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Efficient 2,3-Butanediol/Acetoin Production Using Whole-Cell Biocatalyst with a New NADH/NAD(+) Regeneration System

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Abstract: An auto-inducing expression system was developed that could express target genes in *S. marcescens* MG1. Using this system, MG1 was constructed as a whole-cell biocatalyst to produce 2,3-butanediol/acetoin. Formate dehydrogenase (FDH) and 2,3-butanediol dehydrogenase were expressed together to build an NADH regeneration system to transform diacetyl to 2,3-butanediol. After fermentation, the extract of recombinant *S. marcescens* MG1ABC *(pETDuet-bdhA-fdh)* showed 2,3-BDH activity of 57.8 U/mg and FDH activity of 0.5 U/mg. And 27.95 g/L of 2,3-BD was achieved with a productivity of 4.66 g/Lh using engineered *S. marcescens* MG1*(Pswnb+pETDuet-bdhA-fdh)* after 6 h incubation. Next, to produce 2,3-butanediol from acetoin, NADH oxidase and 2,3-butanediol dehydrogenase from *Bacillus subtilis* were co-expressed to obtain a NAD+ regeneration system. After fermentation, the recombinant strain *S. marcescens* MG1ABC *(pSWNB+pETDuet-bdhA-yodC)* showed AR activity of 212.4 U/mg and NOX activity of 150.1 U/mg. We obtained 44.9 g/L of acetoin with a productivity of 3.74 g/Lh using *S. marcescens* MG1ABC (*pSWNB+pETDuet-bdhA-yodC*). This work confirmed that *S. marcescens* could be designed as a whole-cell biocatalyst for 2,3-butanediol and acetoin production.

Keywords: 2,3-butanediol; acetoin; *Serratia marcescens*; whole-cell biocatalysts; NADH/NAD$^+$ regeneration system

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1. Introduction

As promising bulk chemicals, 2,3-butanediol and acetoin (Figure 1) are proposed to have extensive applications in a variety of fields such as the fuel, chemical and food industries [1]. These chemicals can be produced by chemical synthetic, fermentative, enzymatic or biocatalytic technologies [2,3]. However, because of difficulties in chemical synthesis, the large-scale production of 2,3-butanediol and acetoin is limited using this technology. Several microorganisms have been shown to be capable of producing 2,3-butanediol or acetoin, such as *Klebsiella, Gluconobacter, Bacillus* and *Serratia* [3]. However, in most cases, 2,3-butanediol and acetoin are co-produced, necessitating a downstream purification step. It is therefore difficult to obtain pure 2,3-butanediol or acetoin by fermentation [2]. Enzymatic or biocatalytic technologies have been proposed as appropriate alternative production

methods. As the conversion between 2,3-butanediol and acetoin is reversible and coupled with NADH/NAD+ transformation, the production of 2,3-butanediol or acetoin could be improved by regulating the level of intracellular cofactors [4,5].

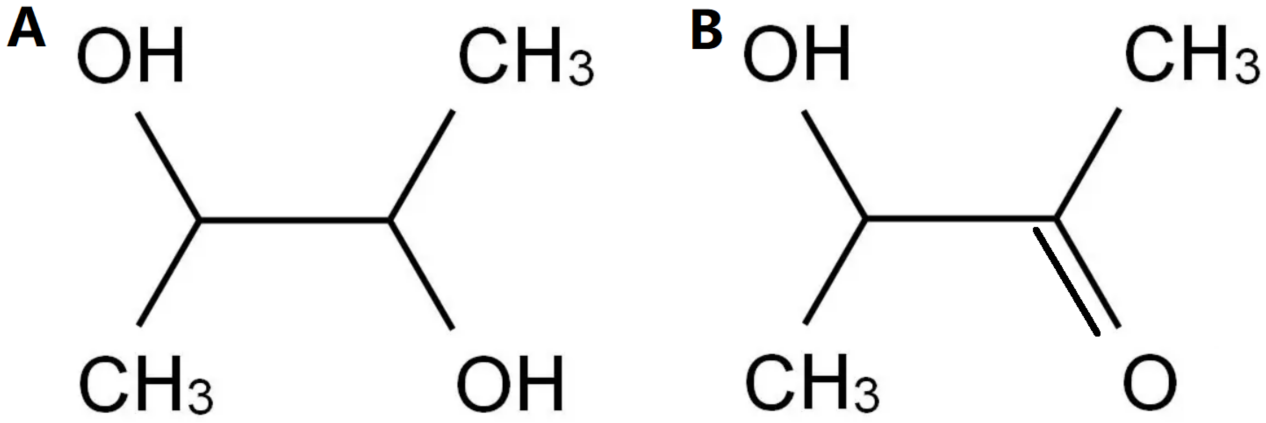

Figure 1. The structure of 2,3-butanediol (**A**) and acetoin (**B**).

Serratia marcescens is a Gram negative, bacillus-shaped bacterium that belongs to the family Enterobacteriaceae [6]. It is reported to possess the potential for industrial 2,3-butanediol or acetoin production, due to its characteristics of resistance to bacterial pollution, relatively simple metabolic pathways, a broad substrate spectrum and cultural adaptability.

Quorum sensing is a bacterial phenomenon that enables bacteria to communicate and work together [7–10]. *S. marcescens* MG1 employs an AHL-type quorum sensing system, to control various functions, including swarming motility, and the production of extracellular enzymes and 2,3-butanediol [6]. This system depends on two proteins, SwrI, which conducts the synthesis of the signal molecules N-butanoyl-L-homoserine lactone and N-hexanoyl-L-homoserine lactone, and SwrR, which, after binding with the signal molecules, is thought to activate or repress transcription of target genes. In *S. marcescens* MG1, the swr system combined with a LysR-like regulator, SlaR, mediate the production of 2,3-butanediol. SlaR is an essential activator for the transcription of genes related to 2,3-butanediol production and disruption of this regulator means that the strain cannot produce acetoin and 2,3-butanediol. The swr system functions in two phases, at logarithmic phase it represses 2,3-butanediol production, while this repression is removed when the strain enters into stationary phase [6].

In this study, An auto-inducing expression system was developed that could express target genes in *S. marcescens* MG1. Using this system, *S. marcescens* MG1 was constructed as a whole-cell biocatalyst to produce 2,3-butanediol/acetoin. Formate dehydrogenase (FDH) and 2,3-butanediol dehydrogenase were expressed together to build an NADH regeneration system to transform diacetyl to 2,3-butanediol. After fermentation, the extract of recombinant *S. marcescens* MG1ABC *(pETDuet-bdhA-fdh)* showed 2,3-BDH activity of 57.8 U/mg and FDH activity of 0.5 U/mg. And 27.95 g/L of 2,3-BD was achieved with a productivity of 4.66 g/Lh using engineered *S. marcescens* MG1*(pSWNB+pETDuet-bdhA-fdh)* after 6 h incubation. Next, to produce 2,3-butanediol from acetoin, NADH oxidase and 2,3-butanediol dehydrogenase from *Bacillus subtilis* were co-expressed to obtain a NAD+ regeneration system. After fermentation, the recombinant strain *S. marcescens* MG1ABC *(pSWNB+pETDuet-bdhA-yodC)* showed AR activity of 212.4 U/mg and NOX activity of 150.1 U/mg. We obtained 44.9 g/L of acetoin with a productivity of 3.74 g/Lh using *S. marcescens* MG1ABC (*pSWNB+pETDuet-bdhA-yodC*). This work confirmed that *S. marcescens* could be designed as a whole-cell biocatalyst for 2,3-butanediol and acetoin production.

2. Results and Discussion

2.1. Construction of the Auto-Inducing Expression System

The auto-inducing expression system is based on the pET system and the swr quorum sensing system in *S. marcescens* MG1. Here, a two-step genetic switching circuit was constructed that consists of two plasmids: plasmid pSWNB (Figure 2A) that expresses T7 RNA polymerase that amplifies the transcriptional strength of promoter slaA, and plasmid pET-X that encodes the proteins of interest. This system works as follows (Figure 2B): at logarithmic phase, SwrR produced by *S. marcescens* MG1 represses the expression of the slaA promoter. This effectively prevents the transcription of T7 RNA polymerase. As the cell density increases, AHL is produced by SwrI and alleviates the repressive effects of SwrR. Meanwhile, sufficient acetate has accumulated and binds to SlaR to trigger full activation of the slaA promoter, allowing the downstream gene encoding T7 RNA polymerase to be transcribed when sugar is present. Then, T7 RNA polymerase amplifies the transcriptional strength of the slaA promoter and activates the transcription of genes under the control of the T7 promoter. Thus, the target proteins are produced. To validate the auto-inducing system, several genes were expressed in *S. marcescens* MG1 using this system and the results are presented in the following sections.

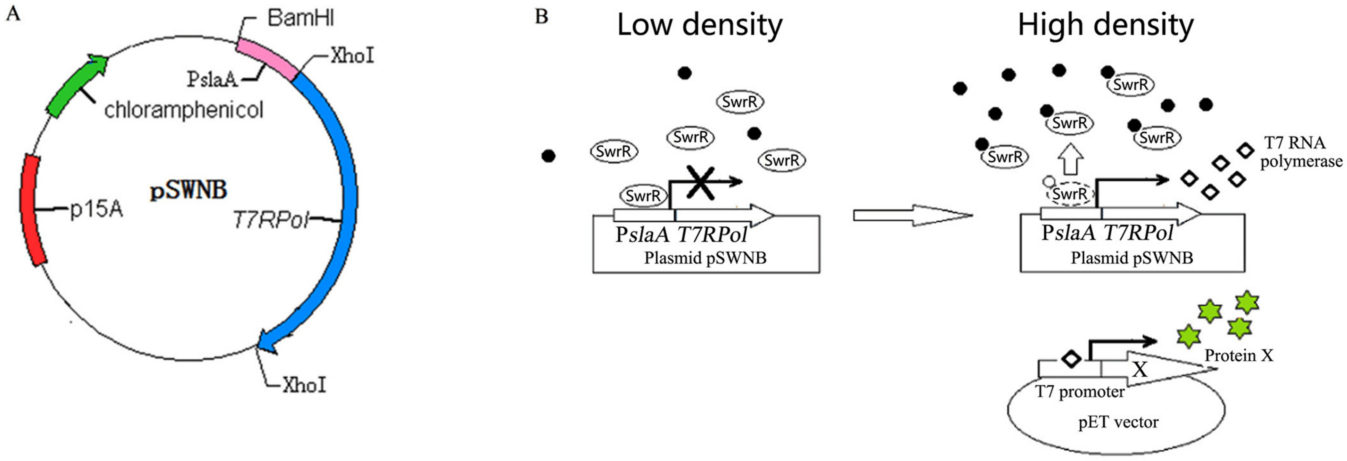

Figure 2. (**A**) Structure of the plasmid pSWNB; (**B**) the mechanism of the auto-inducing system.

2.2. Expression Profile of Green Fluorescent Protein (GFP) Using S. marcescens

The gfp gene was expressed to investigate the expression profile of proteins and to determine whether the system works in a population-dependent manner. The recombinant strain *S. marcescens* MG1 (pSWNB+pET28a-gfp) was cultured in LB (1% glucose) media and harvested at different times. The cell growth profile and expression levels of GFP were measured as described in the Materials and methods section. As shown in Figure 3A, compared with the control strain *S. marcescens* MG1 (pSWNB+pET28a), GFP expression levels increased when strain *S. marcescens* MG1 (pSWNB+pET28a-gfp) entered late logarithmic phase. As the large amount of exogenous protein expression imposed a burden on the host strain, the growth of strain *S. marcescens* MG1 (pSWNB+pET28a-gfp) subsequently decreased (Figure 3B). In particular, GFP began to accumulate gradually after 4 h and reached a maximum at 12 h. This revealed that the system could highly express target proteins from logarithmic phase in *S. marcescens* MG1.

To validate that the expression of the target proteins was responding to the AHL signal and SlaR, the vectors pSWNB and pET-gfp were also co-transformed into the swrI mutant strain *S. marcescens* MG1I and the slaR mutant strain *S. marcescens* MG1R resulting in strains *S. marcescens* MG1I (pSWNB+ pET28a-gfp) and *S. marcescens* MG1R (pSWNB+pET28a-gfp). These two strains were cultured in LB (1% glucose) media and the products were assessed, as shown in Figure 3A. Strain *S. marcescens* MG1R (pSWNB+pET28a-gfp) did not express

GFP, whereas strain *S. marcescens* MG1I (pSWNB+pET28a-gfp) produced a certain amount of the target protein. After the addition of 0.5 mM AHL (Sigma Aldrich) to the culture of *S. marcescens* MG1I (pSWNB+pET28a-gfp) in mid-log phase (OD600 = 0.4–0.6), the production ability of *S. marcescens* MG1I (pSWNB+pET28a-gfp) was restored. This finding was consistent with the fact that the slaA promoter is controlled by the quorum sensing system and regulator SlaR. In addition, it confirmed that the proteins produced by the auto-inducing system were induced by the AHL signal and SlaR.

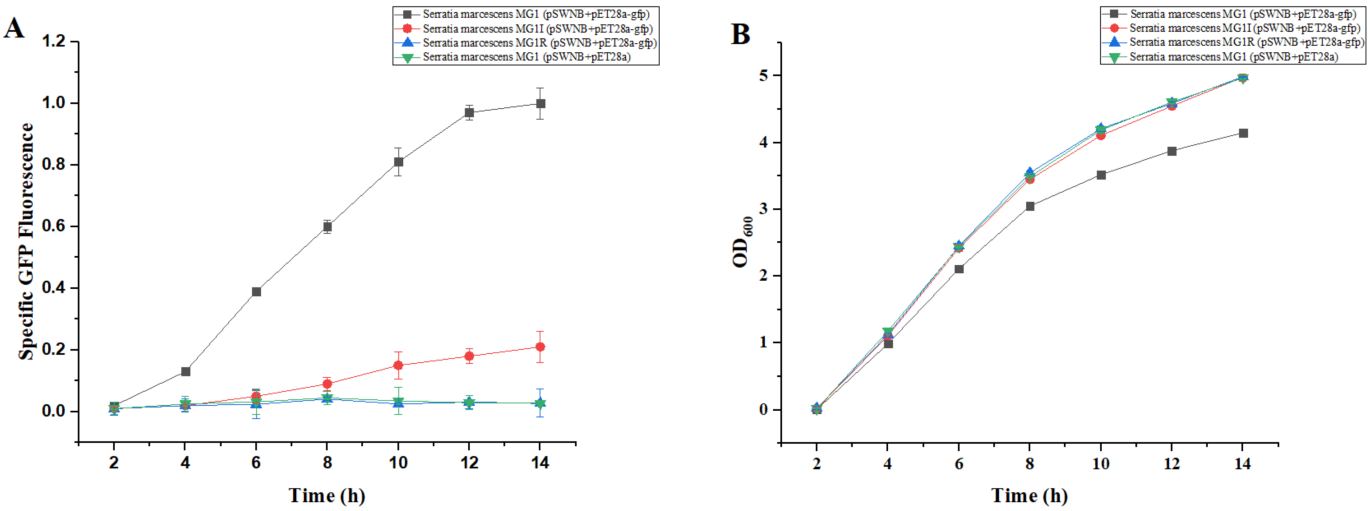

Figure 3. Comparison of specific GFP levels (**A**) and cell growth (**B**) and in LB medium. Square, *Serratia marcescens* MG1 (pSWNB+pET28a-gfp); Sphere, *Serratia marcescens* MG1I (pSWNB+pET28a-gfp); Regular Triangle, *Serratia marcescens* MG1R (pSWNB+pET28a-gfp); Inverse triangle, *Serratia marcescens* MG1 (pSWNB+pET28a).

The overexpression of proteins is an effective way to rebuild the metabolic pathway for a variety of applications. Here, we constructed an auto-inducing expression system by employing the slaA promoter, which is dual-regulated during the process of acetoin biosynthesis in *S. marcescens*. As in Vibrio cholerae, SwrR and SlaR are essential components of this process. SlaR, which belongs to the LysR-type protein family, is the central activator of transcription. When SlaR was disrupted, the slaA gene (encoding alpha-acetolactate decarboxylase) and slaB gene (encoding alpha-acetolactate synthase), controlled by the slaA promoter, couldn't be expressed. slaC represents the 2,3-butanediol dehydrogenase. Moreover, SlaR cannot activate the acetoin operon transcription without acetate. SwrR, which acts as a receptor of the signal molecules, exerts a negative regulatory effect on transcription of the acetoin operon. In detail, SwrR efficiently represses transcription of the slaA promoter at low cell density. As the cell density increases, the repressive effects of SwrR are gradually alleviated. When sufficient acetate has accumulated, full activation of the slaA promoter is triggered, allowing for high levels of transcription of the slaA and slaB genes. Because transcription of the slaA promoter is directly linked to cell-density, in this study, it was used to regulate T7 RNA polymerase expression, which activates the transcription of target genes. Our results confirmed that this regulation mechanism is effective at controlling gene expression during 2,3-butanediol production in *S. marcescens*. The auto-inducing system constructed does not require specific medium or growth conditions to guarantee auto-induction. It responds directly to cell density and grows to saturation without the need to monitor culture growth or add an inducer at a specific time-point during the process.

To validate the auto-inducing system, the gene encoding GFP was expressed in *S. marcescens*. The results showed that only when the recombinant strain reached high cell density did the GFP expression levels increase significantly. Furthermore, the *S. marcescens* mutant strain that was unable to synthesize signal molecules or the regulator SlaR, expressed none or only a certain amount of GFP after being transformed with pSWNB and

pET-GFP. These experiments were not only performed in flasks, but were also carried out at fermentor scale. The results clearly indicated that the auto-inducing system works as intended.

2.3. Bioconversion of Diacetyl to 2,3-Butanediol

In strain *S. marcescens* MG1ABC, genes slaA, slaB and slaC were all inactivated. This strain was therefore unable to produce acetoin and 2,3-butanediol and was consequently suitable for application as a whole-cell biocatalyst to achieve bioconversion.

The genes encoding 2,3-BDH from *B. subtilis* 168 and FDH from *C. boidinii* NCYC 1513 were cloned and recombinant strains were constructed. The enzymatic activities of the recombinant strains were measured, as shown in Table 1. After fermentation, the extract of *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA-fdh) showed 2,3-BDH activity of 57.8 U/mg and FDH activity of 0.5 U/mg. Compared with using *E. coli* as a host strain [11], higher enzymatic activities could be obtained using *S. marcescens*. SDS-PAGE analyses showed that these two proteins were expressed in *S. marcescens* MG1ABC (Figure 4). Obviously these two genes could be successfully co-expressed in *S. marcescens* and thereby used to transform diacetyl to 2,3-butanediol; however, as BDH and FDH have similar molecular weights, they were poorly resolved when co-expressed in *S. marcescens* MG1ABC.

Table 1. Activities of 2,3-BDH and FDH.

Strain	2,3-BDH Activity (U/mg)	FDH Activity (U/mg)
S. MG1ABC (pETDuet)	ND	ND
S. MG1ABC (pETDuet-bdhA)	108.1 ± 2.1	ND
S.MG1ABC (pETDuet-bdhA-fdh)	57.8 ± 1.6	0.5 ± 0.03

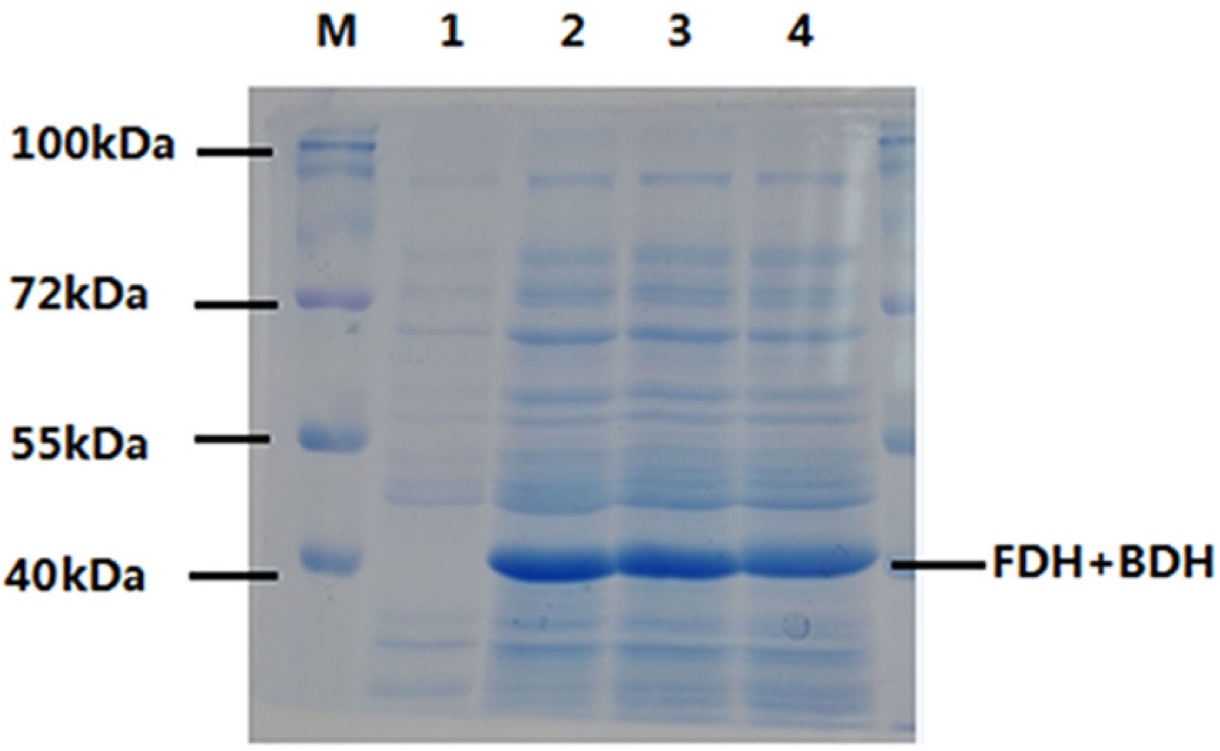

Figure 4. Identification of the expression of 2,3-BDH and FDH in *Serratia marcescens* by SDS-PAGE. M, maker; lane 1, *Serratia marcescens* MG1ABC (pSWNB+pETDuet); lane 2, *Serratia marcescens* MG1ABC (pSWNB+pETDuet-bdhA); lane 3, *Serratia marcescens* MG1ABC (pSWNB+pETDuet-fdh); lane 4, *Serratia marcescens* MG1ABC (pSWNB+pETDuet-bdhA+fdh).

The bioconversion experiments were first performed in flasks under the conditions described in the Materials and methods section. The initial DA concentration was 30 g/L. Cells containing plasmids pETDuet-bdhA and pETDuet-bdhA-fdh were used as whole-cell biocatalysts to achieve the bioconversion, and 27.95 g/L of 2,3-BD was achieved with a productivity of 4.66 g/Lh using *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA-fdh) after 6 h incubation (Table 2). This level of productivity (4.66 g/Lh) was 1.3-fold higher than that achieved using *E. coli* as a whole-cell biocatalyst, confirming that a higher 2,3-BD concentration, productivity and yield could be achieved using *S. marcescens*. This provided evidence that *S. marcescens* is appropriate for use as a whole-cell biocatalyst to convert DA to 2,3-BD.

Table 2. The products of batch bioconversion using different cofactor regeneration systems.

Strain	2,3-BD (g/L)	AC (g/L)	Productivity of 2,3-BD (g/(Lh))	Yield of 2,3-BD (%)
S. MG1ABC (pETDuet)	ND	ND		
S. MG1ABC (pETDuet-bdhA)	22.7 ± 0.45	1.8 ± 0.09	3.78 ± 0.06	75.6 ± 1.23
S. MG1ABC (pETDuet-bdhA-fdh)	27.95 ± 0.52	1.35 ± 0.12	4.66 ± 0.11	93.1 ± 1.45

The introduction of FDH into *S. marcescens* was intended to regenerate cofactors. We examined the intracellular concentrations of NADH and NAD+ in recombinant strains to confirm this (Figure 5A–C). Compared with strain *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA), the intracellular NADH/NAD+ concentration was higher in strain *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA+fdh), which exhibited a high level of NADH and a low level of NAD+. After several hours, the NADH level and the ratio of NADH/NAD+ increased continuously, confirming that NADH was indeed reproduced in strain *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA+fdh). This indicated that the NADH regeneration efficiency in *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA+fdh) was higher than that in *S. marcescens* MG1ABC (pSWNB+pETDuet-bdh). This may accelerate the production of 2,3-BD.

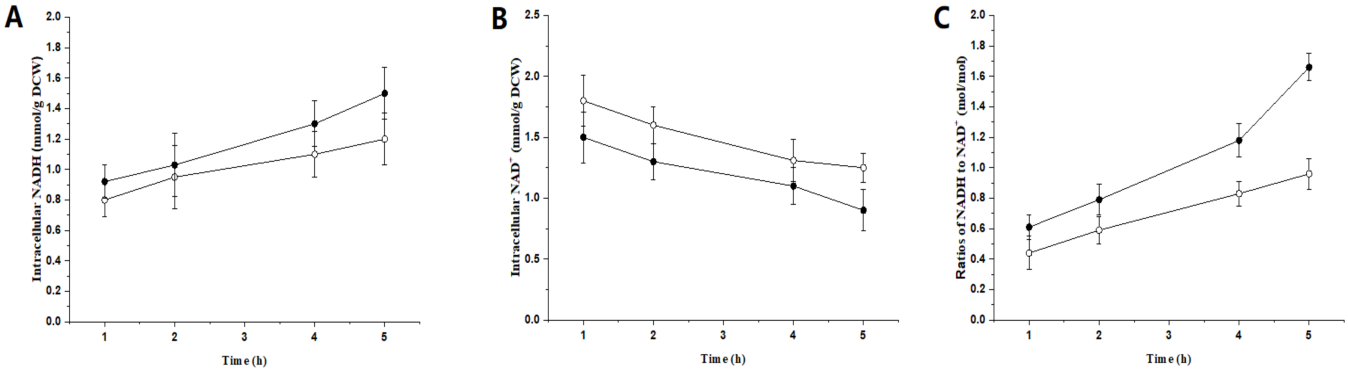

Figure 5. Effects of NADH regeneration system on levels of intracellular NADH, NAD+ and ratios of NADH to NAD+. Circular (Solid) represents *Serratia marcescens* MG1ABC (pSWNB+pETDuet-bdhA-fdh). Circular (Hollow) represents *Serratia marcescens* MG1ABC (pSWNB+pETDuet-bdhA). (**A**) Effects of NADH regeneration system on levels of intracellular NADH; (**B**) Effects of NADH regeneration system on levels of intracellular NAD+; (**C**) Effects of NADH regeneration system on ratios of NADH to NAD+.

To improve the concentration of 2,3-BD, fed-batch conversion experiments were performed (Figure 6). The initial DA concentration was 30 g/L, and a further 10 g/L was added at 2, 4 and 6 h. Figure 6 shows that 54.9 g/L of 2,3-BD were produced with a productivity of 3.43 g/Lh. The yield of 2,3-BD on DA was high and only 1.3 g/L of AC accumulated. Almost no formate was detected at the end of the reaction, indicating that it had been used up.

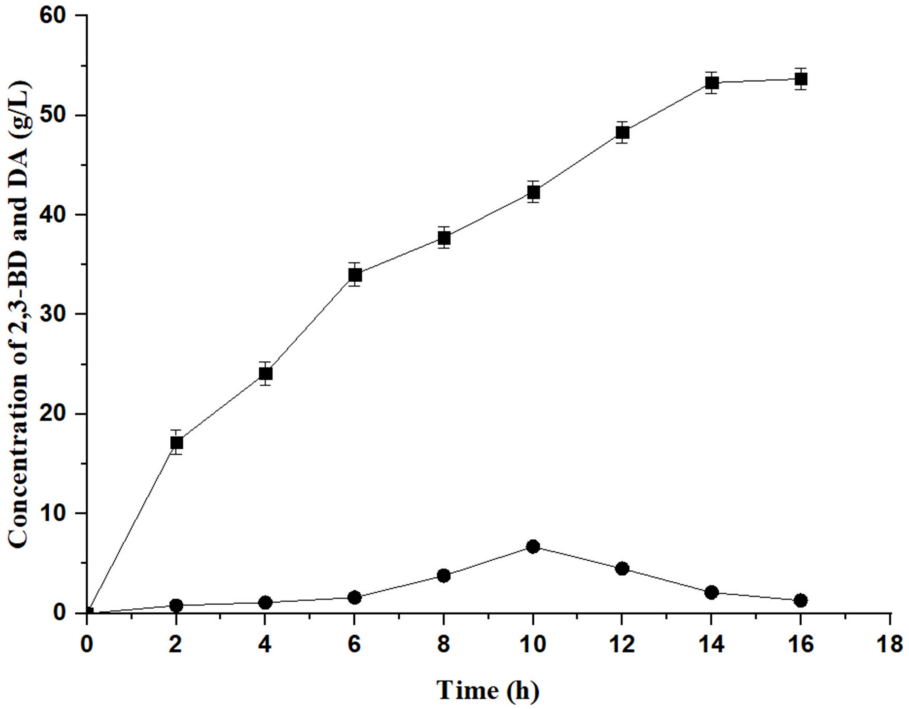

Figure 6. Time course of fed-batch bioconversion by *Serratia marcescens* MG1ABC (pSWNB+pETDuet-bdhA-fdh). Circular represents acetoin. Square represents 2,3-butanediol.

Unlike several microorganisms that are susceptible to the toxicity of many organic chemicals, *S. marcescens* exhibits remarkable tolerance to 2,3-butanediol and acetoin. In previous studies, *S. marcescens* was reported to possess the potential for industrial acetoin and 2,3-butanediol production [2,6]. However, these studies all focused on fermentative technologies and fermentation parameter optimization did not significantly improve production of the target products. Here, we tried to produce acetoin and 2,3-butanediol using *S. marcescens* as a whole-cell biocatalyst. The bioconversion reaction between 2,3-BD and acetoin is coupled with NADH/NAD+ transformation. It is therefore necessary to manipulate the level of intracellular cofactors. Considering that internal cofactor regeneration is not always sufficient, researchers introduce other enzymes to accelerate cofactor regeneration.

We co-expressed 2,3-BDH and FDH to convert diacetyl to 2,3-butanediol in *S. marcescens*. Using the auto-inducing system, it was straight forward to co-express two proteins. To achieve this, 2,3-BDH from *B. subtilis* and FDH from *C. boidinii* NCYC 1513 were subcloned into the expression plasmid pETDuet, resulting in recombinant plasmid pETDuet-bdhA-fdh. The results showed that *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA) could convert diacetyl to 2,3-butanediol. However, as the NADH was consumed gradually, it was difficult to maintain the intracellular NADH concentration. To improve NADH regeneration efficiency, FDH from *C. boidinii* NCYC 1513 was co-expressed in *S. marcescens*. The introduction of FDH, improved the intracellular NADH/NAD+ level.

After culturing, the enzyme activities of 2,3-BDH and FDH produced by *S. marcescens* were all higher than those in *E. coli.* Consistent with this, a higher 2,3-butanediol concentration, productivity and yield were obtained using *S. marcescens*, confirming that *S. marcescens* is a suitable candidate to produce 2,3-butanediol.

2.4. Bioconversion of 2,3-Butanediol to Acetoin

The bdhA and yodC genes from *B. subtilis* 168 were cloned and recombinant strains were constructed. Their enzymatic activities were measured, as shown in Table 3. After fermentation, strain *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA-yodC) showed AR (acetoin reductase) activity of 212.4 U/mg and NOX activity of 150.1 U/mg. SDS-PAGE

analyses (Figure 7) of the recombinant proteins confirmed that these two genes could be successfully co-expressed in *S. marcescens*, where they can effectively convert 2,3-butanediol to acetoin.

Table 3. Enzyme activity and the whole-cell biocatalytic activity of different strains.

Strains	AR Activity (U/mg)	BDH Activity (U/mg)	NOX Activity (U/mg)
S. MG1ABC (pETDuet)	ND	ND	ND
S. MG1ABC (pETDuet-bdhA)	221.3 ± 2.3	103.2 ± 1.7	ND
S. MG1ABC (pETDuet-bdhA-yodC)	212.4 ± 1.4	89.2 ± 0.7	150.1 ± 2.3

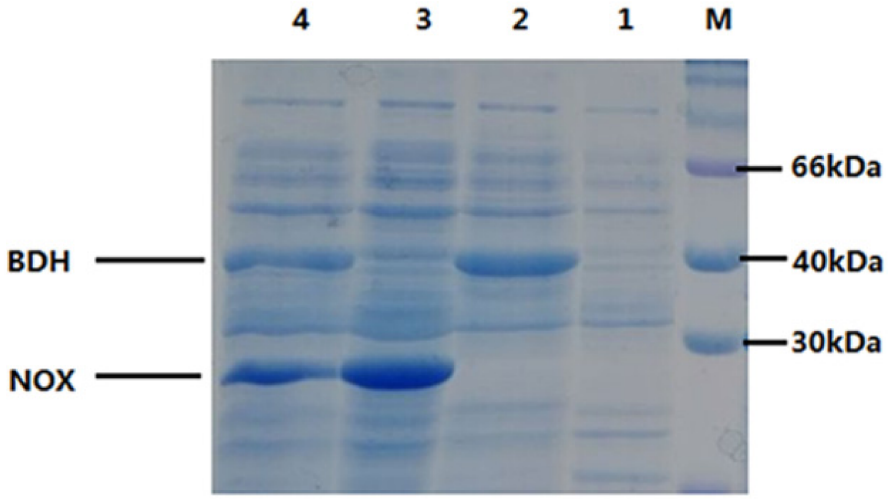

Figure 7. Validation of the expression of 2,3-BDH and NOX in *Serratia marcescens* through SDS-PAGE. M, maker; lane 1, *Serratia marcescens* MG1ABC (pSWNB+pETDuet); lane 2, *Serratia marcescens* MG1ABC (pSWNB+pETDuet-bdhA); lane 3, *Serratia marcescens* MG1ABC (pSWNB+pETDuet-yodC); lane 4, *Serratia marcescens* MG1ABC (pSWNB+pETDuet-bdhA+yodC).

The bioconversion experiments were performed in flasks under the conditions described in the previous section. The initial 2,3-BD concentration was 50 g/L. We used the cells containing plasmid pETDuet-bdhA-yodC as whole-cell biocatalysts to achieve the bioconversion. As shown in Table 4, we obtained 44.9 g/L of acetoin with a productivity of 3.74 g/Lh using *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA-yodC). The conversion yield of 2,3-butanediol on acetoin was 89.8%. These findings confirmed that a high acetoin concentration, productivity and yield could be obtained using *S. marcescens*, providing evidence of the suitability of *S. marcescens* for use as a whole cell biocatalyst to convert 2,3-butanediol to acetoin.

Table 4. The products of batch bioconversion with different cofactor regeneration systems.

Strain	2,3-BD(g/L)	AC(g/L)	Productivity of AC (g/(Lh))	Yield of AC (%)
S. MG1ABC (pETDuet)	ND	ND		
S. MG1ABC (pETDuet-bdhA)	10.9 ± 0.76	38.5 ± 0.45	3.2 ± 0.06	77 ± 1.23
S. MG1ABC (pETDuet-bdhA- yodC)	4.1 ± 0.78	44.9 ± 0.52	3.74 ± 0.11	89.8 ± 1.45

The introduction of NOX into *S. marcescens* MG1ABC was intended to increase the overall intracellular NAD+pool. We examined the intracellular concentrations of NADH and NAD+ in recombinant strains to confirm this (Figure 8A–C). Compared with strain *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA), the intracellular NADH/NAD+ concentration was significantly lower in strain *S. marcescens* MG1ABC (pSWNB+pETDuet-

bdhA-yodC). This indicated that the NAD+ regeneration efficiency in strain *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA-yodC) containing the yodC gene was higher than that in *S. marcescens* MG1ABC (pSWNB+pETDuet-bdhA), potentially accelerating the production of acetoin.

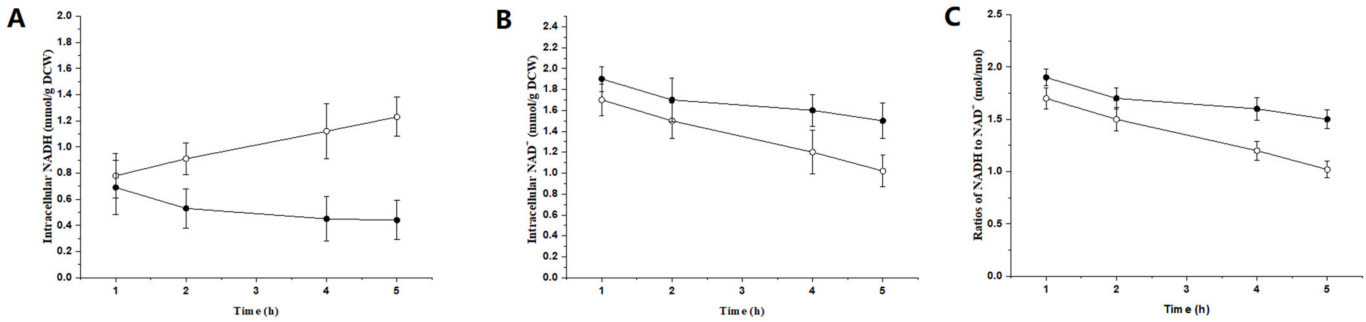

Figure 8. Effects of NAD+ regeneration system on levels of intracellular NADH, NAD+ and ratios of NADH to NAD+ **Circular (Solid)** represents *Serratia marcescens* MG1ABC (pSWNB+pETDuet-bdhA-yodC). **Circular (Hollow)** represents *Serratia marcescens* MG1ABC (pSWNB+pETDuet-yodC). (**A**) Effects of NAD+ regeneration system on levels of intracellular NADH; (**B**) Effects of NAD+ regeneration system on levels of intracellular NAD+; (**C**) Effects of NAD+ regeneration system on ratios of NADH to NAD+.

To improve the concentration of acetoin, fed-batch conversion experiments were performed (Figure 9). The initial 2,3-BD concentration was 50 g/L, and a further 30 g/L was added by 8 h. In total, 58.5 g/L of acetoin was produced with a productivity of 2.92 g/Lh. The yield of acetoin on 2,3-BD was high. In another study, *B. subtilis* was constructed as whole-cell biocatalyst which was used to transform 120 g/L 2,3-BD to 91.8 AC [4]. The yield of AC was 76.5%, which was similar with our *Serratia marcescens*.

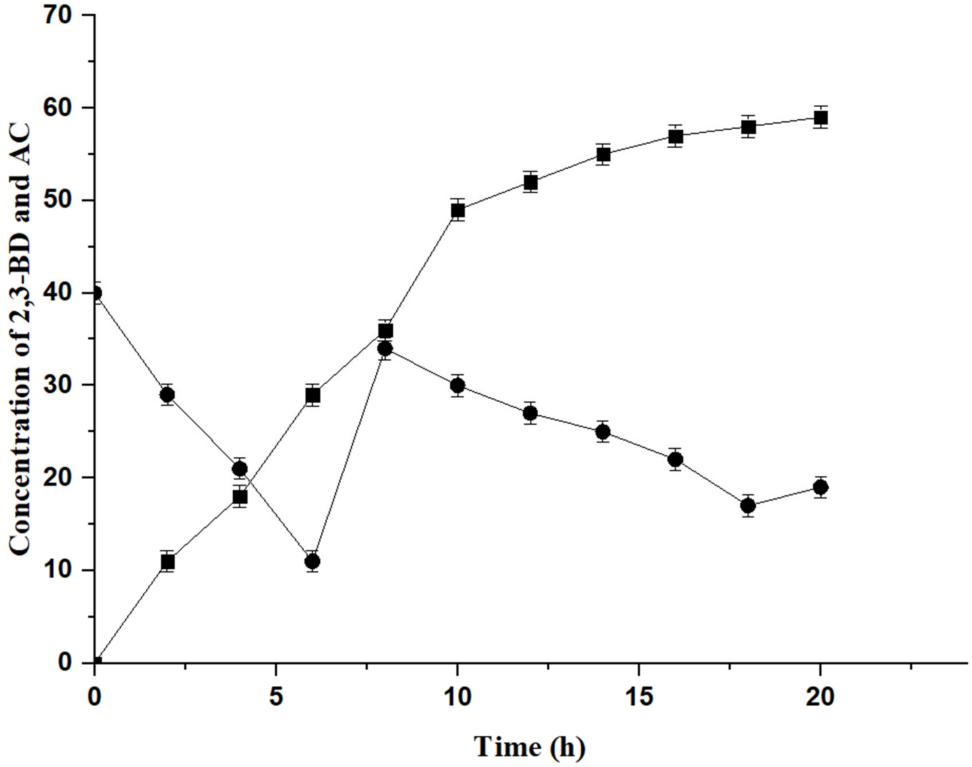

Figure 9. Time course of fed-batch bioconversion by *Serratia marcescens* MG1ABC (pSWNB+pETDuet-bdhA-yodC). Circular represents 2,3-butanediol. Square represents acetoin.

As discussed in the previous section, we used *S. marcescens* to convert diacetyl to 2,3-butanediol. As BDH is a reversible enzyme, it can also be used to convert 2,3-butanediol to acetoin. However, as the reduced NAD+ pool may restrict the process of bioconversion, here, NOX was expressed to maintain low NADH/NAD+ levels. Our experiments showed that the introduction of yodC could indeed reduce intracellular NADH/NAD+ levels. After culturing, a higher acetoin concentration, productivity and yield could be obtained using *S. marcescens*, confirming the suitability of this organism for producing acetoin.

3. Materials and Methods

3.1. Construction of Recombinant Strain S. marcescens MG1ABC and Plasmid pSWNB

Using the method described by Rao Ben [6], we constructed *S. marcescens* mutant strain MG1ABC (Table 5) in which genes slaA, slaB and slaC were all inactivated. The vector pSWNB was constructed by the following steps: first, we obtained plasmids pBT-1 and pBT-2 [6,11]. The promoter slaA was cloned into the BamHI/XhoI sites of pBT-2 before the T7 RNA polymerase gene to control its expression and target plasmid pSWNB (Figure 2) was constructed using primers pSWNBf/pSWNBr (Table 6).

Table 5. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant Characteristics	Reference or Source
Serratia marcescens MG1	The source of other strains in this study	Rao et al. (2012)
Serratia marcescens MG1I	*Serratia marcescens* MG1 swrI- mutant	Rao et al. (2012)
Serratia marcescens MG1R	*Serratia marcescens* MG1 slaR- mutant	Rao et al. (2012)
Serratia marcescens MG1ABC	*Serratia marcescens* MG1 slaABC- mutant	In this study
pBT-1	pBT vector without λcI gene	Rao et al. (2012)
pBT-2	pBT-1 vector carrying T7 RNA polymerase gene	Rao et al. (2012)
pSWNB	Newly constructed "switch" vector	In this study
pET28a	T7-based expression vector	Invitrogen
pETDuet	T7-based expression vector	Invitrogen
pET28a-gfp	pET28a vector containing gfp expression cassette	In this study
pETDuet-bdhA	pETDuet vector containing bdhA expression cassette	In this study
pETDuet-bdhA- yodC	pETDuet vector containing bdhA and yodC expression cassettes	In this study
pETDuet-bdhA-fdh	pETDuet vector containing bdhA and fdh expression cassettes	In this study

Table 6. Primers used in this study.

Primer	Sequence
pSWNBf	attggatccGAGCCGCCTGCGGAGTTGAT
pSWNBr	attctcgagGGCAATGGTGGTTTCACCCTC
Pgfpf	attcatatgATGAGTAAAGGAGAAGAACTTTCACTGG
Pgfpr	attggatccTCACTTGTACAGCTCGTCCATGCC
PbdhAf	attagatctATGAAGGCAGCAAGATGG
PbdhAr	attctcgagATGACGAATA CTCTGGAT
Pnoxf	attccatggGTTAGGTCTAACAAGG
Pnoxr	attgtcgacCAGCCAA GTTGATAC
Pfdhf	attagatctAAGATCGTTTTAGTCTTATATGATGCTGGTA
Pfdhr	attctcgagTTATTTCTTATCGTGTTTACCGTAAGCTTTG

3.2. Construction of Expression Plasmids

A fragment containing the green fluorescent protein gene was amplified from plasmid pQBI63 (Quantum Biotechnologies Inc, NY, USA) using primers Pgfpf/Pgfpr and ligated into NdeI-BamHI-linearized vector pET28a resulting in vector pET28a-gfp.

The bdhA gene encoding 2,3-butanediol dehydrogenase was amplified from *B. subtilis* 168 genomic DNA using primers PbdhAf/PbdhAr and ligated into NcoI-SalI-linearized vector pETDuet-1, resulting in vector pETDuet-bdhA.

The yodC gene encoding NADH oxidase was amplified from *B. subtilis* 168 genomic DNA using primers Pnoxf/Pnoxr and ligated into BglII-XhoI-linearized vector pETDuet-bdhA, resulting in vector pETDuet-bdhA-yodC.

The fdh gene fragment was amplified from Candida boidinii NCYC 1513 genomic DNA using primers Pfdhf/Pfdhr and ligated into BglII-XhoI-linearized vector pETDuet-bdhA, resulting in vector pETDuet-bdhA-fdh.

3.3. Culture Conditions in Flask and Bioconversion Conditions

The auto-inducing vector pSWNB and the recombinant plasmid pET-X carrying target genes were co-introduced into *S. marcescens* by electroporation. Plasmids pSWNB and pET were transformed into *S. marcescens*, resulting in the control strain.

To study the expression of green fluorescent protein, overnight recombinant *S. marcescens* MG1 (pSWNB and pET-gfp) cultures were inoculated into fresh LB media supplemented with 1% glucose to an OD600 of 0.1 in flasks and then incubated.

To convert diacetyl to 2,3-butanediol, overnight recombinant *S. marcescens* (pSWNB and pETDuet-bdhA-fdh) cultures were inoculated into LB media supplemented with 1% glucose to an OD600 of 0.1 in flasks and then incubated. The cells were obtained by centrifugation at 10,000× *g* rpm for 5 min at 4 °C. After washing, the cell pellets were resuspended in buffer. The whole-cell biocatalyst reaction was performed as follows: 50 mL of mixture was reacted at 30 °C. The pH was controlled at 7.0. 20 g/L of diacetyl and 32 g/L of formate were used as substrates.

To convert 2,3-butanediol to acetoin, overnight recombinant *S. marcescens* (pSWNB and pETDuet-bdhA-yodC) cultures in LB media were inoculated into fresh LB media supplemented with 1% glucose to an OD600 of 0.1 in flasks and were then incubated at 30 C with shaking at 250 rpm. The cells were harvested by centrifugation at 10,000× *g* rpm for 5 min at 4 °C. After washing, the cell pellets were resuspended in 50 mL of 50 mM sodium phosphate buffer (pH 8.0) containing 40 g/L 2,3-butanediol. The whole-cell biocatalyst reaction was performed at 37 °C and 220 rpm in 500 mL flasks.

3.4. Analytical Procedures

Protein expression was analyzed by 12% (*w/v*) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Cell growth was monitored by measuring the absorbance at 600 nm (OD600) with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, NY, USA). The fluorescence intensity was measured by determining the 485 nm (excitation) and 515 nm (emission) values with a fluorometer (Molecular Devices, model: SpectraMax M2, NY, USA), using a 96-well clear bottom black plate (Coaster Co). The products (acetoin and 2,3-butanediol) in the broth were extracted using ethyl acetate containing n-butanol as the internal standard and then quantified using a GC system (Agilent GC9860; FID detector, DB-5 column, NY, USA). Determination of the NADH and NAD+ concentrations and assays of the 2,3-BDH, NOX and FDH activities were performed according to previous studies [4].

4. Conclusions

In this study, we developed an auto-inducing expression system that could be used to construct various whole-cell biocatalyst systems. As quorum sensing is a general response to high cell density at stationary growth phase, which may not be ideal for the production of bio-based chemicals or compounds, we produced enzymes using an auto-inducing

expression system. Then, we produced 2,3-butanediol and acetoin using the constructed whole-cell biocatalyst system. By constructing different cofactor regeneration systems, *S. marcescens* could be used in different bioconversion processes. Our findings confirm the suitability of *S. marcescens* for the production of 2,3-butanediol and acetoin.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AHL: N-acyl homoserine lactones; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GFP, green fluorescent protein; slaA, acetoactate decarboxylase; slaB, acetolactate synthase; slaC, 2,3-butanediol dehydrogenase.

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