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ORIGINAL PAPER

Microbial production of 2,3-butanediol by a surfactant (serrawettin)-deficient mutant of *Serratia marcescens* H30

Liaoyuan Zhang · Jian'an Sun · Yingli Hao · Jiawen Zhu · Ju Chu · Dongzhi Wei · Yaling Shen

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Abstract Serrawettin W1 produced by *Serratia marcescens* is a surface-active exolipid resulting in a lot foam formation during the 2,3-butanediol (2,3-BD) fermentation process. In order to avoid excessive addition of antifoam agent and microbial contamination, *S. marcescens* mutants deficient in serrawettin W1 formation were successfully constructed through insertional inactivation of the *swrW* gene coding for serrawettin W1 synthase. The shake flask and batch experiments suggested that disruption of the *swrW* gene led to significant reduction of the foam formation and improved 2,3-BD production a little. Ultimately, fed-batch culturing of the mutant afforded a maximum 2,3-BD concentration of 152 g l^{-1} with a productivity of 2.67 g l^{-1} h^{-1} and a yield of 92.6% at 57 h.

Keywords 2,3-Butanediol · Serrawettin W1 synthase · Inactivation · *Serratia marcescens* · Fed-batch

L. Zhang · J. Sun · Y. Hao · J. Chu · D. Wei (✉) · Y. Shen (✉)
State Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, People's Republic of China
e-mail: dzhwei@ecust.edu.cn

Y. Shen
e-mail: ylshen@ecust.edu.cn

J. Zhu
Chemical Engineering Research Center, East China University of Science and Technology, Shanghai 200237, People's Republic of China

Introduction

The production of bio-based bulk chemicals from renewable resources has recently attracted increasing attention due to petroleum resources depletion and environmental pollution. 2,3-Butanediol (2,3-BD) is one such promising bulk chemical owing to its extensive industrial applications [6, 14]. The dehydration of 2,3-BD yields the industrial solvent methylethyl ketone, and further dehydration produces 1,3-butadiene for the manufacture of synthetic rubber [12]. As a result of its low freezing point of −60°C, 2,3-BD is used as an antifreeze agent [2]. It can also be easily dehydrogenated into acetoin (AC) and diacetyl which are flavoring agents used in dairy products, margarines, and cosmetics [5]. In addition, 2,3-BD has potential applications in the manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, and as a carrier for pharmaceuticals [14].

2,3-BD can be produced from carbohydrates via the mixed acid fermentation pathway by many bacterial species such as *Klebsiella pneumoniae* [15], *Bacillus polymyxa* [4], and *Serratia marcescens* [1]. In a previous study, we reported that *S. marcescens* H30, a pigmented Gram-negative enteric organism, was a potential strain for efficient production of 2,3-BD [16]. Its production by *S. marcescens* is at least partially regulated by *N*-acyl-L-homoserine lactone-dependent quorum sensing [13]. However, the strain produces 2,3-BD with simultaneous production of lipopeptide surfactants named serrawettins [9, 10], resulting in a lot of foam formation during the 2,3-BD fermentation process. Foam formation creates potential for microbial contamination, leads to the need for addition of large amounts of antifoam agent, and decreases bacterial activity. Previous reports showed that three serrawettins (serrawettin W1, W2, and W3) were produced by

S. marcescens [7]. Each serrawettin produced by respective *S. marcescens* strains is restricted to one molecular species (e.g., W1 from strain 274, ATCC 13880; W2 from strain NS 25; and W3 from strain NS 45) [7, 10]. The genes, named *swrW* and *swrA*, responsible for serrawettin W1 and W2 biosynthesis from *S. marcescens* 274 and MG1 have been identified successfully by insertion mutagenesis with Tn5-derived transposon. The determination results showed that inactivation of the two genes could reduce serrawettin W1 and W2 production significantly [7, 8]. In the present study, to investigated the effect of serrawettin W1 on foam formation and 2,3-BD production, a *swrW* mutant strain deficient in serrawettin W1 formation by *S. marcescens* H30 was constructed and characterized.

Materials and methods

Strains, plasmids, primers, and culture conditions

The strains, plasmids, and primers used in this study are listed in Table 1. Luria–Bertani (LB) medium was used as seed culture for *E. coli* S17-1 λpir, *S. marcescens* H30, and its *swrW* mutant strain. *S. marcescens* H30 was grown at 30°C, and *E. coli* was grown at 37°C. Antibiotics were added in the following amounts (per ml) if necessary: 50 μg kanamycin for *E. coli* S17-1 λpir, 100 μg ampicillin and 200 μg kanamycin for the *swrW* mutant strain.

For seed preparation, a full loop of *S. marcescens* H30 strains or its *swrW* mutant strain from the slants was inoculated into 250-ml shake flasks containing 30 ml fresh seed medium and cultivated on a rotary shaker for 12 h at 200 rpm and 30°C. Seed culture (5%, v/v) was then inoculated into the fermentation medium which consisted of (g l^{-1}): sucrose 90, yeast extract (Angel Yeast Co. Ltd, China) 33.36, sodium citrate 10, sodium acetate 4, $NH_4H_2PO_4$ 1, $MgSO_4$ 0.3, and $MnSO_4$ 0.1 at pH 7.2. The flask experiments were conducted in 250-ml shake flasks containing 50 ml fresh fermentation medium for 24 h at 200 rpm and 30°C. All flask experiments were performed in parallel triplicate tests. Batch and fed-batch fermentations were carried out in a 3.7-l bioreactor (KLF2000, Bioengineering, Switzerland) with an initial broth volume of 1.8 l. All cultivation was performed at 30°C with an aeration rate of 1.0 vvm and agitation speed of 600 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 500 rpm, respectively, for 2,3-BD production. When the pH decreases to 6.0, it was maintained at 6.0 by automatic addition of 4 M H_3PO_4 or 4 M KOH using a computer-coupled peristaltic pump. Fed-batch fermentation was conducted by feeding sucrose solution (80%, w/v) when the residual sucrose in the fermentation broth decreased to about 20 g l^{-1}.

Construction of *swrW* mutants

The genomic DNA of *S. marcescens* H30 was extracted with the Bacterial Genomic DNA Mini-prep Kit (BioDev Corp., Beijing, China). A 1,128-bp fragment of the *swrW* gene (GenBank accession number AB193098.2) was PCR-amplified with the genomic DNA as template using primer pairs (Kswrw1 and Kswrw2) with *Kpn* I and *Sca* I recognition sites, which were designed according to the sequence information of *swrW* from *S. marcescens* 274. The PCR products were purified by using the DNA Fragment Purification Kit (BioDev Corp., Beijing, China) and digested by the corresponding restriction enzymes under the recommended conditions. The digested PCR products were subsequently purified and ligated into the suicide vector pUTkm1, which had been treated with the same restriction enzymes, generating the marker-exchange plasmid

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

Strain, plasmid, or primer	Relevant genotype and/or characteristics	Reference or source
S. marcescens H30	*S. marcescens* H30 was created by mutating *S. marcescens* CICC20066 using ultraviolet ray	Laboratory collection
S. marcescens H303	*S. marcescens* H30 *swrW*$^-$ mutant, Km^R	This study
E. coli S17-1 λpir	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7	[3]
pUTKm1	Ap^R Km^R *ori*R6K *ori*TRP4	[3]
pUT-*swrw*	pUTKm1 with a *swrW* fragment from *S. marcescens* H30 inserted into the *Kpn*I-*Sca*I sites of the multiple cloning site; Km^R	This study
Kswrw1[a]	GGGGTACCGAAGCCATACGGCAAATAC	This study
Kswrw2[a]	GTGAGTACTTGCGTTCACCGTCTTCCT	This study
Kswrw3	GAAGTAAGTTGGCCGCAGTG	This study
Kswrw4	CCGCTTATTCCCTGACGACC	This study

[a] Restriction sites introduced by PCR primers are underlined

pUT-*swrw*. The insertion fragment in pUT-*swrw* was confirmed by commercial DNA sequencing and double enzyme digestion. Marker exchange with pUT-*swrw* was carried out by using a similar protocol to that of de Lorenzo et al. [3]. Transconjugants were screened on LB medium agar plate containing 100 μg ml^{-1} ampicillin and 200 μg ml^{-1} kanamycin. The selected mutant was designated as *S. marcescens* H303 for further characterization.

Foam properties assays and surface tension measurement

Four milliliter supernatants from shake flask cultures of the wild *S. marcescens* strain and the mutant strain were poured into two 15-ml tubes, respectively. After being vigorously vortexed on Shakers for 1 min, the foam formation in the tubes was observed for characterization of foam properties. For batch cultures, the heights of foam were recorded for the wild strain and the mutant strain during the batch culture process. When the foam height reached 10 cm, the antifoam agent was added to break it. The surface tension was measured by the capillary rise method as previously described [11].

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 by the optical density (OD) measured at 600 nm in a spectrophotometer (UV-2008 h, Unic) and correlated with dry cell weight (DCW).

The products (AC and BD) in the broth were extracted by using ethyl acetate containing *n*-butanol as the internal standard and then quantified by using a GC system (Agilent GC9860; FID detector, DB-5 column). The operation conditions were as follows: N_2 was used as the carrier gas at a flow rate of 1.5 ml min^{-1}; the injector temperature and the detector temperature were 215 and 245°C, respectively; the column temperature was maintained at 50°C for 1.5 min, then raised to 180°C at a rate of 25°C min^{-1}. The concentration of the products was determined by using calibration curves [16].

Results

Construction of *swrW* mutants by homologous recombination

PCR amplification produced the products of approximately 1,100 bp with primer pairs (Kswrw1 and Kswrw2) designed according to the sequence information of the *swrW* gene. The commercial sequencing results showed the obtained sequence from *S. marcescens* H30 shared 99% identity with that of *S. marcescens* 274, indicating that the PCR amplification fragment for homologous recombination was obtained successfully. The digested and purified PCR products were cloned into the plasmid pUTkm1 for construction of the suicide vector pUT-*swrw* (Fig. 1). The recombinant suicide vector was confirmed by double enzymes digestion and commercial DNA sequencing (data not shown). The pUT-*swrw* vector was transformed into *E. coli* S17-1 λpir for conjugation with *S. marcescens* H30. Transconjugants were screened on LB medium agar plate containing 100 μg ml^{-1} ampicillin and 200 μg ml^{-1} kanamycin. The disruption of the locus was confirmed by PCR analysis using primers Kswrw3 and Kswrw4 complementary to the Km^R cassette and to the *swrW* locus outside of the region used in the marker exchange (data not shown).

Effects of *swrW* inactivation on cell growth and 2,3-BD production

The wild strain H30 and the mutant strain were cultured in parallel in 250-ml flasks containing 50 ml fresh fermentation medium. As shown in Fig. 2, no growth differences between the strains were observed in the initial growth phase (up to 15 h). After 15 h the growth of the mutant strain was slightly slower compared with the wild strain. This implied that the cell growth of *S. marcescens* was somewhat impaired due to disruption of the *swrW* gene. Furthermore, the foam properties of the wild strain and the mutant strain were determined. As shown in Fig. 3, a lot of foam was produced for the wild strain, whereas only a little foam was produced for the selected mutant strain. This indicated that inactivation of the *swrW* gene could result in the reduction of foam formation significantly. For AC and

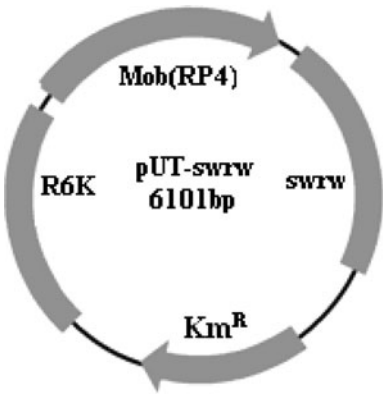

Fig. 1 Structure of the suicide vector pUT-*swrw* constructed in this study

Fig. 2 Foam property assays of the fermentation supernatants from the wild strain *S. marcescens* H30 (*1*) and the *swrW* mutant strain (*2*)

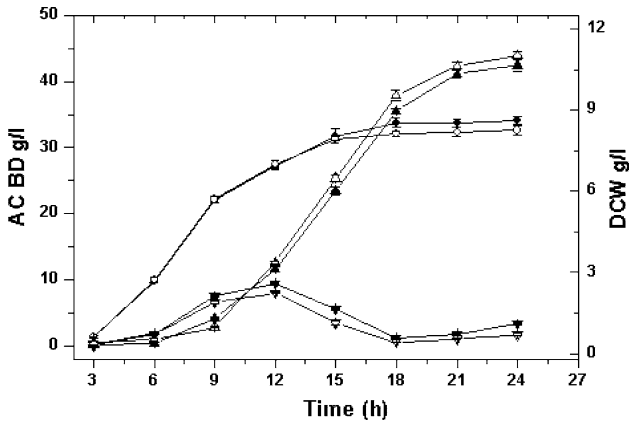

Fig. 3 Time course of cell growth and products formation of the wild strain *S. marcescens* H30 (*filled symbols*) and the *swrW* mutant strain (*unfilled symbols*). DCW (*circles*), AC (*inverted triangles*), and BD (*upright triangles*)

2,3-BD production (Fig. 2), the 2,3-BD yield of the mutant strain was slightly higher than that of the wild strain. In contrast, the AC yield of the mutant strain was lower than that of the wild strain during this process.

Surface activity of supernatants from batch cultures

To further characterize the properties of fermentative broth, the batch cultures were performed for measurement of the foam height and surface tension for the wild and mutant strain in the 3.7-l bioreactor. As shown in Table 2, the foam height for the wild strain increased sharply during cell growth. At 15 h, the foam height reached 10 cm, resulting in addition of antifoam agent. In contrast, the foam heights for the mutant strain remained at a lower level during the whole fermentation process. This indicated that foam formation during the fermentation process by the wild strain of *S. marcescens* H30 was at least partly due to serrawettin W1 production. Table 2 also shows that the supernatant from the wild strain was able to reduce the surface tension of water from 72 to nearly 35 dyne cm^{-1}. In contrast, the supernatant from the mutant strain only reduced the surface tension to nearly 47 dyne cm^{-1}. This implied the mutant strain could reduce serrawettin W1 production. To control foam formation in the batch culture, the added volume of antifoam agent amounted to nearly 2.5 ml for the wild strain, whereas no antifoam agent was added for the mutant strain.

Production of 2,3-BD by fed-batch culture of the *swrW* mutant strain

The *swrW* mutant strain was used to perform fed-batch culture in a 3.7-l bioreactor with an initial broth volume of 1.8 l. As shown in Fig. 4, the maximum DCW was as high as 14.96 g l^{-1} at 15 h, which was almost double the value obtained by shake flask culture. Figure 4 also shows that the production of AC and 2,3-BD was increased sharply with the rapid growth of the mutant strain. This indicated that the products' formation was associated with the growth of the mutant strain. When the residual sucrose concentration decreased to nearly 20 g l^{-1}, a fed-batch culture by feeding sucrose solution was performed to maintain the residual sucrose concentration of 15–25 g l^{-1}. Ultimately, a maximum 2,3-BD concentration of 152 g l^{-1} with a productivity of 2.67 g l^{-1} h^{-1} and yield of 92.6% was obtained at 57 h. In addition, the acetoin concentration was only 0.3 g l^{-1}.

Discussion

Serrawettin W1, W2, and W3, produced by *S. marcescens*, are surface-active cyclodepsipeptides. As described in the "Introduction", serrawettin produced by respective *S. marcescens* strains was restricted to one molecular species. Serrawettin W1 was produced by many pigmented *S. marcescens* strains, whereas serrawettin W2 and W3 was produced by nonpigmented strains of *S. marcescens* [10]. Physiologically, serrawettins act as wetting agents on various surfaces, enhancers of flagellum-independent expansion of bacterial population on agar medium, and accelerators of swarming on semisolid agar medium [7]. However, serrawettin produced by *S. marcescens* as a potential 2,3-BD-producing strain results in a lot of foam formation during the fermentation process. Foam formation, which is very harmful in industrial fermentation

Table 2 Time-course analysis of foam property and surface tension for the wild *S. marcescne* H30 and its *swrW* mutant in batch cultures

Strain	Time (h)	Cell concentration (g dry cell l^{-1})	Foam height (cm)	Surface tension (dyne cm^{-1})	Volume of antifoam agent (ml)
S. marcescens H30	9	9.80	4.5	41.76	2.5
	12	13.19	10	37.60	
	15	13.60	Efflux	39.90	
	18	13.56	9.5	35.52	
	21	13.55	8.5	35.10	
S. marcescens H303	9	9.67	0.2	53.58	No addition
	12	12.20	0.3	50.07	
	15	13.19	0.3	50.23	
	18	13.25	0.35	49.53	
	21	13.11	0.3	47.54	

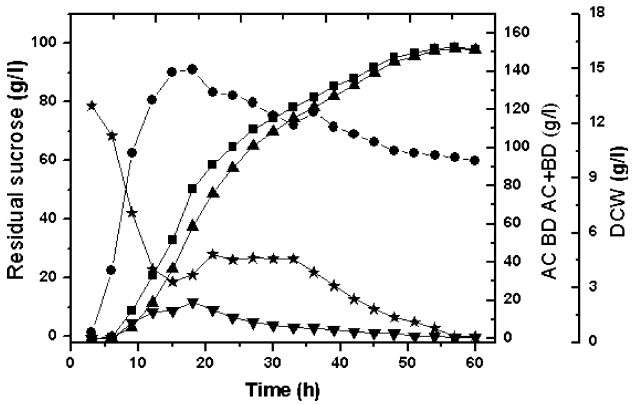

Fig. 4 Time course of 2,3-butanediol production by fed-batch culture of the *swrW* mutant strain. DCW (*filled circles*), residual sucrose (*filled stars*), AC (*filled inverted triangles*), BD (*filled upright triangles*), and AC + BD (*filled squares*)

processes, creates potential for microbial contamination, leads to the need for addition of large amounts of antifoam agent, and decreases bacterial activity. Thus, in this paper, engineered *S. marcescens* strains deficient in serrawettin W1 formation were constructed through insertional inactivation of *swrW*. The mutant strain, which lost the ability to biosynthesize serrawettin W1, exhibited significant reduction of the foam formation and improved 2,3-BD production a little. Moreover, formation of the byproduct, AC, was limited to a lower level.

During the fed-batch culture process, the foam height for the wild strain grew rapidly so that nearly 5 ml antifoam agent had to be added to break it down. In contrast, only two drops (nearly 0.1 ml) of antifoam agent were added for the mutant strain culture in this process. This approach would therefore be very useful for maintenance of bacterial activity and industrial scale-up of 2,3-BD production by *S. marcescens*. So in the fed-batch culture, the maximum 2,3-BD concentration afforded by the mutant strain probably contributed to maintenance of bacterial activity and increase of fermentation time. In addition, The maximum AC concentration of 19.37 g l^{-1} achieved at 18 h suggested that oxygen supply was too high for 2,3-BD production. So aeration rate and agitation speed were decreased to a low level to limit AC accumulation and promote 2,3-BD production. The AC concentration of only 0.3 g l^{-1} afforded by the mutant strain at 57 h would be very helpful during the separation and purification of 2,3-BD.

In conclusion, in this present study, a serrawettin W1 mutant strain of *S. marcescens* was constructed for elimination of the foam formation during the 2,3-BD fermentation process. The results showed that the foam formation and surface tension were reduced significantly in shake flask and batch cultures by using the mutant strain. Fed-batch culturing of the mutant afforded a final 2,3-BD concentration of 152 g l^{-1} with a productivity of 2.67 g l^{-1} h^{-1} and yield of 92.6% at 57 h.

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