3-BD production, overexpression of *budA* and *budB* gene gave 14.6 and 15.7 % increases, respectively, higher than 4.4 % increase with *budC* gene overexpression. In strain G12M, over expression of *budA* or *budB* genes did not enhance 2,3-BD production significantly; while for AC production, only 5.8 % increase was observed in the strain with *budB* gene overexpression (Fig. 5).

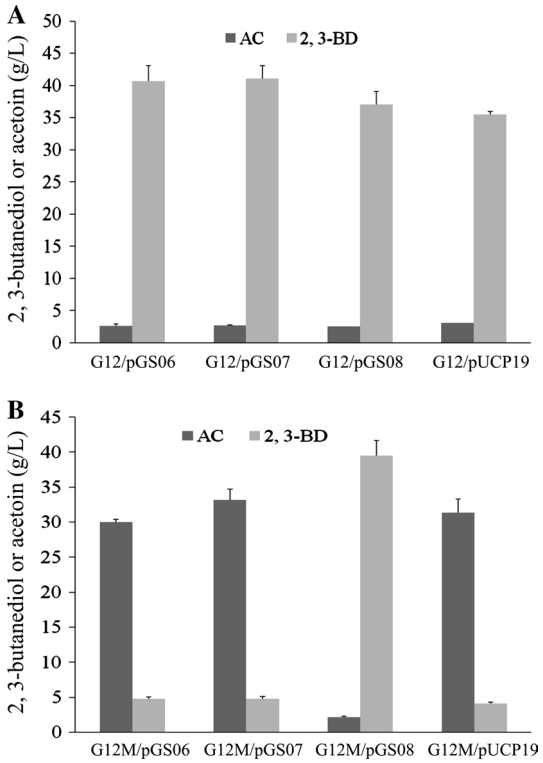

Fig. 5 Overexpression assays. **a** Overexpression assay in strain *S. marcescens* G12, **b** overexpression assay in strain *S. marcescens* G12M. G12: the parent strain *S. marcescens* G12. *G12M* the *budC* gene disrupted mutant *S. marcescens* G12M, *pUCP19* the broad-host-range vector plasmid, *pGS06* the plasmid carrying gene *budA* driven by P_{lac} promoter on the vector, *pGS07* the plasmid carrying gene *budB* driven by its own promoter, *pGS08* the plasmid carrying gene *budC* driven by its own promoter

Discussion

The genes encoding the key enzymes of the 2,3-BD biosynthetic pathway have been characterized in a variety of microorganisms [2, 10, 12]. Quorum sensing system has been confirmed regulating 2,3-BD synthesis in several species, including *Aeromonas hydrophila* AH-1N [20], *S. subtilis* [10], *Vibrio cholera* [5], *S. plymuthica* RVH1 [8], and *S. marcescens* MG1 [21]. Regulators are also identified for modulation the expression of the key enzymes of 2,3-BD biosynthetic pathway. In *Serratia* species, BudR (SlaR) and SwrR are the major regulators controlling the expression of *budB* and *budA* gene [8, 12].

AC and 2,3-BD were synthesized simultaneously during fermentations, though the ratio of AC to 2,3-BD might be varied among studies with different fermentation strategies. In present work, *budC* gene was inactivated by insertion of an antibiotic selection marker Ω fragment. AC was the end product of 2,3-BD biosynthetic pathway of the strain G12M. In the fed-batch fermentations, G12M produced the relatively high amount of AC 47.5 g/L with the optimization of glucose and sodium acetate concentration. However, the engineering strain G12M did not yield AC at the level as high as expected. The underlying mechanism was investigated by analyzing the expression of the related genes. In G12M, knockout of *budC* gene of G12M caused the accumulation of the intermediate product AC inside the bacterial cells, and the feedback inhibition might happen and down regulated the expression of *budB* gene in G12M (Fig. 4a). *BudA* gene was located downstream of *budB* gene in the same operon and its expression was assumed to be at the same level as *budB* gene. The decrease of the two key enzymes might possibly explain the relatively low AC production in G12M. On the other hand, AC was the substrate of 2,3-BD dehydrogenase and its accumulation might have induction effect and up regulated the expression of *budC* gene (Fig. 4b), which was separately located from *budB* and *budA* genes [12]. Additionally, low pH values was observed in fermentation cultures of G12M (Figs. 1b, 2b), indicating carbon flux distribution was switched to the pathways for acids production possibly due to the negative feedback caused by AC accumulation.

Sodium acetate increased *budB* gene expression in both parent and mutant strains (Fig. 4a), while *budC* gene expression was not affected by sodium acetate in the parent

strain G12 (Fig. 4b). The results were consistent with the previous findings [12]. However, acetate also had reverse effects on 2,3-BD production, high concentration of acetate (200 mM) had been shown to inhibit 2,3-BD synthesis in *Paenibacillus polymyxa* [9]. In our work, *budC* gene expression was significantly reduced in the presence of sodium acetate in G12M, indicating the acetate inhibition could be working through down-regulation of *budC* gene expression via an unknown regulatory pathway (Fig. 4b).

In strain G12, overexpression of *budA*, *budB*, or *budC* genes gene gave 14.6, 15.7, or 4.4 % increase of 2,3-BD production, respectively (Fig. 5a). The increases were much less than overexpression of the counterpart gene *bdhA*, in *B. subtilis* [1]. The regulatory mechanisms on 2,3-BD production might be different between the two species. In strain G12M, over expression of *budA* or *budB* genes did not affect AC production (Fig. 5b), also suggesting the metabolic balance existing in AC production. In the fermentation cultures of G12M, 2,3-BD was still detected at relatively low and fixed levels. Similar results have been reported in *B. subtilis* and *S. marcescens* strains, indicating there might be another unknown 2,3-BD biosynthetic pathway or another undiscovered protein with 2,3-BD dehydrogenase activity [10, 12]. For pure AC production, its probably necessary to unveil and block the hypothetical alternative pathway to 2,3-BD synthesis in microbial conversions.

Conclusion

In this work, AC synthesis was achieved by knocking out *budC* gene in *S. marcescens* G12. The regulatory mechanisms on AC production were also investigated. Our results indicated that more extensive genetic research may be needed for further improvement of AC production in microbial fermentations.

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