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Overexpression of *afsR* and Optimization of Metal Chloride to Improve Lomofungin Production in *Streptomyces lomondensis* S015 S

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As a global regulatory gene in *Streptomyces*, *afsR* can activate the biosynthesis of many secondary metabolites. The effect of *afsR* on the biosynthesis of a phenazine metabolite, lomofungin, was studied in *Streptomyces lomondensis* S015. There was a 2.5-fold increase of lomofungin production in the *afsR*-overexpressing strain of *S. lomondensis* S015 N1 compared with the wild-type strain. Meanwhile, the transcription levels of *afsR* and two important genes involved in the biosynthesis of lomofungin (i.e., *phzC* and *phzE*) were significantly up-regulated in *S. lomondensis* S015 N1. The optimization of metal chlorides was investigated to further increase the production of lomofungin in the *afsR*-overexpressing strain. The addition of different metal chlorides to *S. lomondensis* S015 N1 cultivations showed that $CaCl_2$, $FeCl_2$, and $MnCl_2$ led to an increase in lomofungin biosynthesis. The optimum concentrations of these metal chlorides were obtained using response surface methodology. $CaCl_2$ (0.04 mM), $FeCl_2$ (0.33 mM), and $MnCl_2$ (0.38 mM) gave a maximum lomofungin production titer of 318.0 ± 10.7 mg/l, which was a 4.1-fold increase compared with that of *S. lomondensis* S015 N1 without the addition of a metal chloride. This work demonstrates that the biosynthesis of phenazine metabolites can be induced by *afsR*. The results also indicate that metal chlorides addition might be a simple and useful strategy for improving the production of other phenazine metabolites in *Streptomyces*.

Keywords: Lomofungin, *afsR*, *Streptomyces lomondensis*, bioactive phenazine metabolite, metal chloride addition, fermentation technology

Introduction

Phenazine-containing natural products are important microbial secondary metabolites produced by *Pseudomonas*, *Streptomyces*, and a few other genera from soil or marine habitats [1, 22, 29]. More than 100 natural phenazine compounds have been identified and reported to possess a broad range of biological properties, including antibacterial, antifungal, antitumor, antimalarial, antiparasitic, and neuronal cell-protecting activities [1, 6, 8, 20, 22]. Lomofungin is an olive-yellow phenazine metabolite, which was first isolated by Bergy [2] from *S. lomondensis* sp. n. UC-5022. Lomofungin has been reported to exhibit many interesting biological properties, including its ability to inhibit the growth of gram-positive and gram-negative bacteria [11, 21, 22], inhibit the growth of human pathogenic fungi [21], and inhibit the activity of angiotensin-converting enzyme, which is a target for the treatment of hypertension [22]. Furthermore, lomofungin has recently been reported as a precursor for potential antitumor drugs [4].

Phenazine metabolites are generally produced at low levels in wild-type strains. Over the past four decades, attention was paid to enhancing phenazine metabolites production by medium optimization and genetic engineering. Previous reports showed that phenazine metabolite fermentation in many *Pseudomonas* strains was sensitive to some nutritional factors, especially metal salts [7, 37, 38, 40]. Considerable research efforts have been focused on the use of genetic engineering to regulate the biosynthesis of phenazine metabolites in *Pseudomonas* [18, 35]. Although

many of the regulatory genes involved in the biosynthesis of secondary metabolites in *Streptomyces* have been studied [27], only *ppzV* has been reported to be involved in regulating the biosynthesis of phenazine metabolites [36].

As a global regulatory gene for the formation of secondary metabolites in *Streptomyces*, *afsR* was first cloned from *S. coelicolor* A3 (2) by Horinouchi *et al*. [14]. High expression levels of *afsR* have been proven to activate the biosynthesis of many secondary metabolites such as actinorhodin and undecyl-prodigiosin in *S. lividans* [15], a calcium-dependent antibiotic in *S. lividans* [16], clavulanic acid in *S. clavuligerus* [5, 19], doxorubicin in *S. peucetius* [26], and validamycin A in *S. hygroscopicus* [43]. The structures of these secondary metabolites contain a wide variety of different moieties, including polyketides, β-lactams, tripyrroles, and aminoglycosides. Although several studies have been conducted towards developing a deeper understanding of the role of *afsR* in the biosynthesis of secondary metabolites, it remains unknown whether *afsR* could regulate the biosynthesis of phenazine metabolites in *Streptomyces*. In light of their interesting biological and structural properties, there has been considerable research interest in evaluating the effects of *afsR* on the biosynthesis of phenazine metabolites.

S. lomondensis S015 was isolated in our laboratory from a rhizosphere soil sample collected from a suburb of Shanghai in China, and this strain was capable of biosynthesizing lomofungin [41]. Studies on the fermentative conditions of *S. lomondensis* S015 showed that metal salts, such as $FeCl_3$, could be used to increase the production of lomofungin in this strain [41]. Herein, we describe our most recent effort towards further improving the production of lomofungin in *S. lomondensis* S015 by the overexpression of *afsR* and the optimization of the metal chloride used in the culture medium. This study on the optimization of metal chloride concentrations was conducted using response surface methodology (RSM) and the central composite design (CCD) approaches with the *afsR*-overexpressing strain *S. lomondensis* S015 N1. This study represents the first reported account of the effects of *afsR* on the production of phenazine metabolites, and it is therefore envisaged that the results of this study could be used for the efficient production of other bioactive phenazine compounds, especially in *Streptomyces*.

Materials and Methods

Strains, Plasmids, and Culture Conditions

Wild-type *S. lomondensis* S015 (CCTCC No: M2013140), *afsR*-overexpressing *S. lomondensis* S015 N1 (CCTCC No: M2013141), and the vector control *S. lomondensis* S015 N0 were cultivated at 28°C according to the conditions described by Wang *et al*. [41]. For the seed culture, approximately 5 mm^2 sections of the agar slants grown on mannitol soybean (MS) medium (2% mannitol, 2% soybean powder, 2% agar, pH 7.2) for 12 h were punched out with a sterilized cutter and transferred to a 250 ml Erlenmeyer flask containing 50 ml of yeast malt (YM) medium (0.4% yeast extract, 1% malt extract, 0.4% glucose, pH 7.2). After 24 h of cultivation, a 3 ml sample of the seed culture broth was transferred to a 500 ml flask containing 150 ml of the YM medium for mycelia growth and antibiotic production. This particular flask was cultivated on a rotary shaker at 180 rpm.

Escherichia coli ET12567/pUZ8002 and plasmids pSET152 and pHL851 were provided by Prof. Meifeng Tao (Shanghai Jiao Tong University, China). Plasmid pSET152 is a shuttle vector that can replicate in *E. coli* and integrate itself in a site-specific manner into *Streptomyces* chromosomes with an apramycin resistance gene for selection [3]. The strong constitutive *ermE** promoter for the expression of the cloned *afsR* gene was cloned into the integrative vector pSET152 to give pHL851 [5]. *E. coli* was grown on Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.2) at 37°C.

Conjugation

Experiments for the intergeneric conjugation of plasmids pHL851 and pSET152 from *E. coli* ET12567/pUZ8002 into *S. lomondensis* S015 to obtain *S. lomondensis* S015 N1 and *S. lomondensis* S015 N0 were conducted according to the methods described by Chen *et al*. [5] and Mazodier *et al*. [30], respectively. Exconjugants were confirmed through cultivating on MS solid medium with apramycin (50 μg/ml) and nalidixic acid (20 μg/ml).

Measurement of Dry Cell Weight (DCW) and Lomofungin Production

Samples containing up to 5 ml of the culture broth were centrifuged at 12,000 ×*g* for 8 min. The precipitants were washed at least three times with distilled water before being dried to a constant weight at 60°C to give material for a DCW assay.

The supernatant was adjusted to pH 2.0 with a 6 N solution of aqueous HCl and mixed with 5 ml of pure butanone. The resulting mixture was centrifuged at 12,000 ×*g* for 5 min and the upper layer was collected. The water layer (lower layer) was extracted a second time with 5 ml of pure butanone, and the combined extracts were dried using a rotary vacuum dryer (Christ RVC 2-18, Osterode, Germany) at 33°C, and the resulting residue was dissolved in 5 ml of HPLC-grade solvent (*i.e*., a 1:1 (v/v) mixture of 0.1% formic acid and acetonitrile) and filtered through a 0.22 μM PVDF syringe filter (Millipore, Shanghai, China). A 20 μl sample of the resulting filtrate was analyzed by HPLC using an Agilent 1260 HPLC system (Algient, Beijing, China) equipped with a DAD detector, and an Agilent Eclipse Plus C18 column (250 × 4.6 mm; 5 μm), which was used at 30°C. The mobile phase consisted of solvents A (0.1% formic acid) and B (acetonitrile),

which were used with the following gradient profile: 0 to 4 min, 80% to 60% A; 4 to 20 min, 60% A; and 20 to 30 min, 60% to 80% A. The HPLC system was operated at a constant flow rate of 1 ml/min. Lomofungin was monitored at 270 nm and identified by comparison with an authentic sample [41].

Optimization of Metal Chloride Species and Individual Metal Chloride Concentrations

Several different metal chloride species, including $FeCl_3$, $FeCl_2$, $CaCl_2$, $MnCl_2$, $MgCl_2$, $CuCl_2$, and $ZnCl_2$, were individually added to the culture medium at a concentration of 0.1 mM to evaluate their effects. Different concentrations (*i.e.*, 0.05, 0.1, 0.3, 0.5, 0.8, and 1 mM) of $CaCl_2$, $FeCl_2$, and $MnCl_2$ were used to investigate the effects of the metal chloride concentration. The fermentation broth was sampled on day 4 to analyze its DCW and lomofungin production.

Optimization of Metal Chloride Concentrations by RSM

Levels of the three metal chlorides ($CaCl_2$, $FeCl_2$, and $MnCl_2$) were further optimized to enhance lomofungin production by RSM using CCD [24, 25, 34]. Version 8.0.6 of the Design-Expert statistical software package (StatEase, Minneapolis, MN, USA) was used for the experimental design, regression analysis of the data, and creation of the response surface plots. Each factor in the design was studied at five coded levels ($-\alpha$, -1, 0, +1, $+\alpha$) and the actual values for each metal chloride concentration at various levels were chosen according to information obtained from the single factor experiments. A second-order polynomial model was used to fit the response surface, as shown in Eq. (1):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response, x_i and x_j are the coded independent variables that influence the response variable Y, β_0 is the intercept, β_i represents the linear effect of x_i, β_{ii} represents the quadratic effect of x_i, and β_{ij} represents the interaction between x_i and x_j.

The quality of the quadratic model equations was checked by determining R^2. The statistical significance of the models was determined by Fisher's test, and the regression coefficients were determined by Student's test. The optimum concentrations of the variables were calculated through differentiation of the quadratic model.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

The transcriptional levels of the core genes involved in the biosynthesis of lomofungin (*i.e.*, *phzC* (GenBank Accession No. KF144611) and *phzE* (GenBank Accession No. KF144612)) and the regulatory gene *afsR* (GenBank Accession No. KF144615) were analyzed by qRT-PCR according to the methods described by Fan *et al*. [10] and Xie *et al*. [42].

Total RNA from the fresh cells was extracted using an RNAprep pure Cell/Bacteria kit (TianGen, Shanghai, China), and its concentration was determined using a Biophotometer Plus (Eppendorf, Shanghai, China). After DNase treatment, 1 μg of total RNA from each sample was reverse-transcribed using a Quantscript RT kit (TianGen).

Table 1. Sequences of the primers used for qRT-PCR.

Target gene		Primer sequences (5′–3′)
16S rRNA	Forward	5′-TGTCGTGAGATGTTGGGT-3′
	Reverse	5′-TTCATGGGGTCGAGTTGC-3′
*phz*C	Forward	5′-ACCTGCTGTCCGATGTGG-3′
	Reverse	5′-GTGCGGTGATGGTGTTGC-3′
phzE	Forward	5′-CCGAAGGCCGCGGTTACT-3′
	Reverse	5′-CGGGCGATGCCGTTGTTG-3′
afsR	Forward	5′-GCGGCAGGAAGCCGCAGGC-3′
	Reverse	5′-GCTGGACAACGCCCGGGAC-3′

The RealMasterMix (Tiangen) was used to perform qRT-PCR. Table 1 shows the sequences of the primers used in the current study for the amplification of the genes. The 16S rRNA gene (GenBank Accession No. KF144610) was used as an internal control gene because its expression was found to be stable under our experimental conditions. The expression levels of the different genes were normalized with respect to the level of 16S rRNA expression. For each gene, an expression level of 1 was assigned to the samples collected from the control culture at each harvest time point, and the expression levels under the other conditions were presented as fold changes relative to this reference.

Statistical Analysis

All of the experiments were conducted in at least triplicates and the associated data presented as the average of three independent sample measurements. The error bars represent the standard deviation (SD) from the mean value of each set of triplicate data points. These data were analyzed using Student's t-test. Differences between contrasting treatments were considered significant when $p < 0.05$ following a two-tail analysis.

Results

Overexpression of *afsR* in *S. lomondensis* S015

The *afsR* gene was identified in *S. lomondensis* S015 through PCR amplification, and an *afsR*-overexpressing strain of *S. lomondensis* S015 N1 was subsequently constructed for use in this paper to study its effects on the biosynthesis of lomofungin.

The dynamic cell growth and lomofungin production profiles of wild-type *S. lomondensis* S015, *afsR*-overexpressing *S. lomondensis* S015 N1, and the vector control *S. lomondensis* S015 N0 are shown in Fig. 1. No obvious differences were seen in the biomass in Fig. 1A, which suggested that neither of the two plasmids had any effect on cell growth. All three strains reached their maximum DCW of about

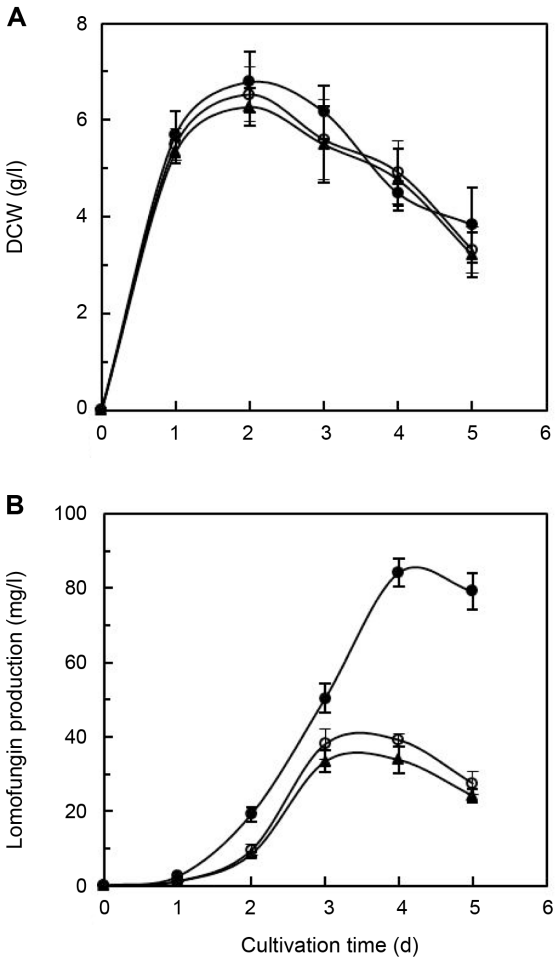

Fig. 1. Effects of the overexpression of *afsR* on cell growth (**A**) and lomofungin production (**B**).
Symbols: wild-type *S. lomondensis* S015 (dark triangle), *afsR*-overexpressing *S. lomondensis* S015 N1 (dark circle), and vector control *S. lomondensis* S015 N0 (open circle). The error bars in the figure represent the standard deviations from three independent samples.

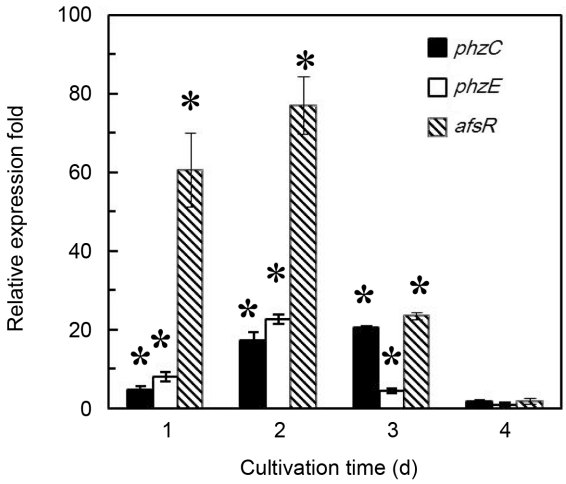

Fig. 2. Transcription levels of the three genes (*i.e.*, *afsR*, *phzC*, and *phzE*) in *S. lomondensis* S015 N1 cells with *afsR* overexpression.
The culture medium containing the wild-type *S. lomondensis* S015 was regarded as a control culture for each gene at each sample point with an expression level of 1.0, and the expression levels in the *afsR*-overexpressing strain *S. lomondensis* S015 N1 have been displayed as fold increases relative to the control. The error bars in the figure represent the standard deviations from three independent samples. *indicates statistical significance ($p < 0.05$) compared with the control culture.

6.5 g/l on day 2, with the value then decreasing until day 5. Fig. 1B showed that the lomofungin yield increased significantly under all conditions after 2 days of cultivation. The *afsR*-overexpressing strain *S. lomondensis* S015 N1 gave its maximum lomofungin production of 84.2 ± 3.7 mg/l on day 4, representing a 2.5-fold increase compared with that of the wild-type strain. No discernible differences were observed in the production of lomofungin between the vector control *S. lomondensis* S015 N0 and the wild-type strain.

In *Streptomyces* and *Pseudomonas*, phenazine metabolites are biosynthesized through the phenazine biosynthetic pathway using a conserved core gene cluster known as *phz*. Two of the most important genes belonging to the *phz* cluster are the *phzC* and *phzE* genes [28, 29, 31]. In this study, the transcription levels of *afsR*, *phzC*, and *phzE* were investigated by qRT-PCR with samples taken on days 1, 2, 3, and 4. The relative expression folds of these three genes in the *afsR*-overexpressing strain compared with the wild-type strain are shown in Fig. 2.

The regulatory (*afsR*) and structural (*phzC* and *phzE*) genes involved in the biosynthesis of lomofungin were all up-regulated on days 1, 2, and 3. The maximum transcription levels of the *afsR*, *phzC*, and *phzE* genes in *S. lomondensis* S015 N1 were 76.1- (day 2), 19.5- (day 3), and 21.6-times (day 2) greater than those in the wild-type strain control culture, respectively. The relative expression folds of these three genes in the vector control *S. lomondensis* S015 N0 had no statistical significance ($p < 0.05$) compared with those in the wild-type strain (data not shown).These results indicated that the global regulatory gene *afsR* could be used to enhance the biosynthesis of phenazine metabolites by activating the expression of the core genes involved in the biosynthesis of phenazines in *Streptomyces*.

Optimization of Metal Chloride Species and Individual Metal Chloride Concentrations

The pathways involved in the biosynthesis of numerous

natural phenazine metabolites could be enhanced by the addition of different metal salts [37, 38, 40]. We recently demonstrated that the production of lomofungin could be increased by the addition of a 0.1 mM solution of $FeCl_3$ to a culture medium containing *S. lomondensis* S015 [41]. In this study, we have examined the effects of a variety of different metal chlorides on the production of lomofungin in *S. lomondensis* S015 N1 cultivations at a concentration of 0.1 mM. The maximum level of lomofungin production achieved in this strain following the addition of an individual metal chloride was obtained on day 4, as shown in Table 2.

In all cases, the addition of a metal chloride solution to the culture medium had very little impact on cell growth (data not shown). In contrast, the addition of different metal chlorides to the culture medium had a significant effect on the accumulation of lomofungin. The production of lomofungin increased significantly following the addition of $FeCl_2$ and $FeCl_3$, with $FeCl_2$ appearing to be more effective than $FeCl_3$. $CaCl_2$ and $MnCl_2$ also led to significant increases in lomofungin production, whereas $MgCl_2$, $ZnCl_2$, and $CuCl_2$ all had a positive impact on lomofungin accumulation. These results suggested that the chloride anions of these salts were not critically involved in enhancing lomofungin production.

Taken together, these results suggested that $CaCl_2$, $FeCl_2$, and $MnCl_2$ were playing a significant role in stimulating the biosynthesis of lomofungin in cultures containing *S. lomondensis* S015 N1, and further studies were therefore conducted to assess the impact of their dosage on the production of lomofungin. Solutions containing the three different metal chlorides were prepared at six different final concentrations (*i.e.*, 0.05, 0.1, 0.3, 0.5, 0.8, and 1.0 mM) and added to cultures containing *S. lomondensis* S015 N1 on day 0. The lomofungin production levels were enhanced in all cases on day 4 (Fig. 3) following the addition of the three different metal chlorides to the medium. Furthermore,

Table 2. Effects of various metal chlorides on the production of lomofungin by *S. lomondensis* S015 N1 on day 4.

Metal chlorides	Lomofungin production (mg/l)
$FeCl_3$	124.8 ± 8.9
$FeCl_2$	147.7 ± 6.4
$CaCl_2$	124.8 ± 10.3
$MnCl_2$	105.2 ± 7.0
$MgCl_2$	32.6 ± 3.8
$CuCl_2$	Not detected
$ZnCl_2$	Not detected
Control	74.8 ± 5.2

All the metal chlorides were added at a concentration of 0.1 mM on day 0. No metal chlorides were added to the control. The data shown in the table represent the mean values of three independent experiments with the associated standard deviations.

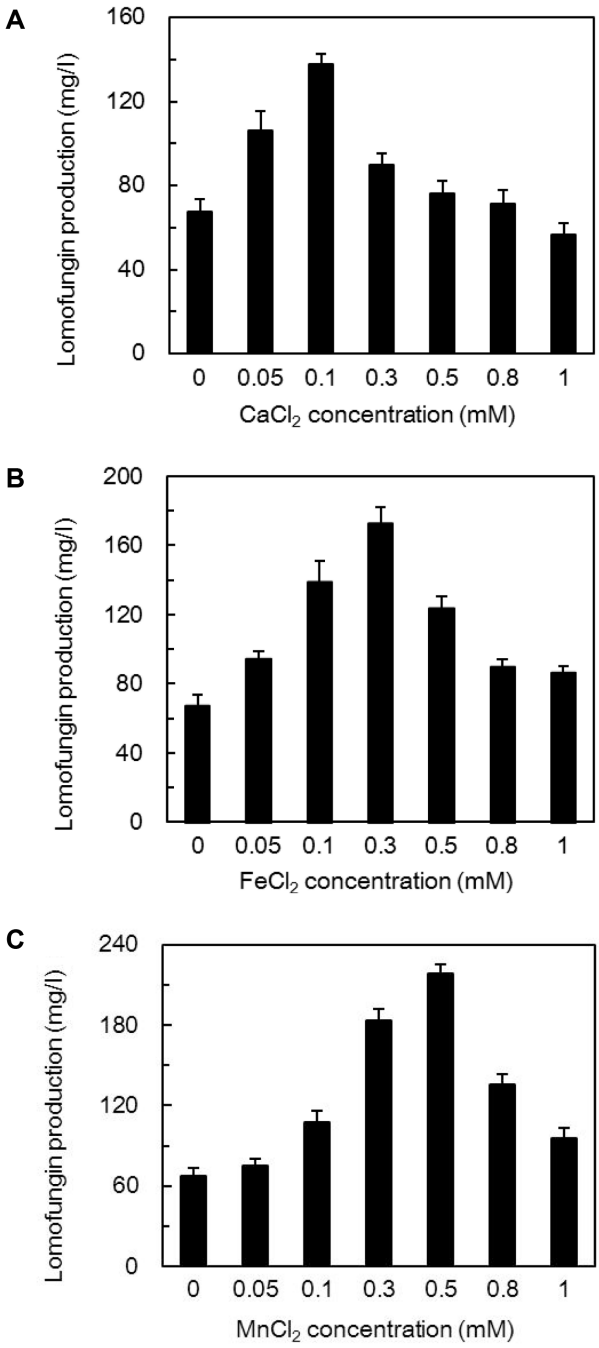

Fig. 3. Effects of the addition of individual metal chlorides (**A**: $CaCl_2$; **B**: $FeCl_2$; and **C**: $MnCl_2$) on lomofungin production in a culture medium containing *S. lomondensis* S015 N1 on day 4. The error bars in the figure represent the standard deviations from three independent samples.

increasing the concentration of these metal chlorides initially led to further increases in the production of lomofungin, although higher concentrations led to a decrease in production. The maximum lomofungin production levels achieved in this system were 137.7 ± 5.0, 172.8 ± 9.5, and 218.6 ± 7.0 mg/l following the addition of 0.1, 0.3, and 0.5 mM of $CaCl_2$, $FeCl_2$, and $MnCl_2$, respectively, which were approximately 2.0, 2.6, and 3.2 times the production level obtained in the control cultures without the addition of a metal chloride.

Optimization of Metal Chloride Concentration by RSM

The concentrations of $CaCl_2$, $FeCl_2$, and $MnCl_2$ were optimized by RSM, where interactions between these parameters were analyzed. The design matrix and the corresponding results of RSM experiments are shown in Table 3. The data were analyzed using ver. 8.0.6 of the Design Expert software package, and a second-order polynomial model equation was given as Eq. (2):

$$Y = 299.0 - 13.0x_1 + 3.6x_2 - 14.3x_3 - 18.8x_2x_3 + 24.5x_1x_3 - 15.7x_1^2 - 36.4x_2^2 - 31.1x_3^2 \quad (2)$$

where Y represents the response (predicted lomofungin production, mg/l), x_1 represents $CaCl_2$, x_2 represents $FeCl_2$, x_3 represents $MnCl_2$. This quadratic equation includes three linear terms, two two-factorial interactions, and three quadratic terms. The nonsignificant term of P values more than 0.1 (i.e., x_1x_2) was excluded from the model (Table 4).

The fitness of the model was verified by analysis of variance (ANOVA), which was tested by Fisher's statistical analysis, as shown in Table 4. The *F* value of the model was 60.98 and the Prob>*F* value was <0.0001, indicating that the model is highly significant. The coefficient of determination (R^2) was 0.9821, denoting that more than 98% of the sample variation was attributed to the variables. The predicted R^2 (0.8831) and adjusted R^2 (0.9660) validated the significance of the model.

To determine the optimal levels of each variable for maximum lomofungin production, 3D response surface plots were constructed (Fig. S1). According to the optimized mathematical model, the optimum levels of the three metal chlorides were 0.04 mM of $CaCl_2$, 0.33 mM of $FeCl_2$, and 0.38 mM of $MnCl_2$, and the corresponding predicted maximum lomofungin production was 310.9 mg/l.

Several experiments using both the optimized (*i.e.*, addition of metal chlorides as predicted) and non-optimized (*i.e.*, without the addition of a metal chloride) media were performed to confirm the predictions described above. A maximum lomofungin production titer of 318.0 ± 10.7 mg/l was obtained after 4 days of cultivation under the optimized medium, representing a 4.1-fold increase over the non-optimized medium (Fig. S2).

Table 3. Experimental design and results of CCD.

Run	Coded levels (real values, mM)			Lomofungin production (mg/l)	
	x_1 ($CaCl_2$)	x_2 ($FeCl_2$)	x_3 ($MnCl_2$)	Actual	Predicted
1	–1 (0.04)	–1 (0.18)	–1 (0.32)	241.6	242.5
2	1 (0.16)	–1 (0.18)	–1 (0.32)	178.4	173.0
3	–1 (0.04)	1 (0.42)	–1 (0.32)	303.0	292.8
4	1 (0.16)	1 (0.42)	–1 (0.32)	216.3	212.4
5	–1 (0.04)	–1 (0.18)	1 (0.68)	198.3	202.6
6	1 (0.16)	–1 (0.18)	1 (0.68)	220.4	231.0
7	–1 (0.04)	1 (0.42)	1 (0.68)	171.7	177.5
8	1 (0.16)	1 (0.42)	1 (0.68)	195.5	195.1
9	–1.682 (0.00)	0 (0.30)	0 (0.50)	276.8	276.5
10	1.682 (0.20)	0 (0.30)	0 (0.50)	233.0	232.8
11	0 (0.10)	–1.682 (0.10)	0 (0.50)	196.2	190.2
12	0 (0.10)	1.682 (0.50)	0 (0.50)	196.8	202.2
13	0 (0.10)	0 (0.30)	–1.682 (0.20)	223.8	235.1
14	0 (0.10)	0 (0.30)	1.682 (0.80)	198.8	187.0
15	0 (0.10)	0 (0.30)	0 (0.50)	296.5	299.0
16	0 (0.10)	0 (0.30)	0 (0.50)	307.4	299.0
17	0 (0.10)	0 (0.30)	0 (0.50)	301.4	299.0
18	0 (0.10)	0 (0.30)	0 (0.50)	291.5	299.0
19	0 (0.10)	0 (0.30)	0 (0.50)	301.9	299.0
20	0 (0.10)	0 (0.30)	0 (0.50)	295.4	299.0

The actual values for the production of lomofungin represent the mean values of three different experiments.

Table 4. ANOVA for the full second-order polynomial quadratic model.

Source	Sum of squares	Mean square	*F* value	*P* value
Model	44,383.79	4,931.53	60.98	< 0.0001
x_1 ($CaCl_2$)	2,308.62	2,308.62	28.55	0.0003
x_2 ($FeCl_2$)	175.16	175.16	2.17	0.0719
x_3 ($MnCl_2$)	2,794.17	2,794.17	34.55	0.0002
x_1x_2	58.86	58.86	0.73	0.4136
x_1x_3	4,797.1	4,797.10	59.31	< 0.0001
x_2x_3	2,838.81	2,838.81	35.1	0.0001
x_1^2	3,553.54	3,553.54	43.94	< 0.0001
x_2^2	19,042.38	19,042.38	235.45	< 0.0001
x_3^2	13,954.71	13,954.71	172.54	< 0.0001

R^2 = 0.9821, adjusted R^2 = 0.9660, predicted R^2 = 0.8831, CV = 3.71

Discussion

The *afsR* gene is a global regulatory gene in *Streptomyces* that can increase the production of a series of secondary metabolites, such as polyketides, β-lactams, tripyrroles, and aminoglycosides [5, 15, 39, 43]. The results of the current study demonstrate that *afsR* could be used to enhance the biosynthesis of phenazine metabolites through the overexpression of *afsR* in *S. lomondensis* S015. In most *Streptomyces*, the gene cluster responsible for the biosynthesis of phenazine metabolites contains six core genes, including *phzB*, *phzC*, *phzD*, *phzE*, *phzF*, and *phzG* [28]. The biosynthesis of phenazine metabolites begins with shikimic acid [22, 31]. Gene *phzC* encodes a 3-deoxy-D-aranimoheptulosonate 7-phosphate synthase, which catalyzes the first step of the shikimate pathway. The subsequent steps in the shikimate pathway give rise to chorismic acid, which is converted to 2-amino-2-desoxyisochorismic acid by PhzE [28]. The *S. lomondensis* S015 N1 mutant strain prepared in the current paper expressed the *phzC* and *phzE* genes at a much higher level than the wild-type stain. The lomofungin production levels suggested that the *afsR* gene could be used to enhance the biosynthesis of phenazine metabolites by activating the shikimate pathway. Further studies towards determining whether *afsR* could modulate other genes involved in the biosynthesis of phenazine metabolites are currently under way in our laboratory and will be published in due course.

In this work, the addition of a metal chloride salt (*i.e.*, $CaCl_2$, $FeCl_2$, or $MnCl_2$) to the culture medium was found to have a positive effect on the production of lomofungin. Ferric and manganese salts represent positive nutrition in the culture medium for many phenazine metabolites production [37, 38, 40]. However, few reports have been published on calcium salt stimulation of phenazine biosynthesis. Magnesium and zinc salts have also been reported to have a positive impact on the production of phenazine metabolites in cultures containing different *Pseudomonas* strains [37, 38, 40]. The results of the current study, however, showed that the addition of a 0.1 mM solution of $MgCl_2$ led to a 60% decrease in the production of lomofungin compared with the control culture containing no metal salt, and no lomofungin was detected in the culture medium following the addition of a 0.1 mM solution of $ZnCl_2$. These results suggested that the impact of different metal salts could vary depending on the nature of the phenazine-producing strain and the structure of the phenazine product.

Price-Whelan *et al*. [33] suggested that Fe^{2+} could be used to enhance phenazine production by influencing the precursors involved in the biosynthesis of phenazine compounds. Lomofungin could be activated by chelation to Mn^{2+} [11, 12, 32], and this chelate might have an influence on the concentration of lomofungin in the culture broth, which could result in an increase in the production of lomofungin. Both iron and manganese can regulate secondary metabolite production through gene *fur* [9, 13, 17, 23]. The expression levels of *fur* increased in *S. lomondensis* S015 N1 cultivated with $FeCl_2$ or $MnCl_2$ addition (Fig. S3). This finding suggests that both $FeCl_2$ and $MnCl_2$ can regulate phenazine biosynthesis through gene *fur*. In contrast to $FeCl_2$ and $MnCl_2$, no obvious changes of *fur* expression level were observed in cultivation with $CaCl_2$ addition. The relationship between the minerals and the biosynthesis of lomofungin warrants further investigation.

The whole genome sequence of *S. lomondensis* S015 has recently been completed, and a single gene cluster responsible for the biosynthesis of phenazine metabolites has been found, containing *phzBCDEFG*. The mechanism responsible for the observed increase in the biosynthesis of phenazine metabolites following the addition of a metal chloride to the culture medium or the overexpression of regulatory genes (*i.e.*, *afsR*) in *S. lomondensis* S015 are currently being investigated in our laboratory.

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