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Enhancement of Herboxidiene Production in *Streptomyces chromofuscus* ATCC 49982 S

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Structurally, herboxidiene contains the tetrahydropyran acetic acid moiety and a side chain including a conjugated diene, and has been isolated from *Streptomyces chromofuscus* ATCC 49982. Its production was significantly elevated nearly 13.5-fold (0.74 g/l) in a medium supplemented with glycerol (medium No. 6A6), and was more efficacious (1.08 g/l; 19.8-fold) in fed-batch fermentation at 36 h in medium No. 6A6, from *Streptomyces chromofuscus*. For further enhancement, regulatory genes *metK1-sp* and *afsR-sp* from *Streptomyces peucetius* were overexpressed using an expression vector, pIBR25, and similarly *ACCase* from *Streptomyces coelicolor* and two genes, *metK1-sp* and *afsR-sp*, were also overexpressed using an integration vector, pSET152, under the control of the strong *ermE** promoter in *Streptomyces chromofuscus*. Only the recombinant strains *S. chromofuscus* SIBR, *S. chromofuscus* GIBR, and *S. chromofuscus* AFS produced more herboxidiene than the parental strain in optimized medium No. 6A6 with an increment of 1.32-fold (0.976 g/l), 3.85-fold (2.849 g/l), and 1.7-fold(1.258 g/l) respectively.

Keywords: Herboxidiene, *Streptomyces chromofuscus*, glycerol, fed-batch fermentation, metabolic engineering

Introduction

Actinomycetes that produce potent antibiotics with broad pharmacological and agricultural profiles have received special attention for resolving the problem of antibiotic resistance to conventional drugs [2, 30]. The polyketide herboxidiene was isolated from *Streptomyces chromofuscus* ATCC 49982 (*Streptomyces* sp. A7847) and was found to control (>90%) several important biannual weeds at relatively low application rates (<250 g/hectare) without damaging wheat [5, 17].

Herboxidiene is structurally characterized by the tetrahydropyran acetic acid moiety and a side chain including a conjugated diene [6]. Herboxidiene activates the synthesis of low-density lipoprotein (LDL) receptor by up-regulating the gene expression of the LDL receptor, which effectively reduces plasma cholesterol [12], and exhibits strong cytotoxic activity by up-regulating luciferase receptor gene expression as well as inducing both G1 and G2/M arrest in the WI-38 human tumor cell line [24, 8]. Recently, the 53 kb biosynthesis gene cluster for herboxidiene was analyzed by genome sequencing and gene inactivation [27]. As herboxidiene bioactivity was found to be high against several herbs, a human tumor cell line, and cholesterol, the chemical syntheses of herboxidiene and analogs have been explored [4, 7, 18, 23, 31]. The major problem associated with the industrial production of herboxidiene is its high production cost. Thus, taking into consideration of previous evidence on the production of teicoplanin in *Actinoplanes teichomyceticus* [21] and clavulanic acid in *Streptomyces clavuligerus* [13], the effect of glycerol on herboxidiene production was assessed. It is presumable that biosynthesis of herboxidiene production can be prolonged by supplementation of glycerol in the medium, as acetyl-CoA is the starter unit of herboxidiene and further extended as a fatty acyl chain *via* malonyl-CoA-which can use glyceraldehyde-3-phosphate as the precursor.

In the era of molecular microbiology and recombinant

DNA technology, it is easy to alter the metabolic flux distribution of different precursors, which can be another target in product enhancement. Acetyl-CoA carboxylase is as example used for enhanced production of actinorhodin and flaviolin *via* modified precursor supplies [16]. The positive regulator *S*-adenosylmethionine synthetase (MetK) and a global regulatory gene, *afsR*, can enhance secondary metabolites by heterologous expression. The effect of *S*-adenosylmethionine synthetase (MetK) on the production of secondary metabolites from different *Streptomyces* species has been previously reported [11, 14, 15, 19, 29, 32]. Another regulatory protein (*afsR),* which is a pleiotropic global regulator, controls the production of multiple secondary metabolites production in *Streptomyces* [9, 26]. The overexpression of *afsR-sp* in *Streptomyces lividans*, *S. clavuligerus*, *S. griseus*, and *S. venezuelae* leads to overproduction of actinorhodin, clavulanic acid, streptomycin, and pikromycin, respectively [15, 20]. Here, efforts were made to analyze the effect of glycerol and develop recombinants for the enhancement of herboxidiene production. To this end, cell mass, pH, and herboxidiene production were evaluated.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Escherichia coli XL1-Blue (MRF) (Stratagene, USA) was used for DNA amplification and *E. coli* JM110 was used to propagate non-methylated DNA. *E. coli* strains were grown at 37°C in Luria–Bertani medium in both liquid broth and agar plates supplemented with the appropriate antibiotics when necessary (100 µg/ml ampicillin and 100 µg/ml apramycin). Standard methods were used for DNA cloning, plasmid isolation, and restriction enzyme digestion [25]. *S. chromofuscus* was cultured for seed, and transformation of recombinant plasmids in ISP medium 2 and on R2YE plate, respectively. For the production, 5% seed of *S. chromofuscus* wild strain or each transformants was inoculated into a baffled 500 ml flask containing 50 ml of the production medium No. 6A6 and grown at 28°C on a rotatory shaker at 235 rpm for 8 days. The bacterial strains and plasmids used in this study are listed in Table 1.

Construction of Recombinant Plasmids

The expression vector pIBR25 [28] and integrative vector pSET152 [3], under the control of the strong *ermE** promoter, were used for cloning. The *metK1-sp* from *S. peucetius* was cloned into the *BamH*I and *Hin*dIII sites of pIBR25 and *BamH*I and *Bgl*II sites of pSET152 to form the recombinant plasmids pSIBR [15] and pSAM152 [22], respectively. Similarly, *afsR-sp* from *S. peucetius* was cloned into the *EcoR*I and *Hin*dIII sites of pIBR25 and *Spe*I and *Bgl*II sites of pSET152 to form the recombinant plasmids pGIBR [20] and pAFS152 [22], respectively. The integrative plasmids pACC152, pASA152 [16], and pSA152 [22] were also used.

Optimization of Glycerol

To evaluate the effect of glycerol on herboxidiene production, seed (4%) was cultured in various levels (%) of different carbon (corn starch 3.5%, maltose 3.5%, and sucrose 3.5%) and nitrogen (ProFlo 0.8%, peptone 0.8%, tryptone 0.8%, and soybean flour 0.8%) sources along with sources of carbon, nitrogen, and minerals of known herboxidiene production medium, medium No. 1 (pH 7.2) [17], and incubated at 28°C for 8 days in a shaking incubator at 235 rpm. Finally, medium No. 6A6 (Table 2), in which the strain exhibited optimum levels of herboxidiene production, was selected for further analysis.

Table 1. Strains and plasmids used in this study.

Bacterial strain	Description	Sources
S. chromofuscus 49982	Wild-type herboxidiene producer	ATCC
S. chromofuscus IBR25	Expression of pIBR25 vector into *S. chromofuscus*	This study
S. chromofuscus SIBR	pIBR25 with *metK1-sp* expressed into *S. chromofuscus*	This study
S. chromofuscus GIBR	pIBR25 with *afsR-sp* expressed into *S. chromofuscus*	This study
S. chromofuscus ACC	pSET152 with *ACCase* integrated into *S. chromofuscus*	This study
S. chromofuscus SAM	pSET152 with *metK1-sp* integrated into *S. chromofuscus*	This study
S. chromofuscus AFS	pSET152 with *afsR-sp* integrated into *S. chromofuscus*	This study
S. chromofuscus AS	pSET152 with *metK1-sp* and *afsR-sp* integrated into *S. chromofuscus*	This study
S. chromofuscus ASA	pSET152 with *ACCase*, *metK1-sp*, and *afsR-sp* integrated into *S. chromofuscus*	This study
E. coli		
XL1 Blue	General cloning host	Stratagene
JM110	Demethylation host	Stratagene

Table 2. Sources used for the production of herboxidiene.

Ingredient	Medium No. 1 (%)	Medium No. 6A6 (%)
Corn starch	3.5	…..
Glycerol	…..	5
ProFlo	0.8	0.25
$MgSO_4 \cdot 7H_2O$	0.1	0.1
KH_2PO_4	0.2	0.2
KNO_3	0.2	…..
NaCl 0.05	0.05	0.05
$CaCO_3$	0.015	0.015
$ZnSO_4 \cdot 7H_2O$	0.001	0.001
Fe-EDTA	0.018	0.018

Analysis of Growth Rate, pH, and Herboxidiene Production

To analyze the production of herboxidiene, 0.15 L of medium No. 6A6 was inoculated with 0.0045 g/l of 36-h-old mycelia in 2 L Erlenmeyer flasks. After 24 h of incubation, the cell pellets were collected at 24 h intervals by centrifuging 10 ml of culture broth at 3,000 rpm for 15 min. The supernatants obtained after removing the cell pellets were used to study the change in pH. The cell pellets were collected, washed with deionized water, and centrifuged twice. They were then placed at 80°C until their mass was constant to analyze the growth rate of *Streptomyces chromofuscus*, which was employed to prove the effectiveness of feeding glycerol and nitrogen to determine the best feeding time**.** Feeding experiments were carried out at 24, 36, and at 48 h of batch culture of *Streptomyces chromofuscus* (Table 3). Fermentation products were centrifuged and filtered through Whatman filter paper. The resulting ethyl acetate extracts were concentrated by a Rota-vapor and resuspended in methanol. Samples were analyzed by TLC, using chloroform:methanol in a ratio of 9:1. The Rf value, as determined by ultraviolet (UV) and *p*-anisaldehyde reaction, was 0.44 [17]. Finally, samples were analyzed by HPLC using a reverse phase C_{18} column (4.6 × 250 mm, 50 µm; KANTO Reagents, Japan) with a solvent system consisting of 0.05% trifluoroacetic acid and 100% acetonitrile with a flow rate of 1 ml/min (Rt 28 min) in the binary condition.

Protoplast Transformation

Protoplast transformation was done as previously described [10]. Briefly, plasmids pIBR25, pSIBR, pGIBR, pACC152, pSAM152, pAFS152, pSA152, pASA152, and pSET152 were introduced into *S. chromofuscus* by protoplast transformation from *E. coli* JM110 carrying the non-methylated plasmid. For protoplast transformation, 36-h-old mycelia of *S. chromofuscus* were harvested by centrifugation (3,200 rpm and 4°C for 12 min) and then washed with 15 ml of sucrose solution (10.3%), centrifuged (3,200 rpm and 4°C for 12 min), and washed again with 15 ml of P-buffer. Finally, 10 ml of lysozyme solution (2 mg/ml in P-buffer) was added to the cell pellets and the content incubated for 50 min at 37°C. After incubation, it was filtered and centrifuged for 12 min at 6,000 rpm, then washed with P-buffer twice, and mixed with 1 ml of P-buffer. From the resulting mixture, 100 µl was mixed with 20 µl of plasmid DNA and 200 µl of 40% polyethylene glycol (PEG) 1000, mixed and centrifuged for 1 min to discard the supernatant partially, then mixed with 100 µl of P-buffer, and finally plated on R2YE plate. The plates were incubated at 28°C for 24 h and then overlaid with 0.3% agar solution containing 10 µg/ml thiostreptone and 60 µg/ml apramycin to select *Streptomyces chromofuscus* recombinant with expression and integrative vector, respectively. After 1 week, thiostreptone- and apramycin-resistant colonies were selected and cultured in liquid ISP medium 2. Transformation was confirmed by isolation, and PCR of the plasmid from each strain. The transformants were designated as *S. chromofuscus* IBR25, *S. chromofuscus* SIBR, *S. chromofuscus* GIBR, *S. chromofuscus* ACC,

Table 3. Feeding effect of glycerol, ProFlo, and glycerol:ProFlo on herboxidiene production in fed-batch fermentation on medium No. 6A6.

No. of test	Fed at 24 h (%)	Fed at 36 h (%)	Fed at 48 h (%)	Herboxidiene (g/l)
1	…..	…..	…..	0.74
2	Glycerol 2.5	…..	…..	0.66
3	…..	Glycerol 2.5	…..	0.70
4	…..	…..	Glycerol 2.5	0.58
5	ProFlo 0.125	…..	…..	0.62
6	…..	ProFlo 0.125	…..	0.51
7	…..	…..	ProFlo 0.125	0.75
8	Glycerol : ProFlo 2.5 : 0.125	…..	…..	0.91
9	…..	Glycerol : ProFlo 2.5 : 0.125	…..	1.08
10	…..	…..	Glycerol : ProFlo 2.5 : 0.125	0.87

S. chromofuscus SAM, *S. chromofuscus* AFS, *S. chromofuscus* SA, *S. chromofuscus* ASA, and *S. chromofuscus* SET152, respectively, (Table 1).

Product Confirmation

The product analysis was carried out in optimized medium No. 6A6 (Table 2). To confirm the enhanced product, HPLC, ESI-QTOF mass, ^{1}H-NMR, and ^{13}C-NMR analyses were performed.

Results

Optimization of Glycerol

For the purpose of enhancement, glycerol was selected as the carbon source and another three different carbon sources were selected for analysis to verify the superiority of glycerol over the carbon sources used: glycerol 3.5% (0.58 g/l), corn starch 3.5% (0.055 g/l), maltose 3.5% (0.037 g/l), and sucrose 3.5% (0.027 g/l). Previously, ProFlo was favored as the nitrogen source for herboxidiene production. Hence, to assess the efficacious effect of nitrogen sources in combination with glycerol for enhancement of herboxidiene, ProFlo along with three other nitrogen sources were evaluated with 3.5% glycerol: ProFlo 0.8% (0.58 g/l) peptone 0.8% (0.3 g/l), tryptone 0.8% (0.2 g/l), and soybean flour 0.8% (0.38 g/l). The next stage involved the selection of minerals; among different mineral components in medium No. 1 [17], KNO_3 was excluded and the effect was significant (0.59 g/l without KNO_3). Finally, after verification of the efficacious effect of glycerol at various levels with different levels of nitrogen source (ProFlo), and exclusion of mineral (KNO_3), the new production medium was designated as medium No. 6A6 (Table 2).

Calibration Curve and Feeding Time

To investigate a suitable time for feeding in medium No. 6A6, a calibration curve between dry weight mycelia and the pH was plotted (Fig. 2). As a result, feeding experiments were carried out at 24, 36, and 48 h in medium No. 6A6 with neutral pH into the shaking flask at different fermentation times (Table 3). Samples were withdrawn from each flask and analyzed by HPLC (Table 3). The results presented in Table 3 indicate that it was better to feed glycerol:ProFlo (2.5%:0.125%) from 24 h to 48 h, with 36 h being optimal.

Effects of *afsR-sp*, *metK1-sp*, and ACCase on Herboxidiene Production

To enhance the production of herboxidiene from *S. chromofuscus*, pSAM152 and pSIBR were transformed into *S. chromofuscus* by protoplast transformation to generate *S. chromofuscus* SAM and *S. chromofuscus* SIBR, respectively (Table 1). The production of herboxidiene was then recorded and compared against that of the wild type in production medium No. 6A6 (Table 4).

Table 4. Enhancement of herboxidiene production in medium No. 6A6 by metabolic engineering.

Strains	Production (g/l)
S. chromofuscus ATCC 49982	0.74
S. chromofuscus IBR25	0.70
S. chromofuscus SIBR	0.976
S. chromofuscus GIBR	2.849
S. chromofuscus ACC	0.042
S. chromofuscus SAM	0.74
S. chromofuscus AFS	1.258
S. chromofuscus AS	0.66
S. chromofuscus ASA	0.64

Similarly, the introduction of pAFS152 and pGIBR into *S. chromofuscus* generated *S. chromofuscus* AFS and *S. chromofuscus* GIBR, respectively. The herboxidiene production was recorded to be higher in *S. chromofuscus* AFS and highest in *S. chromofuscus* GIBR at 8 days in production medium No. 6A6 (Table 4).

We also tried to enhance the carbon flux through acetyl-CoA to malonyl-CoA. The genes encoding the ACCase subunits, *accA2*, *accB*, and *accE*, were cloned in an integration vector under the control of the strong promoter *ermE** to generate pACC152. Introduction of pACC152 into *Streptomyces chromofuscus* did not significantly enhance the production of herboxidiene (Table 4).

To assess the combined effect of ACCase, *metK1-sp*, and *afsR-sp*, the construct pASA152 that incorporated all three genes was applied. Similarly, to ascertain the combined effect of *afsR-sp* and *metK1-sp*, construct pAS152 that incorporated the two genes was also applied. The recombinant strains *S. chromofuscus* ASA and *S. chromofuscus* AS were obtained by introducing pASA152 and pAS152 into *S. chromofuscus*, respectively. No significant changes were observed (Table 4).

Finally, the enhanced product was confirmed to be herboxidiene by ESI-QTOF mass (Supplementary Fig. S1A), ^{1}H-NMR (Fig. S1B), and ^{13}C-NMR analysis (Fig. S1C) analyses.

Discussion

Streptomyces chromofuscus ATCC 49982 produces herboxidiene, which is a polyketide natural product with important

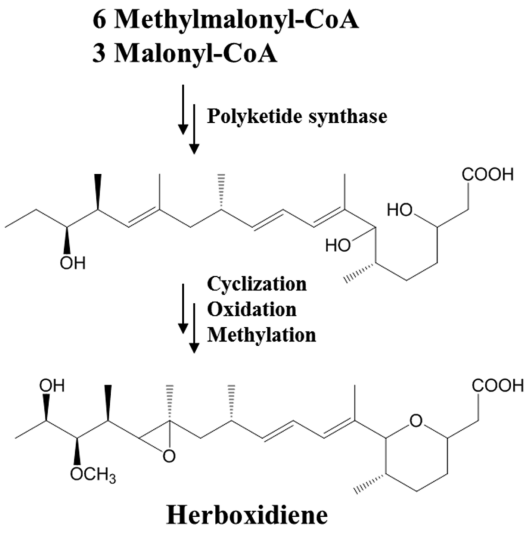

Fig. 1. Diagrammatic sketch of various approaches used for enhancement of herboxidiene production.

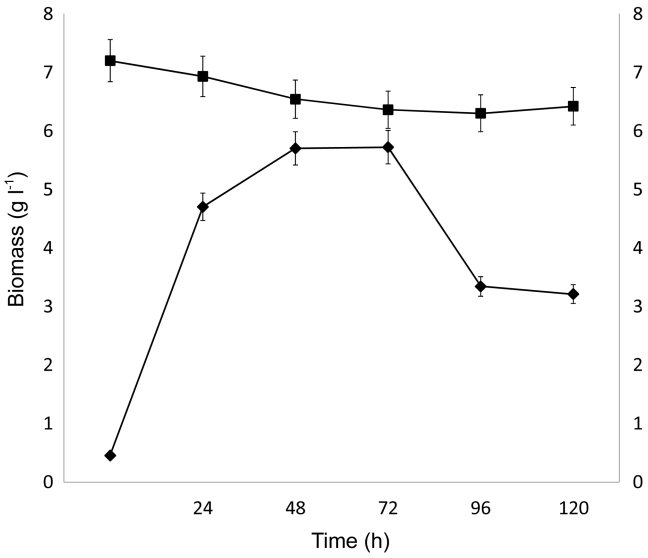

Fig. 2. Time course profiles of biomass and pH, after 24 h of batch fermentation of *S. chromofuscus*, the cell pellets were collected at 24 h intervals until 5 days to measure mass (◆) and pH (■).

applications that include herbicide, and the treatment of cancer, infectious diseases, and cardiovascular diseases. Industrially, herboxidiene is the most important polyketide. To improve the production of herboxidiene, we focused on implication of glycerol as the carbon source, the feeding process, and metabolic engineering. In previous studies on the effect of glycerol, it was observed that the production of teicoplanin and clavulanic acid in *Actinoplanes teichomyceticus* and *Streptomyces clavuligerus* was significantly increased [21, 13]; thus, the effect of glycerol on herboxidiene production was assessed. It is presumable that biosynthesis of herboxidiene production can be prolonged by supplementation of glycerol in medium, as acetyl-CoA is the starter unit of herboxidiene and this is further extended as a fatty acyl chain *via* malonyl-CoA, which can use glyceraldehyde-3-phosphate as the precursor (Fig. 1). Another possibility is that glycerol supports the development of cell mass, which is then followed by herboxidiene production. Previously, production was reported to be 0.0549 g/l herboxidiene [17], which is very little and comes with a high production cost in terms of the industrial scale. For the same purpose, glycerol was selected as the carbon source and another three different carbon sources were selected for analysis to verify the superiority of glycerol over the carbon sources. Previously, ProFlo was favored as the nitrogen source for herboxidiene production. ProFlo is the sole source of different amino acids, which may be partially involved in the formation of acetyl-CoA, the precursor for herboxidiene biosynthesis. The next stage involved the selection of minerals to improve the efficacious effect of glycerol on herboxidiene production. Potassium is required in carbohydrate metabolism, phosphate is a key element in the regulation of cell metabolism present in nucleic acid, sulfur and magnesium are cofactors for enzymes, iron plays a regulatory role in the fermentation process, and other minerals like salt, calcium, and zinc are necessary. KNO_3 is an inorganic nitrogen source, based on the knowledge that inorganic nitrogen sources such as nitrates, nitrite, and several ammonium salts can sometimes restrict antibiotic production [1]. A calibration curve between dry weight mycelia and the pH was plotted (Fig. 2). With a large concentration of mycelia, little pH difference in the calibration curve was evident during the lag phase (Fig. 2). As a result, feeding experiments were carried out at 24, 36, and 48 h in medium No. 6A6. The results presented in Table 3 indicate that it was better to feed from 24 h to 48 h, with 36 h being optimal; for this, there are several possible explanations. The large increase of cell mass (Fig. 3) and the feeding of ProFlo or glycerol:ProFlo can lower the pH of the fermentation broth. The low pH might be more suitable for herboxidiene production (Fig. 2). Moreover, it is better to feed the ProFlo or glycerol:ProFlo in the neutral or sub-acid pH condition. Thus, ProFlo or glycerol:ProFlo could be used for the production of herboxidiene. Finally, we approached metabolic engineering for further enhancement of herboxidiene

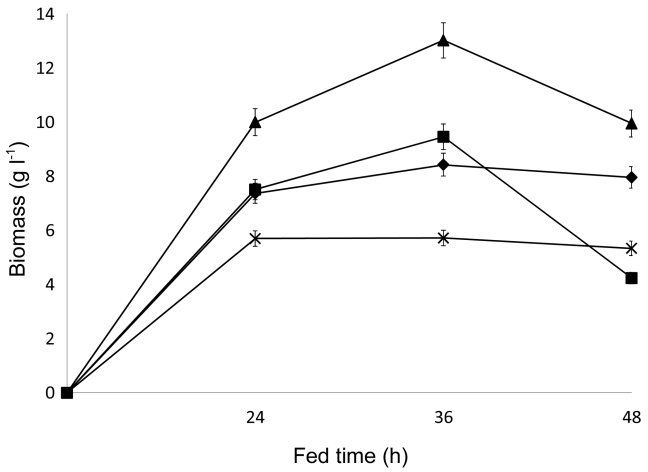

Fig. 3. Time-course profiles of biomass, after 24 h of fed-batch fermentation of *Streptomyces chromofuscus*.
The cell pellets were collected to measure the mass from media No. 6A6 (×) and No. 6A6 fed with ProFlo (◆), glycerol (■), and glycerol:ProFlo (▲) at 24, 36, and 48 h, respectively.

production. Introduction of pSAM152 and pSIBR into *S. chromofuscus* generated *S. chromofuscus* SAM and *S. chromofuscus* SIBR, respectively, which play an important role in the conversion of ATP and L-methionine to *S*-adenosyl-L-methionine (SAM), and may act as a methyl donor for the transmethylation reaction in the herboxidiene, as the gene cluster includes methyltransferases (*herF*) that enable herboxidiene efflux and therefore produce a higher amount in production medium No. 6A6. Introduction of pAFS152 and pGIBR into *S. chromofuscus* generated *S. chromofuscus* AFS and *S. chromofuscus* GIBR, respectively, which may play the major role in regulation of genes involved in production of herboxidiene, as it is a global regulator gene in production medium No. 6A6 . We also tried to enhance the carbon flux through acetyl-CoA to malonyl-CoA. The *accA2*, *accB*, and *accE* genes encoding the ACCase subunits were cloned in an integration vector under the control of the strong promoter *ermE** to generate pACC152. Introduction of pACC152 into *S. chromofuscus* did not significantly enhance the production of herboxidiene. Similarly, to assess the combined effect of ACCase, *metK1-sp*, and *afsR-sp*, the construct pASA152 that incorporated all three genes was applied, and to assess the combined effect of *afsR-sp* and *metK1-sp*, the construct pASA152 that incorporated both genes was also applied. The recombinant strains *S. chromofuscus* ASA and *S. chromofuscus* AS were obtained by introducing pASA152 and pAS152 into *S. chromofuscus*, respectively. However, there were no significant changes. This observation indicates that enhancement of the carbon flux does not have an influential role for enhancement of herboxidiene in production medium 6A6. On the basis of these results, we conclude that the increased production of herboxidiene is due to the increased pool of biosynthetic precursors, which could be harnessed for the feasible and cost-effective production of herboxidiene at the industrial scale.

Acknowledgments

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