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Enhancement of spinosad production in *Saccharopolyspora spinosa* by overexpression of the complete 74-kb spinosyn gene cluster

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Abstract

Background Spinosad, a secondary metabolite produced by *Saccharopolyspora spinosa*, is a polyketide macrolide insecticide with low toxicity and environmental friendliness. Owing to the high level of DNA methylation and unclear regulatory mechanisms, gene engineering to increase spinosad production is challenging. Limited improvements in yield have been observed with heterologous expression or partial overexpression of the 74-kb spinosyn gene cluster (*spn*), and research on the overexpression of the complete spinosyn gene cluster is lacking.

Results The plasmid pCM265-spn was constructed using CRISPR/Cas9-mediated Transformation-Associated Recombination cloning to enable the overexpression of the complete *spn* gene cluster in *Sa. spinosa*. The engineered strain *Sa. spinosa-spn* achieved a 124% increase in spinosad yield (693 mg/L) compared to the wild type (309 mg/L). The overexpression of the *spn* gene cluster also delayed spore formation and reduced hyphal compartmentalization by influencing the transcription of related genes (*bldD*, *ssgA*, *whiA*, *whiB*, and *fstZ*). Transcriptional analysis revealed significant upregulation of genes in the *spn* gene cluster, thereby enhancing secondary metabolism. Additionally, optimization of the fermentation medium through response surface methodology further increased spinosad production to 920 mg/L.

Conclusions This study is the first to successfully overexpress the complete *spn* gene cluster in *Sa. spinosa*, significantly enhancing spinosad production. These findings have significance for further optimization of spinosad biosynthesis.

Keywords *Saccharopolyspora spinosa*, Spinosad, Overexpression, Spinosyn gene cluster

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Background

Spinosad is a secondary metabolite produced through aerobic fermentation by *Sa. spinosa*, a gram-positive bacterium isolated from soil [1]. In fact, *Sa. spinosa* can produce more than 25 structurally similar compounds, among which the mixture of the two most active components, spinosyn A and D, is known as spinosad. Structurally, spinosad belongs to the class of polyketide macrolide compounds, and is characterized by a 21-carbon macrolactone ring, along with a rhamnose and a forosamine. As a broad-spectrum insecticide, spinosad has low toxicity toward predatory insects and mammals, and is easy to degrade and environmentally friendly, making it highly valuable for research [2].

The biosynthetic gene cluster for spinosad was identified, comprising 23 genes, with 19 genes situated within a large region of approximately 74 kb (abbreviated as the *spn* gene cluster), while the remaining 4 genes are dispersed throughout the genome [3]. The *spnA*, *spnB*, *spnC*, *spnD*, and *spnE* genes encode polyketide synthases responsible for forming the macrolactone backbone [3]. *spnF*, *spnJ*, *spnM*, and *spnL* further modify this backbone into its final structure [4]. Additionally, *spnG*, *spnH*, *spnI*, *spnK*, *gtt*, *gdh*, *epi*, and *kre* are involved in the synthesis and attachment of rhamnose, as well as its methylation [5, 6]. Finally, *spnN*, *spnO*, *spnP*, *spnQ*, *spnR*, and *spnS* play roles in the synthesis and linkage of forosamine [7, 8] (Fig. 1).

The biosynthesis of secondary metabolites from actinomycetes relies on the expression of biosynthetic gene clusters (BGCs) and the regulation of regulatory genes. An increase in biosynthesis can be achieved by the overexpression of BGCs, the upregulation of positive regulatory genes, or the downregulation of negative regulatory genes [9, 10]. However, the high methylation level in *Sa. spinosa* DNA present significant challenges for genetic modification aimed at increasing spinosad production [11]. Moreover, the regulatory mechanisms governing spinosad biosynthesis are not well understood. Recently, Mu's study suggested that the LysR family transcriptional regulator ORF-L16 may act as a positive regulator of spinosad biosynthesis [12]. Therefore, overexpressing spinosad BGCs is considered a potent strategy to enhance yield. Extensive research has focused on genes involved in the synthesis and linkage of rhamnose and forosamine. For example, the engineered strain *Sa. spinosa* pIBR-SPN FR, which overexpressed these genes, exhibited a 13-fold increase in spinosad production compared to the wild type [13]. The overexpression of the *gdh*, *kre*, *gtt*, and *epi* genes led to a 2.6-fold increase in yield [14]. However, owing to the large size of the entire *spn* gene cluster and extremely difficult to perform genetic manipulation in *Sa. spinosa*, most studies have only duplicated individual genes or partial segments within this cluster [15]. Madduri et al. overexpressed a 20-kb gene segment of the *spn* gene cluster in *Sa. spinosa*, but observed no increase in yield [16]. Similarly, Tang et al. duplicated an approximately 18-kb gene segment of the *spn* gene cluster in *Sa. spinosa*, increasing the yield of spinosad from 100 mg/L to 388 mg/L [17]. To date, there has been no research on the overexpression of the complete 74-kb *spn* gene cluster in *Sa. spinosa*. Moreover, while the spinosad BGC has been successfully introduced into various heterologous hosts such as *S. erythraea* [18], *S. coelicolor* [19], and *S. albus* [20], the yields from these expression systems have generally been lower (< 70 mg/L).

In this work, the whole *spn* gene cluster in *Sa. Spinosa* was captured using TAR (Transformation-Associated Recombination) cloning system which relies on the homologous recombination system of yeast, and cloned into integrated plasmid pCM265. We overexpressed the *spn* gene cluster in *Sa. spinosa* to improve the production

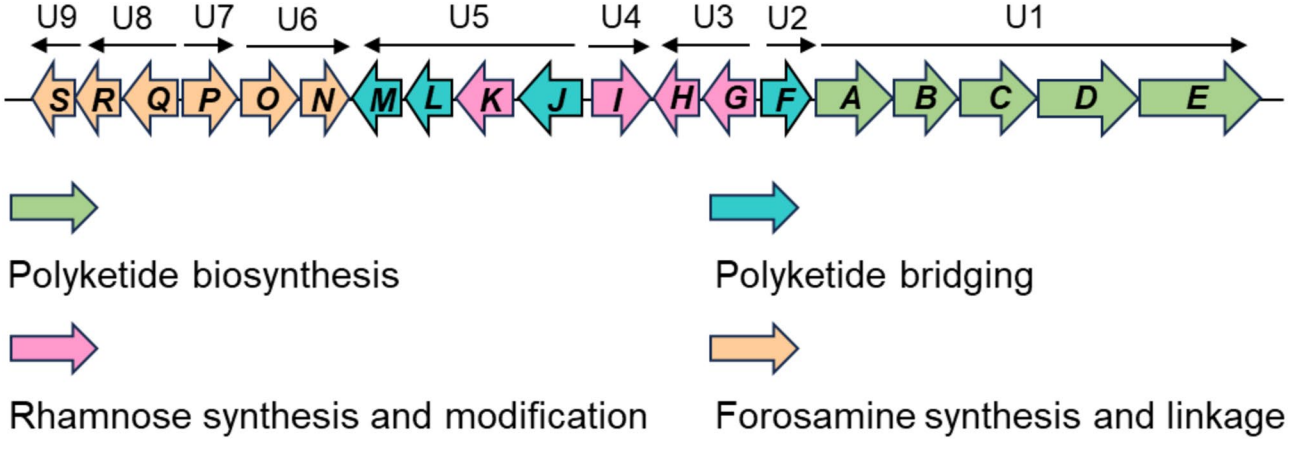

Fig. 1 Schematic diagram of the composition and function of the *spn* gene cluster. The 19 genes of *spn* were divided into 9 co-transcription units (U1, U2, U3, U4, U5, U6, U7, U8, and U9) and are shown by black arrows. According to their functions, the 19 genes can be divided into four groups, each represented by a different color

of spinosad. Further enhancements in production were achieved by optimizing the medium components using response surface methodology (RSM).

Methods

Strains, plasmids, primers, medium and growth conditions

All strains and plasmids used in this study were listed in Table S1, and the primers used were listed in Table S2. The *Sa. spinosa* strain was cultured on solid medium (beef extract 1 g/L, yeast extract 5 g/L, glucose 10 g/L, tryptone 3 g/L, $MgSO_4$ 2 g/L, agar 20 g/L and pH 7.4) at 28℃ to produce spores. Fresh spores were transferred to liquid seed medium (cornstarch 15 g/L, mannitol 20 g/L, cottonseed meal 20 g/L, yeast extract 15 g/L, soybean meal 15 g/L, L-tyrosine 1 g/L, $MgSO_4$ 2 g/L, $(NH_4)_2SO_4$ 0.5 g/L, and pH 7.0) and incubated at 28℃ for 3 days. The seed solution was inoculated into the fermentation medium (soybean oil 12.5 g/L, corn steep powder 10 g/L, yeast extract powder 4 g/L, glucose 60 g/L, soybean meal 15 g/L, cottonseed meal 40 g/L, NaH_2PO_4 2 g/L, $FeSO_4$ 0.05 g/L, $CaCO_3$ 5 g/L and pH 7.4) at an inoculum level of 15% and incubated at 28℃ for 10 days. *Escherichia. coli* EPI300 and DH5α were used as general cloning hosts. *E. coli* S17-1 was used to introduce plasmids into the *Sa. spinosa*. *E. coli* was cultured in LB medium (yeast 5 g/L, tryptone 10 g/L, NaCl 10 g/L) at 37℃. For the conjugative transfer of *Sa. spinosa*, the strains were spread on 2×CMC plates (soluble starch 10 g/L, tryptone 2 g/L, NaCl 1 g/L, $(NH_4)_2SO_4$ 2 g/L, K_2HPO_4 1 g/L, Casamino acid 2 g/L, $MgSO_4{\cdot}7H_2O$ 2 g/L, $CaCO_3$ 2 g/L, agar 20 g/L, pH 7.2) and cultured at 30℃ for about 12 d. All strains containing the pCM265 plasmid required the addition of the apramycin during culture (50 μg/mL).

Construction of recombinant plasmids and engineered strains

The *spn* gene cluster was excised from the chromosome of *Sa. spinosa* by CRISPR/Cas9-mediated TAR according to the reported experimental method [21]. Three Cas9-gRNA complexes were separately used for a 12 h overnight enzymatic digestion of the *Sa. spinosa* genomic DNA to obtain two segments of the *spn* gene cluster, referred to as *spn1* and *spn2*. Next, *spn1* and *spn2* were separately transformed into yeast protoplasts along with linearized pCAP01a-CAP1 and pCAP01a-CAP2 cloning vectors for homologous recombination. Correct yeast transformants containing the desired gene clusters were selected through PCR screening. The plasmids pCAP01a-spn1 and pCAP01a-spn2 were transformed into EPI300 for plasmid amplification. The restriction enzyme *SwaI* was used to cut the pCAP01a-spn1 and pCAP01a-spn2 plasmids to release *spn1* and *spn2*. Then, we utilized the overlap between *spn1* and *spn2*, along with the homologous fragments UP3 and DOWN3, to insert *spn1* and *spn2* into the *PmeI* site of pCM265-CAP (One Step Fusion Cloning Mix, TOROIVD, China). The pCM265-spn was constructed and transformed into *E. coli* DH5α. After pCM265-spn was successfully constructed, it was transformed into *E. coli* S17-1, which was subsequently transformed into *Sa. spinosa* by *E. coli-Streptomyces* intergeneric conjugation using the method of Matsushima et al. [11]. The authenticity of these plasmids was verified through restriction enzyme digestion, PCR, and DNA sequencing.

Analysis of spinosad concentration

After 10 days fermentation, 1 mL of fermentation broth was added to 4 mL methanol, sonicated for 30 min at RT, and centrifuged for 4 min at 9,000 g. Spinosad was determined by high-performance liquid chromatography (HPLC) (Shimadzu, Japan). The chromatographic column used was a Waters Symmetry C18 reversed-phase column (5 μm, 4.6 × 250 mm) with the mobile phase being 45% methanol, 45% acetonitrile, 10% 260 mM ammonium acetate solution at a flow rate of 1 mL/min and a detection wavelength of 250 nm with a sample volume of 10 μL. The spinosad concentration was calculated as the sum of spinosyn A and D. Spinosad was quantified using a regression line generated from a commercially available standard (MedChemExpress HY138800) dissolved in methanol. Each treatment was performed in triplicates to calculate the standard deviations.

RNA extraction and cDNA synthesis

After fermentation, 1 mL of the fermentation broth was centrifuged at 4℃ and 6,000 g for 6 min. 800 μL of *AG RNAex Pro* RNA Extraction Reagent (Accurate biology, China) was added to the precipitate, which was mixed thoroughly, and the mixture was frozen at -20℃ for 4 h. We added 160 μL of trichloromethane, vortexed for 1 min, and then allowed the mixture to stand for 5 min. Following centrifugation at 4℃ and 12,000 g for 15 min, the supernatant was transferred to a new tube containing 400 μL of isopropanol, gently mixed, and then left to stand for 10 min. The mixture was centrifuged for 5 min under the same conditions, and the supernatant was discarded. Next, 800 μL of 80% ethanol (anhydrous ethanol diluted with DEPT water) was added, and the mixture was washed twice. After standing at RT for 15 min, 50 μL of RNase-free water was added to the precipitate, which was then dissolved completely in 37℃-water bath for 15 min. After instantaneous centrifugation, the RNA concentration in the supernatant was measured by Nanodrop (Thermo Fisher Scientific, USA). We used RNase-free DNase I (Takara, Japan) to remove residual DNA. cDNA synthesis was performed with Evo M-MLV Reverse Transcription Reagent Premix (Accurate biology,

China). The efficiency of the cDNA synthesis was verified by PCR amplification.

For RNA extraction from spores, the spores on slants were first resuspended in 5 mL of sterile water by gentle scraping, respectively. The resulting spore suspension was transferred to a sterile flask containing glass beads. After thorough shaking, the spore suspension was filtered through sterile cotton. The filtrate containing the spores was then centrifuged at 9000 g for 5 min, and the spores were collected into a new tube. Subsequent RNA extraction procedures followed the protocol used for RNA extraction from fermentation broth samples.

Quantitative reverse transcriptase PCR (qRT-PCR)

The expression levels of target genes were analyzed by the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, USA) using SYBR Green Pro Taq HS Kit (Accurate biology, China). The sequences of primers used were listed in Table S2. To reduce experimental errors, three samples were taken and qRT-PCR was performed twice for each gene. The 16S rRNA gene was chosen as the internal reference gene to standardize the transcript level measurements and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method [22].

Optimization of the fermentation medium by RSM

Based on preliminary experiments, cottonseed meal, glucose, and soybean oil were identified as key factors influencing spinosad production. Single-factor experiments preliminarily identified the optimal concentrations as 50 g/L for cottonseed meal, 90 g/L for glucose, and 15 g/L for soybean oil (Fig. S4). A three-factor, three-level combination experiment was then designed, with the optimal concentration for each factor set as "0", and levels below and above this optimal set as "-1" and "+1" (Table S4). The detailed experimental combinations and corresponding results are presented in Table S5. The experimental data were analyzed using Design-Expert software, resulting in the regression equation for spinosad production (Y) as a function of cottonseed meal (A), glucose (B), and soybean oil (C).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 9.0), and the unpaired *t*-tests were used to calculate *p* values for comparing results between the experimental groups and controls. All the experiments were performed in triplicate.

Results

Construction of pCM265-spn and *Sa. spinosa-spn*

Given that the complete *spn* gene cluster is about 74 kb in length, multiple attempts to cleave it as a whole using Cas9-gRNA complexes were unsuccessful. It was finally decided to split the *spn* gene cluster into two fragments, designated *spn1* and *spn2*, with a 925 bp overlap between them. The schematic diagram of the entire process is shown in Fig. 2. We used the Cas9-gRNA complexes to obtain the large fragments *spn1* and *spn2* separately, which were then inserted into pCM265-CAP (*PmeI* digested) by homologous recombination. Next, pCM265-spn was introduced into *Sa. spinosa* via conjugative transfer and integrated into its chromosome, successfully resulting in the genetic engineering strain *Sa. spinosa-spn*. During the construction process, we verified the results by PCR amplification and sequencing (Fig. S1).

Effects of overexpression of *spn* on sporulation and morphology

Actinomycetes have a complex lifecycle characterized by two distinct modes of cell division, which include the formation of cross-walls during vegetative hyphal development and the production of aerial hyphae with septa to generate reproductive spores [23]. During our study, we observed significant differences in growth between *Sa. spinosa* and *Sa. spinosa-spn*.

To investigate the effects of overexpressing the *spn* gene cluster on sporulation and morphology, we cultured *Sa. spinosa* and *Sa. spinosa-spn* on solid medium and observed spore development at various time points. We found that the spores of *Sa. spinosa* turned pinkish gray earlier than the spores of *Sa. spinosa-spn* (Fig. 3a). Furthermore, we observed the morphology of single bacterial colonies under the microscope. We found that the colony morphology of *Sa. spinosa-spn* was more circular in shape, and when there were dents and cracks on *Sa. spinosa*, *Sa. spinosa-spn* remained intact (Fig. 3b). These results indicate that the overexpression of *spn* slows down the production of spores and changes their appearance. In addition, after culturing the strains in liquid medium for 60 h, the mycelia were collected and the hyphal morphology was observed using scanning electron microscopy (SEM). We observed that the hyphae of *Sa. spinosa-spn* were finer and longer with reduced septum, whereas *Sa. spinosa* exhibited more pronounced hyphal fragmentation (Fig. 3d and S2). The number of septa in the hyphae of *Sa. spinosa* was estimated to be approximately twice that of *Sa. spinosa-spn*. To further explore the underlying causes of these morphological changes, we selected several genes related to spore growth and mycelial separation for transcript analysis.

The *bldD* gene is a regulatory gene closely associated with the development and differentiation of actinomycetes. In many actinomycetes, the *bldD* gene is considered a "repressor" and its downregulation promotes spore formation [24, 25]. Additionally, proteins encoded by *ssgA*, *whiA* and other genes play crucial roles in sporulation in actinomycetes [26–28]. qRT-PCR analysis

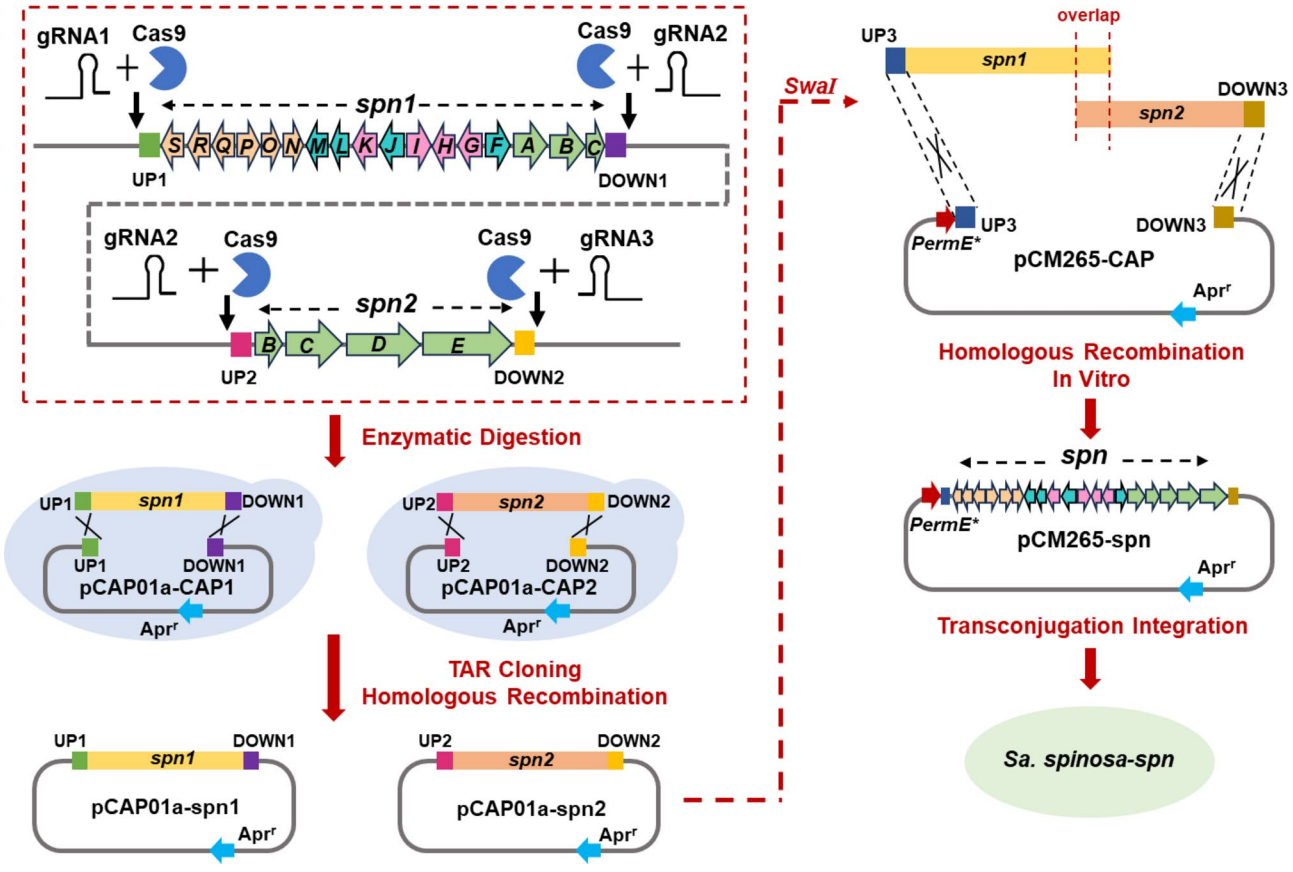

Fig. 2 Schematic diagram of the construction process for *Sa. spinosa-spn*. UP and DOWN represented the regions upstream and downstream of genes, and they contained overlapping sequences to facilitate the DNA assembly. *spn*1 and *spn2* were obtained from the genomic DNA of *Sa. spinosa* by CRISPR/Cas9-mediated TAR, as shown on the left side of the figure. After obtaining the *spn1* and *spn2* fragments separately, they were connected to the pCM265 vector using the 925 bp overlap and the homologous segments UP3 and DOWN3. Then the plasmid pCM265-spn was introduced into *Sa. spinosa* by conjugative transfer

was conducted to examine the transcriptional levels of the *bldD*, *ssgA*, *whiA* and *whiB* genes in *Sa. spinosa* and *Sa. spinosa-spn*. The results showed that the transcription level of the *bldD* gene was upregulated, while the transcription levels of *ssgA*, *whiA* and *whiB* were downregulated in *Sa. spinosa-spn* (Fig. 3c). These findings can explain the delayed spore formation and changes in their appearance observed in *Sa. spinosa-spn*. The *ftsZ* gene encodes a protein that plays a critical role in bacterial cell division. In actinomycetes, FtsZ proteins polymerize to form a ring-like structure (Z-ring) and are involved in regulating branch formation [29, 30]. qRT-PCR analysis revealed that the transcription level of the *fstZ* gene was downregulated in *Sa. spinosa-spn* (Fig. 3c), which may explain the reduced septa and fragmentation in the hyphae of *Sa. spinosa-spn*.

Effects of overexpression of *spn* on spinosad biosynthesis

Spinosad is a secondary metabolite produced by *Sa. spinosa* through aerobic fermentation. To evaluate the spinosad production capabilities of *Sa. spinosa* and *Sa. spinosa-spn*, the concentration of spinosad was determined using HPLC after 10 days culture in shake flask. The results showed that the overexpression of the whole *spn* gene cluster significantly increased the yield of spinosad in *Sa. spinosa-spn* compared to *Sa. spinosa* (Fig. 4a). The introduction of an empty vector, which does not contain foreign genes, may indirectly influence the production of secondary metabolites in actinomycetes. To assess this potential impact, the empty vector pCM265 was introduced into *Sa. spinosa*. The results revealed no significant changes in the fermentation titer of spinosad, suggesting that the increased spinosad yield in *Sa. spinosa-spn* is not attributable to the plasmid vector but rather to the overexpression of the *spn* gene cluster (Fig. 4c).

Although genetic engineering approaches typically enable a significant enhancement in the yield of secondary metabolites, genetic instability frequently occurs, leading to productivity loss and impacting industrial applications [31]. To evaluate the genetic stability of *Sa. spinosa-spn*, multiple batch fermentations were

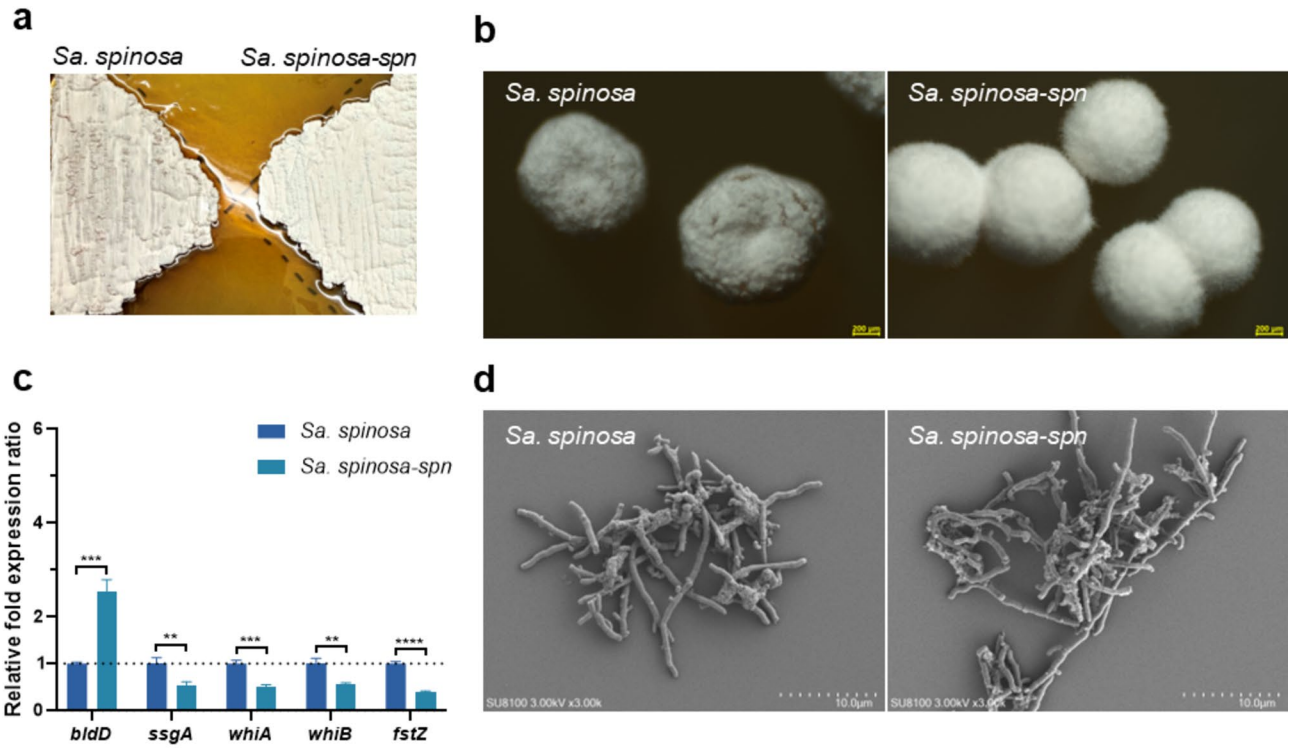

Fig. 3 Effects of overexpression of *spn* on sporulation and morphology. **a** Comparison of morphology on solid media after 7 days of cultivation. **b** Microscopic image of single bacterial colonies of *Sa. spinosa* and *Sa. spinosa-spn* after 7 days of cultivation. **c** Transcriptional analysis of genes related to sporulation and hyphal differentiation by quantitative real-time PCR. RNA samples of the genes *bldD*, *ssgA*, *whiA* and *whiB* were isolated from spores at 3 d, while RNA samples of the gene *fstZ* were isolated from mycelia at 48 h. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. **d** Sanning electron microscopy images of hyphae from *Sa. spinosa* and *Sa. spinosa-spn* after 60 h of cultivation

conducted. The results indicated that the genetically engineered strain *Sa. spinosa-spn* can stably produce spinosad with an average yield of 693 mg/L, representing a 124% increase compared with the original strain's yield of 309 mg/L (Fig. 4b).

There are 19 genes related to spinosad biosynthesis located in the *spn* gene cluster. To investigate the impact of overexpressing the *spn* gene cluster on the transcription levels of these genes, we collected samples on the 7th day of fermentation, extracted total RNA and then synthesized cDNA. Validation of the cDNA synthesis was performed via PCR amplification, and the results are shown in Fig S3. We conducted transcription level analysis using qRT-PCR and used 16S rRNA as the reference gene. In the experimental setup, the gene expression levels in *Sa. spinosa* served as the control group (represented by dashed lines). The analysis results revealed that, compared with those of the control, the transcription levels of all 19 genes were increased to varying degrees in *Sa. spinosa-spn* (Fig. 4d). The elevated transcription levels of the spinosad BGC suggested an enhancement in secondary metabolism, which contributed to the increased yield of *Sa. spinosa-spn*.

Optimization of the fermentation medium by RSM

After overexpressing the *spn* gene cluster, the strain exhibited greater production potential. However, the original fermentation medium could no longer provide adequate nutrients for spinosad biosynthesis. Therefore, we optimized the fermentation medium by RSM.

Significance testing and analysis of variance were performed (Table S6). The model F-value of 20.54 and the *p* value of 0.002 (< 0.05) indicated that the model was significant. The lack of fit F-value of 0.15 implied that the lack of fit was not significant relative to the pure error, indicating that the model was suitable. Additionally, the predicted R^2 of 0.8745 is in reasonable agreement with the adjusted R^2 of 0.9262, and the difference was within 0.2, indicating that the model was valid.

Response surface plots were generated based on the experimental results, with each plot illustrating the pairwise interaction effects among the factors on spinosad production (Fig. 5). The results showed that the yield of spinosad was related to all three factors and adjusting the ratios of these three factors could enhance the production of spinosad. Software predictions identified the optimal levels of the three factors as follows: cottonseed meal 55 g/L, glucose 90 g/L, and soybean oil 10 g/L, with a predicted spinosad yield of 931.3 mg/L. To validate the

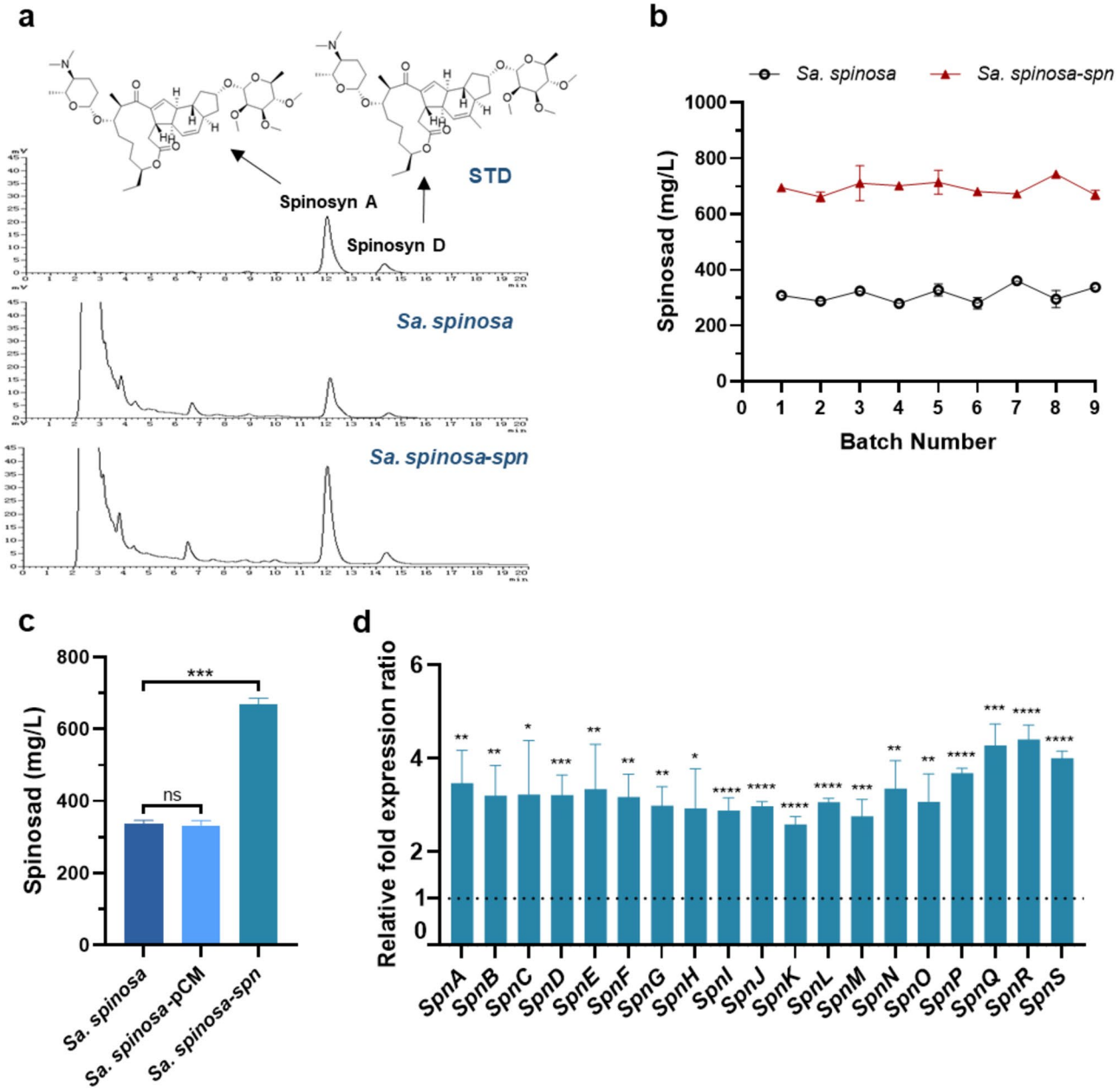

Fig. 4 Effects of overexpression of *spn* on spinosad biosynthesis. **a** The HPLC analysis of fermentation products of *Sa. spinosa* and *Sa. spinosa-spn*. **b** Fermentation analysis of 9 batches of *Sa. spinosa* and *Sa. spinosa-spn* to verify genetic stability. **c** Spinosad production profiles of *Sa. spinosa*, *Sa. spinosa*-PCM, and *Sa. spinosa-spn*. **d** Transcriptional analysis of 19 genes by qRT-PCR. RNA samples were isolated from fermentation at 7 d. The transcription level of *Sa. spinosa* was used as a control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p < 0.001$, ****$p < 0.0001$

reliability of the predicted results, we conducted three replicate experiments under the predicted optimal conditions. The average spinosad yield was 920 mg/L, which closely matched the predicted value, confirming the validity of the model and highlighting its potential for practical applications. By optimizing the fermentation medium for *Sa. spinosa-spn*, the yield of spinosad was further increased by 198% compared with that of *Sa. spinosa* (309 mg/L).

Discussion

Previous studies have demonstrated that the heterologous biosynthesis of spinosad is inefficient (< 70 mg/L) [32], and *Sa. spinosa*, as the host strain, still holds significant advantages. The *spn* gene cluster includes most spinosad biosynthesis genes. However, due to the large size of the Polyketide Synthase (PKS) genes within this cluster has led previous studies to avoid replicating the PKS genes [16, 17]. It is clear that the increase in spinosad production from overexpressing an incomplete

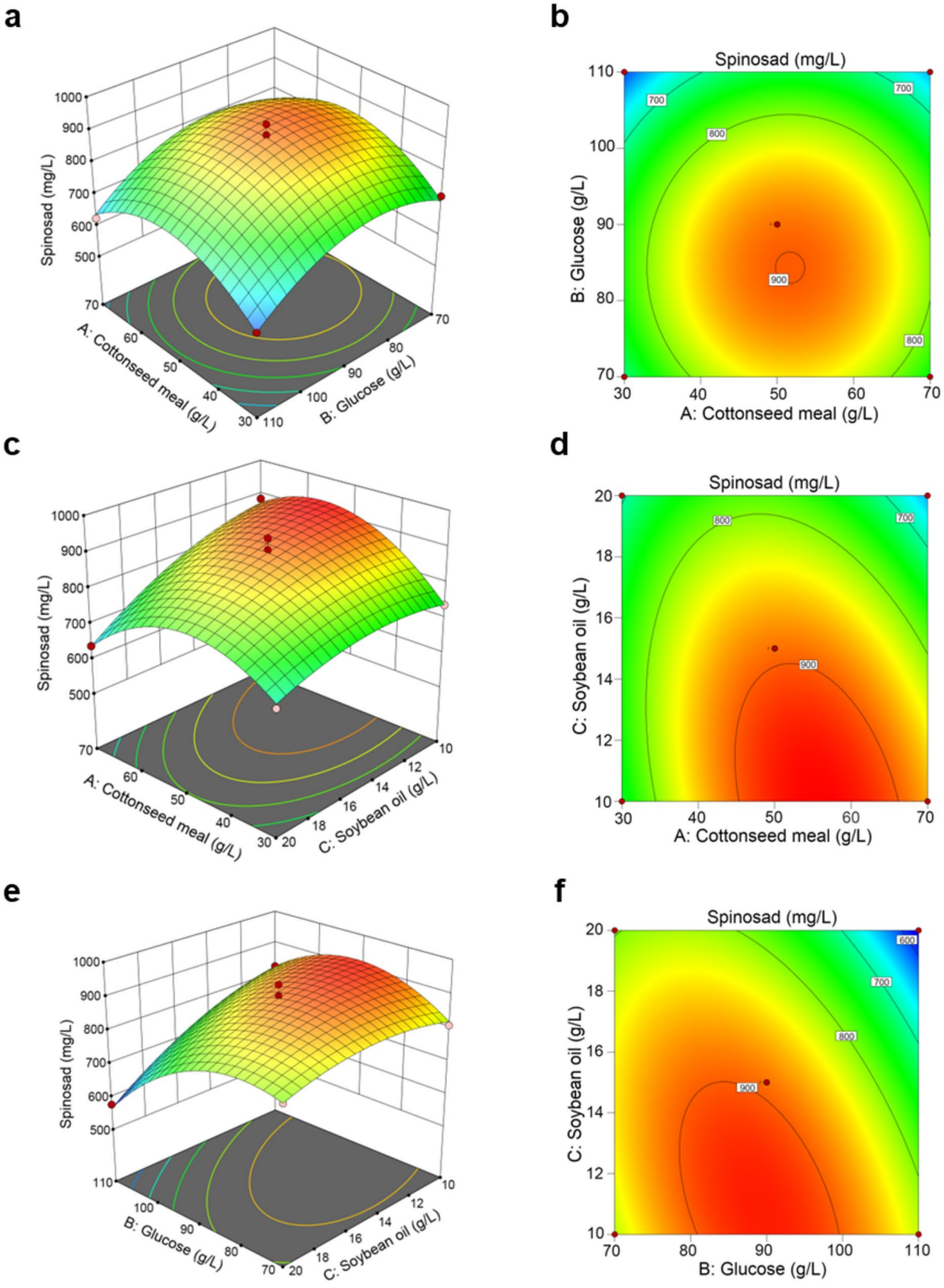

Fig. 5 Optimization of the fermentation medium by RSM. **a**, **b** Effects of cottonseed meal and glucose on spinosad yield. **c**, **d** Effects of cottonseed meal and soybean oil on spinosad yield. **e**, **f** Effects of glucose and soybean oil on spinosad yield

spn gene cluster is limited. To overcome this limitation, we constructed an engineered strain overexpressing the complete *spn* gene cluster in this study and conducted morphological analysis and fermentation optimization.

Conjugative transfer is a commonly used method for introducing foreign genes into actinomycetes. However, due to the significant challenges associated with genetic manipulation in *Sa. spinosa*, selecting an appropriate vector is crucial for successful gene transfer. In previous genetic engineering studies involving *Sa. spinosa*, the pOJ260 vector, derived from *E. coli*, was mainly used to introduce foreign genes into the *S. spinosa* genome [11]. In this study, we adopted the pCM265 vector, a specifically modified plasmid designed for efficient integration of genes into the *S. spinosa* genome. Compared with vectors such as pSET152, which are derived from phiC31, pCM265 is more efficient [33].

In this study, we observed a slowdown in sporulation after overexpression of the *spn* gene cluster. Interestingly, this phenomenon contrasted with the accelerated sporulation observed in genetically engineered strains that overexpressed genes related to rhamnose and forosamine biosynthesis [13]. Early studies have shown that rhamnose was an essential component of *Sa. spinosa* cell wall, and the synthesis of rhamnose in the cell wall was the same as that used for synthesizing rhamnose in the spinosad structure [16]. This suggests that overexpression of genes related to rhamnose synthesis may promote the formation of *Sa. spinosa* cell wall and subsequently accelerate sporulation. However, overexpression of the *spn* gene cluster enhances secondary metabolism while weakens primary metabolism, resulting in slowed sporulation and hyphal differentiation. The relationship between primary and secondary metabolism in microorganisms is highly intricate. While this study provides a preliminary explanation of how the overexpression of the *spn* gene cluster might alter primary metabolism, the underlying mechanisms of its impact require further investigation.

Although the overexpression of the entire *spn* gene cluster and RSM optimization increased spinosad production from 309 mg/L to 920 mg/L, we also noted, as mentioned in a review article [34], that the overexpression of just a few genes could achieve similar improvements. We hypothesize that this variability is partly due to differences in the genetic backgrounds of the original strains. For example, Bridget et al. reported that integrating pIBR-SPN FR into *Sa. spinosa* MUV (UV mutagenesis) resulted in a 36% higher spinosad yield compared to integration into wild-type *Sa. spinosa* [13]. Therefore, applying the method of overexpressing the entire *spn* gene cluster to other strains of *Sa. spinosa* may led to even more significant increases in spinosad production.

Conclusions

This study successfully constructed a genetically engineered strain *Sa. spinosa-spn* that overexpressed a complete *spn* gene cluster. The overexpression of the *spn* gene cluster enhanced secondary metabolism while suppressing primary metabolism, leading to delayed sporulation and altered hyphal morphology. Additionally, fermentation optimization using RSM further enhanced production.

Abbreviations

BGC	Biosynthetic gene cluster
Sa	Saccharopolyspora
spn	Spinosyn gene cluster

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02724-x.

Supplementary Material 1: Additional file 1: supplementary methods Synthesis of gRNA and digestion by Cas9. Construction of pCAP01a-CAP1, pCAP01a-CAP2 and pCM265-CAP. Table S1 Strains and plasmids used in this study. Table S2 Primers used in this study. Table S3 Sequences of the homologous arms. Table S4 Three-factor and three-level experimental design. Table S5 Box-Behnken Design of process variables for experiment and values of experimental data for spinosad.Table S6 ANOVA for response surface quadratic model. Fig. S1 Verification of pCM265-spn and ***Sa. spinosa-spn***. Fig. S2 Microscopic image of hyphae, with arrows indicating the sites of septum. Fig. S3 PCR validation of cDNA and RNA. Fig. S4 Results of single factor experiments.

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Author contributions

LG completed the data analysis and the first draft. LG and ZYZ finished the main experimental research. JTC and ZCS optimized fermentation medium. WJC cultivated the strains. JYL and SXC conceived the study, supervised the project and provided funding and guidance. All authors read and agreed on the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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