Increasing the heterologous production of spinosad in *Streptomyces albus* J1074 by regulating biosynthesis of its polyketide skeleton

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Spinossyns are glycosylated polyketide-derived macroolecules possessing a perhydro-as-indacene core that is formed via a series of intramolecular cross-bridging reactions. From fermentation broth extracts of *Saccharopolyspora spinosa*, a series of spinosyn factors were purified and structurally characterized, the most efficient are spinosyn A and D that together comprise the commercial insecticide spinosad [2], spinosyn J and L can further be chemically catalyzed to spinetoram [3]. Spinosad and spinetoram are biodegradable pesticides that are non-toxic to mammals [4–6]. Therefore, they are permitted as natural pesticides for growing organic food and have won the US President’s Green Chemicals Challenge Award three times (1999, 2008, and 2010).

1. Introduction

Spinossyns are natural broad-spectrum biological insecticides with a double glycosylated polyketide structure that are produced by aerobic fermentation of the actinomycete, *Saccharopolyspora spinosa*. However, their large-scale overproduction is hindered by poorly understood bottlenecks in optimizing the original strain, and poor adaptability of the heterologous strain to the production of spinosyn. In this study, we genetically engineered heterologous spinosyn-producer *Streptomyces albus* J1074 and optimized the fermentation to improve the production of spinosad (spinosyn A and spinosyn D) based on our previous work. We systematically investigated the result of overexpressing polyketide synthase genes (spnA, B, C, D, E) using a constitutive promoter on the spinosad titer in *S. albus* J1074. The supply of polyketide synthase precursors was then increased to further improve spinosad production. Finally, increasing or replacing the carbon source of the culture medium resulted in a final spinosad titer of ~70 mg/L, which is the highest titer of spinosad achieved in heterologous *Streptomyces* species. This research provides useful strategies for efficient heterologous production of natural products.
involved in the reaction pathway; however the most productive component is spinosad. The mechanism of spinosyn A has been elucidated (Fig. 1). A load module and ten extension modules of polyketide synthase (PKS) genes (\textit{spnA}, B, C, D, and E) are required to synthesize the polyketide skeleton starting from malonyl-CoA, methylmalonyl-CoA, and propionyl-CoA, which is then catalyzed by \textit{SpnJ}, F, and M to form aglycone [7,8]. A rhamnose group, which is synthesized by four enzymes (Gtt, Gdh, Epi, and Kre) located outside the spinosad gene cluster, is loaded onto the C-9 group of aglycone by \textit{SpnG} [9]. Subsequently, \textit{SpnL} catalyzes a transannular cyclization reaction between C-3 and C-14 [7], the 2',3',4'-hydroxy group of rhamnose is then O-methylated by \textit{SpnI}, K, and H [10]. Finally, a forosamine group synthesized by \textit{SpnO}, N, Q, R, and S [11–13] is loaded onto the C-17 group of aglycone by \textit{SpnP} and an unknown auxiliary protein to synthesize spinosad [14]. During recent decades, great efforts have been made to improve spinosad production in \textit{Sa. spinosa} by mutagenesis [15], fermentation optimization [16–19], genomic engineering [20,21], and metabolic engineering [22–25]. However, the titer of spinosad has not yet reached a level sufficient for industrial production; therefore, a poorly understood bottleneck may occur when optimizing the original spinosad producer \textit{Sa. spinosa}.

Since the biosynthetic pathway of spinosad is known, heterologous production can be used to unlock the bottleneck. The original BGC of spinosad was heterologously expressed in \textit{Sa. erythraea}, \textit{Streptomyces coelicolor}, and \textit{S. lividans} to obtain the spinosad titer of 1–3 mg/L [26,27]. Metabolic engineering has also been attempted in addition to the replacement of the chassis. Huang et al. (2016) replaced native erythromycin PKS genes with the spinosad BGC in \textit{Sa. erythraea}, performed several steps of metabolic engineering, and multiple rounds of ultraviolet mutagenesis to increase the spinosad titer to 830 mg/L [28]. Song et al. (2019) used five constitutive promoters to reconstitute the spinosad BGC in \textit{S. albus J1074} to obtain 1.11 mg/L spinosad [29]. We also heterologously expressed the original BGC of spinosad from \textit{Sa. spinosa} in \textit{S. albus J1074} using the omics-guide strategy to increase the titer of spinosad to 1.46 mg/L by three targeted engineering steps [30]. Despite \textit{Streptomyces} producing a much lower titer of spinosad than \textit{Sa. erythraea}, it has great potential for heterologous production as a chassis host due to its rapid growth rate, easy genetic manipulation, and the relatively mature research methods of \textit{Streptomyces} species [31]. Spinosad is a type I polyketide, and the efficient expression of PKS may be the key to its heterologous overproduction. However, previous metabolic engineering studies have not conducted in-depth studies on efficient PKS expression in heterologous hosts.

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![Fig. 1. The biosynthetic pathway of spinosyn A. KS: \(\beta\)-ketoacyl synthase. AT: acyltransferase, the loading and extension units including malonyl-CoA, methylmalonyl-CoA, and propionyl-CoA. DH: dehydratase. ER: enoyl reductase. KR: \(\beta\)-ketoreductase. ACP: Acyl carrier protein.](image-url)
erythromycin, which have developed mature industrial production methods, the optimized fermentation method of Sa. erythraea is likely a contributing factor to the greater production of spinosad than the model Streptomyces species. Although Song et al. (2019) confirmed that the previous culture method we used for S. albus J1074 for heterologous production of spinosad is the most efficient among several developed methods [29], it still suggests the need for designing a unique fermentation method to overcome the relatively low yield of heterologous spinosad production in Streptomyces.

To promote the large-scale industrialization of spinosad, we performed further metabolic engineering and fermentation optimization based on our previous work. We systematically investigated the influence of using a constitutive promoter for overexpressing spnA, except for spnB, in S. albus J1074. After selecting the optimal combination of PKS expression, the supply of polyketide precursors was evaluated to assist in spinosad overproduction. The spinosad titer was further improved by increasing the carbon source in the culture medium and replacing it with different sugars.

2. Materials and methods

2.1. Microorganisms and flask fermentation

Streptomyces and its derivatives were cultivated as described previously [30]. Briefly, strains were cultured on soybean flour-mannitol agar plates (2% w/v soybean flour, 2% w/v mannitol, and 2% w/v agar). Spores were collected, suspended in 20% (v/v) glycerol, and stored at 80 °C. For fermentation experiments, spores were grown in trypticase soy broth and the fermentation medium was 4% (w/v) sugar (glucose, sucrose, fructose, mannitol, or maltose), 1% (w/v) glycerol, 3% (w/v) soluble starch, 1.5% (w/v) Difco soytome, 0.2% (w/v) MgSO\textsubscript{4}, 0.2% (w/v) NaCl, and 0.24% (w/v) CaCO\textsubscript{3}. Unless otherwise specified, all the strains were cultured in a 250 mL Erlenmeyer flask at 30 °C and analyzed at the end of the 8-d fermentation unless otherwise specified.

2.2. Gene overexpression plasmid construction

The plasmids and primers used for strain construction are listed in Tables S1 and S2, respectively. In general, all plasmids used in this study were based on pJTU1278, which is an efficient vector for gene disruption and replacement in Streptomyces species [32]. pJTU1278-spnA was constructed for replacement of the natural promoter of the spnABC operon with promoter rpSLp-cf. Primer pair RPSP-CF-F/RPSP-CF-R were used to amplify promoter rpSLp-cf from pH8 [34], and primer pairs spnA-UF/spnA-UR and spnA-DR/spnA-DR were used to amplify upstream and downstream homologous arms from S. albus J1074 (C416-M)-OE3 (OE3) [30], respectively. The amplified fragments were joined by the overlap extension polymerase chain reaction (OE-PCR) [30], respectively. The amplified fragments were joined by OE-PCR using primers spnB-UR/spnB-DR and spnB-DR/spnB-DR were used to amplify the upstream end. All amplified DNA fragments and digested p JTU1278 were joined together using Gibson assembly method to obtain plasmid pZEA01.

pHT603 was constructed to replace anhidrotic PKS expression; the primer pair 001-L-F/001-L-R and 001-D-F/001-D-R was used to amplify upstream and downstream homologous arms from OE3, respectively. Plasmid pJTU1278 was then digested with XbaI and HindIII. All the amplified DNA fragments and digested p JTU1278 were joined together using Gibson assembly method to obtain plasmid pHTE603.

pFF209 was constructed to insert accA2 and accBE between two genes in the chromosome of S. albus J1074. Primer pair 009-DF/009-DR were used to amplify the upstream and downstream homologous arms from OE3, respectively. The promoter kasOp was amplified from pLH10 by PCR using the primer pair 63KasOP-F/63KasOP-R. Primer pairs ScoAsca-F/ScoAsca-R and ScoPcBE-F/ScoPcBE-R were used to amplify accA and accBE from S. coelicolor CH999 genomic DNA, respectively. Three primer pairs (F8-1278-1F/F8-1278-1R, F9-1278-2F/F9-1278-2R, F10-1278-3F/F10-1278-3R) were used to amplify pJTU1278. Primer pair 426-1F/426-1R was used to amplify the yeast helper fragment from the plasmid pRS426. All amplified DNA fragments were joined using DNA assembler method [33] to obtain the plasmid pHTE603.

Plasmid pZEA01 was constructed to insert kasOp+ promoter upstream of spnA, except for spnB. Primer pair spnB-UR/spnB-DR were used to amplify the promoter kasOp+ promoter from pH8 [34], and primer pairs spnB-UR/spnB-DR and spnB-DR/spnB-DR were used to amplify upstream and downstream homologous arms from OE3, respectively. The amplified fragments were joined by OE-PCR using primers spnC-UF/spnC-DR. The final PCR product and pJTU1278 were digested with BamHI and XbaI, purified, and ligated with DNA ligase as described above to construct the plasmid pJTU1278-spnC.

pJTU1278-spnB was constructed to insert the promoter kasOp+rpSLp-cf in front of spnB. Primer pair spnB-UR/spnB-DR and spnB-DR/spnB-DR were used to amplify the upstream and downstream homologous arms from OE3, respectively. The amplified fragments were joined by OE-PCR using primers spnB-UF/spnB-DR. The final PCR product and pJTU1278 were digested with Sacl and EcoRI, purified, and ligated with DNA ligase as described above to construct the plasmid pJTU1278-spnB.

pZEA01 was constructed to interrupt anhidrotic PKS expression; the primer pair 001-L-F/001-L-R and 001-D-F/001-D-R was used to amplify upstream and downstream homologous arms from OE3, respectively. Plasmid pJTU1278 was then digested with XbaI and HindIII. All the amplified DNA fragments and digested pJTU1278 were joined together using Gibson assembly method to obtain plasmid pHTE603.

pFF209 was constructed to insert accA2 and accBE between two genes in the chromosome of S. albus J1074. Primer pair 009-DF/009-DR were used to amplify the upstream and downstream homologous arms from OE3, respectively. The promoter kasOp was amplified from pLH10 by PCR using the primer pair 63KasOP-F/63KasOP-R. Primer pairs ScoAsca-F/ScoAsca-R and ScoPcBE-F/ScoPcBE-R were used to amplify accA and accBE from S. coelicolor CH999 genomic DNA, respectively. Three primer pairs (F8-1278-1F/F8-1278-1R, F9-1278-2F/F9-1278-2R, F10-1278-3F/F10-1278-3R) were used to amplify pJTU1278. Primer pair 426-1F/426-1R was used to amplify the yeast helper fragment from the plasmid pRS426. All amplified DNA fragments were joined using DNA assembler method [33] to obtain the plasmid pHTE603.

All of the constructed plasmids were verified by sequencing.

2.3. Strain construction

The strains used in this study are listed in Table 1. The engineered strains were constructed by triparental conjugation as previously described [30]. Briefly, the plasmid donors Escherichia coli DH10b/plasmid and E. coli ET12567(pUB307) were grown to an OD\textsubscript{600} of 0.4–0.6. Cells were pelleted by centrifugation at 4000 × g for 4 min, washed twice in LB, and resuspended 100 μL of LB. The fresh or frozen Streptomyces spores (stored at −40 °C) were washed twice in LB, suspended in TES (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid) buffer (0.05 M, pH 8.0) and incubated at 50 °C for 10 min to activate germination. An equal volume of double-strength germination medium (1% w/v Oxoid yeast extract, 1% w/v Difco Casamino acids and 0.01 M CaCl\textsubscript{2}) was added and the mixture was incubated at 37 °C for 2–3 h with shaking at 220 rpm. The germinated spores were pelleted by centrifugation as stated above, collected, and resuspended in 100 μL of TES buffer. Approximately 1 × 10\textsuperscript{8} E. coli cells (DH10b/plasmid):ET12567(pUB307):1:1) were added to the prepared spores (not less than 10\textsuperscript{5} spores/conjugation) and the mixture was spread onto a soya flour mannitol (SFM) agar plate containing 30 mM MgCl\textsubscript{2}. The conjugation plates were incubated for 14 h at
Table 1

Genotypic features of the Streptomyces and Escherichia coli strains used in this study.

| Strain         | Features                                      | Source            |
|----------------|-----------------------------------------------|-------------------|
| S. albus J1074 (C416-M)-OE3 | Based on S. albus J1074 (C416-M) in which a BAC plasmid containing the sparsosin biosynthetic gene cluster is integrated into the chromosome of S. albus J1074, an extra spnE gene was overexpressed under the control of rpslp-cf, gdh and ksp were co-overexpressed under the control of kasOp*, spnQ and spnR were co-overexpressed under the control of kasOp*, spnQ and spnR were co-overexpressed under the control of rpslp-cf, spnS was overexpressed under the control of rpslp-p, and the PKS gene spnI was overexpressed by kasOp*. | Tan et al., 2017 [30] |
| S. coelicolor C'H999 | proA1, argA1 redE601 Δ:ermE SCP*, SCP2* | McDaniel et al., [43] |
| AE             | The PKS operon spnIBC was overexpressed under the control of the rpslp-cf promoter in S. albus J1074 (C416-M)-OE3 | This study         |
| ADE            | The PKS gene spnD was overexpressed under the control of the rpslp-cf promoter in AE | This study         |
| ACDE           | The PKS gene spnC was individually overexpressed under the control of the kasOp* promoter in ADE | This study         |
| ABD E          | The PKS operon spnBC was overexpressed under the control of the kasOp*-rpslp-cf promoter in ADE | This study         |
| ABCDE          | The PKS genes spnB and spnC were overexpressed under the control of the kasOp*-rpslp-cf, and kasOp* promoters in ADE, respectively | This study         |
| ABE            | The PKS operon spnBC was overexpressed under the control of the kasOp*-rpslp-cf promoter in AE | This study         |
| DcanP2         | Deletion part of candidin P KS gene in ADE | This study         |
| ADE-AP         | Extra accA and pceB were overexpressed under the control of kasOp*, additional accA2 and accBE were overexpressed under the control of rpslp-cf in ADE, PKS of candidin BGC in ADE was replaced by accA and pceB to interrupt the expression of the candidin P KS gene | This study         |
| E. coli DH10b  | dam dcm hsdR/pUB307 | Gibco-BRL        |
| E. coli ET12567 | dam dcm hsdR/pUB307 | Flett et al., 1997 [44] |

30 °C, then the surfaces of the plates were overlaid with 1 mL of sterile water containing 600 μg trimethoprim, 1.5 mg amprycin, and 300 μg thiotrepton. The plates were incubated for an additional 3–6 d at 30 °C, and exconjugants resistant to thiotrepton and amprycin were selected. After sporulation on SFM medium with apramycin (50 μg/mL), double-crossover mutants were selected based on thiotrepton-resistant and amprycin-resistant and verified by PCR analysis with flanking primers (Fig. S2), primer pairs A-VF/A-VR for verifying AE, primer pairs D-VF/D-VR for verifying ADE, primer pairs C-DVF/C-DVFR and C-UVF/C-UVR for verifying ACDE, primer pairs B-DVF/B-DVFR and B-UVF/B-UVR for verifying ABE, ABDE, and ABCDE, primer pairs 001-VF/001-VR for verifying DcanP2, primer pairs 003-DVF/003-DVFR, 003-UVF/003-UVR, 209-DVF/209-DVFR, 209-UVF/209-UVR for verifying ADE-AP.

2.4. Extraction and analysis of sparsosins

Sparsosins were extracted from the fermentation cultures (1 mL) by mixing with 2 mL of acetonitrile, vortexed for 20 min, incubated for 30 min at room temperature, and centrifuged at 3500 × g for 10 min to remove cell debris. The supernatant was filtered using a 0.22 μm syringe filter and injected into a C18-reversed phase HPLC column (5 μm, 250 × 4.6 mm, Waters, Milford, USA) at 25 °C using isocratic elution with acetonitrile: methanol: 0.05% ammonium acetate buffer (4.5:4.5:1, v/v/v) at a flow rate of 1 mL/min and detection at 250 nm (HPLC with a UV detector, SPD-20A, Shimadzu, Kyoto, Japan), or a C18-reversed phase HPLC column (5 μm, 250 × 4.6 mm, Agilent, California, USA) at same separation condition and detection at 250 nm (HPLC, 1260DAD, Thermo scientific, Waltham, USA coupled with a DAD detector, DAD3000, Dionex, Sunnyvale, USA). Spinosad titer from Streptomyces and its derivatives was determined by comparison with standard spinosyn A and D.

2.5. Extraction and analysis of acyl-CoAs by LC-MS

After 3 d fermentation, 10 mL of S. albus J1074 broth was centrifuged at 3500 × g for 10 min at 4 °C to collect the bacteria, then was frozen with liquid nitrogen and stored at −80 °C for subsequent extraction of acyl-CoAs. Three biological replicates were used. When extracting the acyl-CoAs, the sample was chilled in ice, then two zirconia glass beads (2 mm), 100 μL of zirconia glass beads (1 mm), and 1 mL of ice-cold monopotassium phosphate buffer (67 mM, pH 4.9) were added to the sample, and the bacteria were ground twice using a grinder (JXSTTPR-24L, Jingxin, Shanghai, China, 60 Hz, stop for 10 × after 60 s) that was precooled at −40 °C. Next, 50 μL of precooled isopropanol was added, and the sample was ground again. After grinding, 1 mL of precooled acetonitrile and 60 μL of saturated ammonium sulfate solution (room temperature) was added, and the sample was ground once. After grinding, the tube was placed on ice for 10 min, centrifuged at 12,000 × g at 4 °C for 10 min, the supernatant transferred into a new tube, lyophilized and then stored at −80 °C for subsequent analysis. The sample was resuspended in 200 μL of 50% methanol, vortexed for 1 min, and then water bath sonicated for 2 min. The sample was centrifuged at 12,000 × g at 4 °C for 10 min and the supernatant used for acyl-CoA analysis using a HPLC-high resolution mass spectrometer (HPLC-HRMS, Ultimate 3000 system, Dionex, Sunnyvale, USA coupled with a Q Exactive Orbitrap mass analyzer, Thermo Fisher, Waltham, USA).

Chromatographic separation was achieved at 25 °C on a SeQuant ZIC-pHILIC column (150 × 2.1 mm, 5 μm; Merck, Darmstadt, Germany) coupled with a 20 mm SeQuant ZIC-pHILIC guard column at a flow rate of 0.2 mL/min. A linear gradient of solvent A (5% acetonitrile, 95% 15 mm NH₄HCO₃ pH 8.5) and solvent B (acetonitrile) was performed as follows: 0–1 min 90% B, 1–13 min 90%–30% B, 13–16 min 30% B, 16–17 min 30%–90% B, followed by 8 min of re-equilibration at 90% B. The injection volume was 1 μL and the autosampler was set at 4 °C during the analysis. The Q Exactive mass spectrometer was operated in electrospray ionization (ESI) negative mode. Source parameters were optimized with a spray voltage of 3.2 kV (−). The other parameters were set as follows: capillary temperature, 320 °C; auxiliary gas temperature, 300 °C; sheath gas, 40 Arb; auxiliary gas, 10 Arb; sweep gas, 0 Arb, and the S-lens RF level was set at 50. The Q Exactive detector was operated in full scan mode plus data-dependent MS² (dd MS²) mode. In the full scan mode, the resolution was set at 70,000. The automatic gain control (AGC) target and maximum injection time (IT) were set at 1 × 10⁶ ions capacity and 100 ms, respectively. In data-dependent MS² (dd MS²) mode, the resolution was set to 17,500. The AGC target and maximum IT were set at 2 × 10⁶ ion capacity and 50 ms, respectively. The inclusion list was on. All targeted metabolites m/z at [M – H]⁻ were included in the list and prepared to fragment. The scan range was set at m/z 100–1500. The normalized collision energies (NCE) were 20%, 40%, and 60%. The isolation window was set at 1.2 Da. The apex trigger was set at 5–15 s, the loop count was set at 3, and the dynamic exclusion was set at 5 s.

The standard solutions were prepared at six individual calibration concentrations between 0.1 and 5 mg/L for acetyl-CoA and propionyl-CoA; or 0.2–5 mg/L for malonyl-CoA and methylmalonyl-CoA.
2.6. Measurements of malonyl-CoA using an ELISA kit

Cell suspensions of the strains cultured in 10 mL of fermentation medium for 3 d were removed by centrifuging at 4000 × g for 10 min. The cells were then re-suspended in 4 mL of ddH₂O, and the cell wall was destroyed by ultrasonication, after which the suspensions were centrifuged at 4000 × g for 10 min. The concentration of malonyl-CoA in the supernatant was measured using a microbial malonyl-CoA ELISA kit (Shanghai FANKEL Industrial Co., Ltd., Shanghai, China) according to the manufacturer’s instructions.

2.7. Detection of residual glucose

The fermentation broth was centrifuged and diluted 10 times using sterile water, and the residual glucose concentration was detected by the enzyme electrode method with a glucose analyzer (SBA-40e, Biology Institute Shandong Academy of Sciences, Shandong, China) based on a standard curve of glucose. The analyzer uses immobilized glucose oxidase to convert glucose into gluconic acid and H₂O₂, with the electrode detecting the amount of H₂O₂ produced.

3. Results

3.1. Overexpression of PKS genes using a constitutive promoter in OE3

Previous studies have shown that the original BGC of spinosad contains ten operons; the PKS genes are controlled by two promoters, spnA, B and C are co-expressed by one promoter located before spnA, and spnD and E are co-expressed by another promoter located in before spnD (Fig. 2A) [29,30]. In our previous work, the expression of spnE was found to be enhanced by inserting a strong constitutive promoter (kasOp*) between spnD and spnE with the spinosad titer increasing from 686 μg/L to 1460 μg/L [30], showing that the overexpression of PKS significantly contributes to the overproduction of spinosad in heterologous hosts. In our current study, through the rejuvenation of our former engineered strain OE3, the period of fermentation was extended from 5 to 8 d resulting in an increased spinosad titer from 1.46 mg/L to 2.81 mg/L (Fig. S1). Subsequent results show that further extension of the fermentation period did not increase the production of spinosad. Therefore, 8 d fermentation was used for genetically modified strains in this study unless otherwise specified.

Previously, we have quantified the expressing strength of 20 promoters in S. albus [34], three promoters showed similar strength with kasOp* [35], an engineered promoter of kasOp that encodes a SARP family regulator and is an activator of a cryptic type I PKS responsible for coelimycin P1 production in S. coelicolor A3. Two of them are rpsLp

![Fig. 2. Metabolic engineering of spinosad PKS promoters to increase the titer of spinosad in S. albus J1074. (A) The spinosad biosynthetic genes are located in different operons. Each red arrow indicates the gene(s) in the same operon. (B) The production of spinosad in different engineered strains overexpressing PKS genes using different constitutive promoters. Each sample was performed in triplicate with the error bars stated as mean ± SD. wt: wild-type (natural) promoter. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)](image-url)
promoter from *Cellulomonas flavigena* and *Tsukamurella paurometabola* that controlling the expression of the housekeeping gene, 3OS ribosomal protein S12, named as *rpsL*-promoter, *rpsL*-promoter, respectively [36], the last one is a *kas* derived promoter (*kas*-promoter) which combing *kas*-promoter, RibOJ insulator and the ribosomal binding site of *rpsL*-promoter [37]. To investigate the influence of PKS overexpressing on the production of spinosad in heterologous *S. albus* J1074, we attempt to control the expression of every PKS gene using these four strong constitutive promoters. Replacement of the original promoter of *spnABC* with the *rpsL*-promoter in OE3 to construct the strain AE (Fig. S2A) resulted in spinosad titer increasing by ~11-fold to 30.26 ± 1.69 mg/L compared with OE3 (Fig. 2B). When the original promoter of *spnD* was replaced with the *rpsL*-promoter to construct the ADE strain (Fig. S2C), the spinosad titer increased by 21.22% to 36.68 ± 1.81 mg/L compared with AE (Fig. 2B). These results showed that the use of a strong constitutive promoter to overexpress PKS is an efficient engineering strategy to increase spinosad titer.

Although in the ADE strain, five PKS genes were controlled by strong constitutive promoter, *spnABC* still co-expressed as an operon which controlled by *rpsL*-promoter. To investigate whether individual expression of each PKS gene using the constitute promoter can further enhance the production of spinosad in heterologous host *S. albus* J1074, we attempt to express *spnB* and *spnC* individually using different strong constitutive promoter. When the ADE strain was engineered to insert strong independent constitutive *kas* and *kas*-promoter to *spnABC* and *spnD* genes, strain ACDE and ABDE were constructed, respectively (Figs. S2B and E). As a result, the titer of spinosad decreased from 12.71 ± 5.46 mg/L in ACDE and 2.18 ± 1.00 mg/L in ABDE (Fig. 2B). These results suggest that the overexpression of *spnB* and *spnC* may be harmful to the overproduction of spinosad when the *spnABC* operon is destroyed. Since ACDE showed a smaller negative effect on the reduction of spinosad production compared with ABDE, the balanced expression of *spnA* and *spnBC* seems to be more important than that of *spnAB* and *spnC* for spinosad overproduction.

To verify that the disruption of the balanced expression of the *spnABC* operon leads to a decrease in the production of spinosad in the heterologous strain, two strains (ABDCE and ABE) were constructed (Fig. S2E). When all five PKS genes were individually overexpressed by a strong constitutive promoter (ABDCE), the titer of spinosad decreased from 12.71 ± 5.46 mg/L to 9.43 ± 3.05 mg/L compared to ACDE (Fig. 2B). In the ABE strain, the PKS operon *spnBC* was co-overexpressed under the control of the *kas*-*rpsL*-promoter based on the AE strain. The titer of spinosad in ABE was 3.21 ± 0.45 mg/L compared with 30.26 ± 1.69 mg/L in AE (Fig. 2B). These results reinforce the conclusion that disruption of the *spnABC* operon is detrimental to spinosad overproduction, while the destruction of the co-expression of *spnA* and *spnBC* causes a significant decrease in production. Overall, the results clearly indicated that balanced overexpression of PKS using one strong constitutive promoter to overexpress the *spnABC* operon and two other strong constitutive promoters to individually overexpress *spnD* and *spnE* (ADE strain), significantly increased the production of spinosad.

### 3.2. Overproduction of polyketide precursors to enhance the production of spinosad

A sufficient supply of polyketide precursors is considered an important factor for the overproduction of spinosad in the engineered strain. The first committed step in polyketide biosynthesis involves the biotin-dependent carboxylation of acyl-CoA and is catalyzed by acyl-CoA carboxylases (ACCase), such as acetyl-CoA carboxylase (ACC) and propionyl-CoA carboxylase (PCC) producing malonyl-CoA and methylmalonyl-CoA, respectively (Fig. 3A) [38]. For spinosad synthesis,
the precursors of polyketide include malonyl-CoA, methylmalonyl-CoA, and propionyl-CoA (Fig. 1) and their supply in the host should be considered for optimal production. LC-MS analysis of acyl-CoAs is problematic since acyl-CoAs are structurally complex, easily degraded with observed signal deterioration, and show severe peak tailing with poor detection limits [39,40]. The limit of detection (LoD) of the developed method for detecting acyl-CoAs was evaluated using commercial standards. The LoD was 0.1 mg/L and 0.2 mg/L for malonyl-CoA and methylmalonyl-CoA, and acetyl-CoA and propionyl-CoA respectively (Fig. S3A). Meanwhile, only a small amount of acetyl-CoA, propionyl-CoA, and methylmalonyl-CoA was detected in *S. albus* J1074 (Fig. S3B). We speculate that the absence of malonyl-CoA was due to its low extraction efficiency, which was previously reported to be <20% [41]. We, therefore, employed the ELISA platform to quantify malonyl-CoA and found its concentration in *S. albus* J1074 to be 0.155 ± 0.006 ng/g cdw.

Considerating acyl-CoAs serve as the common precursors for nearly all polyketides, knockout of other PKS genes in *S. albus* J1074 may optimize acyl-CoAs level required for spinosad biosynthesis. Therefore, based on the ADE strain, candinicin expression, which was previously detected by transcriptomics (unpublished), was disrupted to construct the DcanP2 strain (Fig. S2D). Results revealed that the spinosad titer in DcanP2 was 38.89 ± 4.75 mg/L (Fig. 3B), indicating that disruption of candinicin expression slightly enhances spinosad synthesis. Therefore, to further increase the supply of polyketide precursors, ACC and PCC should be overexpressed to increase the levels of malonyl-CoA and methylmalonyl-CoA, respectively (Fig. 3A). The enzymes of ACC and PCC in *S. albus* J1074 have not been identified; however, the components and mechanism of these enzymes in *S. coelicolor* have been determined [42]. In *S. coelicolor*, ACC and PCC share the same biotinylated subunit, AccA2, while the β and ε subunits are specific to each complex. Acetyl-CoA synthetase (AcsA) synthesizes malonyl-CoA using the substrate acetyl-CoA, and may also produce propionyl-CoA (Fig. 3A). Accordingly, AcsA, AccA2, the β and ε subunits of ACC (AccBE), and that of PCC (PccBE) should be overexpressed to increase the supply of precursors (malonyl-CoA, methylmalonyl-CoA, and propionyl-CoA) for spinosad. Therefore, *acsA, accA2, accBE, and pccBE* were inserted into the chromosome of ADE to form ADE-AP (Fig. 2F), the titer of spinosad in ADE-AP was 59.59 ± 3.00 mg/L, which is 62.46% greater than that of ADE (Fig. 3B).

To verify whether the increased spinosad in ADE-AP is related to the polyketide precursor concentration, we quantified the concentration of acyl-CoAs in ADE and ADE-AP. Considering that the extraction efficiency and detector response differed for each acyl-CoA, the concentration of acyl-CoAs in ADE and ADE-AP was measured as their fold change of *S. albus* J1074 in 3-day fermentation. This method of relative quantization revealed higher concentrations in all acyl-CoAs in ADE compared to in *S. albus* J1074, an effect that was particularly apparent for acetyl-CoA (Fig. 3C). Hence, the expression of spinosad genes appears to impact the carbon metabolism of host. Comparing ADE with ADE-AP, the concentration of propionyl-CoA was 6.8-fold higher in ADE than in ADE-AP, while that of methylmalonyl-CoA in ADE was lower than that in ADE-AP (Fig. 3C), indicating that PCC overexpression resulted in more propionyl-CoA being converted to methylmalonyl-CoA. Additionally, the concentration of acetyl-CoA was 1.3-fold higher in ADE-AP than in ADE (Fig. 3C), indicating that AcsA is functioning efficiently. Although we observed no difference in malonyl-CoA concentration between ADE and ADE-AP (Fig. 3C), the spinosad titer increased from 0.21 ± 0.10 mg/g cdw in ADE to 0.34 ± 0.07 mg/g cdw in ADE-AP (Fig. 3D), indicating that ADE-AP utilized more malonyl-CoA to synthesize spinosad. We, therefore, speculated that the overexpression of ACC increases the abundance of malonyl-CoA available for spinosad synthesis. Collectively, these results indicate that a targeted increase in spinosad precursors leads to efficient overproduction of spinosad.

### 3.3. The relationship between residual sugar in the medium with spinosad production

The selection of an efficient supply of carbon atoms as building blocks of polyketides and spinosad synthesis may be closely related to sugar type and concentration, which is the main carbon source in the culture medium. Residual sugar was detected in the samples on days 1, 3, 5, 7, 8, 9, and 10 of fermentation in both ADE and ADE-AP. In the ADE strain, the spinosad titer increased from day 1 to day 8, while the concentration of residual glucose during this period decreased from 29.4 to 0 g/L (Fig. 4A). Meanwhile the residual sugar on the first day was much lower than the original concentration (40 g/L), which may be due to the carbonization of glucose during the sterilization process preventing their reaction with glucose oxidase on the detector. From day 8 to day 10, the production of spinosad stopped increasing (the fluctuating titer of spinosad may be due to evaporation of the culture medium and subsequent calculation errors). Similar results were observed in the ADE-AP strain (Fig. 4B) with increased spinosad production from days 1–7 and decreased residual glucose concentration in the medium from 29.3 to 0 g/L. Hence, with no available glucose in the culture medium, spinosad production did not increase after day 7. These results showed that spinosad production is negatively correlated with the concentration of residual glucose in the culture medium.

The effect of glucose concentration on spinosad titer was measured by transferring the same amount of cells grown in the seed media to culture media containing two different concentrations of glucose (4% and 4.8%). The additional 0.8% glucose was either added to the fermentation medium from the beginning or after 4 d. The spinosad titer decreased in 4.8% glucose media in the ADE strain regardless of when the additional glucose was added (Fig. 4C), while the opposite was observed in the ADE-AP strain (Fig. 4D). A maximum of 68.39 ± 9.20 mg/L was achieved when an additional 0.8% glucose was added to the culture medium of ADE-AP at the beginning of fermentation, which is 25.49% higher than that at 4% glucose. We propose that the difference in performance between the two strains may be due to differing efficiency in the use of glucose. In the ADE strain, additional glucose seems to prevent carbon atoms from flowing toward spinosad synthesis. However, the overexpression of ACC, PCC and AcsA in ADE-AP allows a range of carbon sources to be involved in spinosad synthesis.

### 3.4. The production of spinosad with different sugars

The supplementation of glucose in different engineered strains of *S. albus* J1074 shows variable titer of spinosad suggesting that it is not a common efficient strategy to increase its production. Four kinds of frequently used sugar sources (sucrose, fructose, mannitol, and maltose) were chosen to replace glucose in the fermentation culture medium to explore whether alternative sugar sources can readily increase the production of spinosad in engineered heterologous strains. The replacement of glucose with sucrose or fructose reduced the production of spinosad in ADE strains, while the replacement of glucose with mannitol slightly increased spinosad titer from 30.88 ± 1.81 mg/L to 33.12 ± 3.74 mg/L (Fig. 5A). Notably, the replacement of glucose with maltose shows a significant increasing from 30.88 ± 1.81 mg/L to 54.96 ± 0.37 mg/L (Fig. 5A). These results suggest that maltose is beneficial for increasing the production of spinosad in ADE.

Similarly, the replacement of glucose with sucrose or fructose in the ADE-AP strain also reduced the production of spinosad from 54.30 ± 14.21 mg/L to 26.52 ± 8.79 mg/L, and 16.84 ± 3.49 mg/L, respectively. Meanwhile the replacement of glucose with mannitol and maltose increased spinosad titer from 54.30 ± 14.21 mg/L to 56.41 ± 5.30 mg/L, and 70.61 ± 3.99 mg/L, respectively (Fig. 5B). These results reinforced that fructose or sucrose are not suitable sugars for production of spinosad, while maltose is beneficial for increasing the production of spinosad in engineered *S. albus*. The titer of spinosad in ADE-AP fermenting maltose-based culture medium (70.61 ± 3.99 mg/L) is very close to the
previous titer (68.39 ± 9.20 mg/L) achieved in culture medium with 4.8% glucose. These results suggest a bottleneck may exist that limits the accumulation of more spinosad in ADE-AP. Nearly all of the spinosad synthesized in ADE-AP was an intracellular product under all culture conditions (the detection of tiny extracellular product may be caused by cell rupture) (Fig. 5C), indicating that the spinosad titer may not continue to increase may due to limited intracellular space or intracellular tolerance to spinosad.

4. Discussion

We systematically investigated the influence of using a constitutive promoter for overexpressing each PKS gene on the titer of spinosad in *S. albus* J1074. The engineered strain ADE, which uses one strong constitutive promoter to co-overexpress *spnABC* operon and two other strong constitutive promoters to individually overexpress *spnD* and *spnE* significantly increased the titer of spinosad by ~12-fold. When the supply of polyketide precursor (acyl-CoAs) was increased by overexpressing ACC, PCC, and AcsA, a higher titer of spinosad in the engineered strain ADE-AP than ADE was achieved. Additional glucose or replacement with maltose as the carbon source in the culture medium resulted in the titer of spinosad increasing to ~70 mg/L, which is the highest titer achieved in heterologous *Streptomyces* in the literature and an improvement of our previous work [27, 29, 30]. Based on the original OE3 strain [30], the production of heterologously expressed spinosad in *S. albus* J1074 was increased by approximately 50-fold.

In this study, the most significant improvement in spinosad production is the use of a strong constitutive promoter that has been characterized in *S. albus* to control the expression of the spinosad PKS gene. We speculated that this effect was mainly due to the use of biological elements that the heterologous host can recognize to efficiently express heterologous products. In our previous work [30], the efficiency of spinosad production in *S. albus* J1074 using its original promoter was low due to the incompatibility of the heterologous host and biological elements. This is a universal problem in heterologous expression of natural products, especially for complex molecules such as polyketides. Our successful strategy herein may help follow-up research to solve the incompatibility of the heterologous host and biological elements.

We also observed that unbalanced expression of PKS genes is harmful to spinosad overproduction. Specifically, disrupting the original *spnABC* operon with one or two strong promoter(s) resulted in decreased spinosad production implying that a finely balanced co-expression of *spnA*, *spnB*, and *spnC* is required. Therefore, we suggest that the original organization of the operon is suitable for expression, unless the expression level of the gene is particularly low, such as *spnE* in our previous work [30]. In our work, we observed that the concentration of acetyl-CoA is significantly increased in ADE-AP than *S. albus* J1074, although we overexpressed ACC, the concentration of malonyl-CoA did not significantly increase, implying that the efficiency of ACC was weak. Therefore, selecting an alternative host with a sufficient supply of malonyl-CoA, as well as other acyl-CoAs, may further increase the spinosad titer in future work.

Optimization of the culture medium is an efficient strategy to increase the titer of heterologous production, and our work shows that supplementation or replacement of the carbon source is useful for increasing the production of spinosad. Further optimization of the culture medium was not performed as we speculate the current bottleneck of spinosad heterologous production in *S. albus* J1074 is due to the limitation of intracellular space or intracellular tolerance. Therefore, the next rational engineering strategy will focus on secreting spinosad through introducing an appropriate transporter, or adding a specific spinosad extractant that has little effect on cell growth during
fermentation. Overall, this research provides an efficient strategy to solve the low production of spinosad in heterologous *S. albus* J1074. This approach may be applied for increasing heterologous production of other natural products.

**CRediT authorship contribution statement**

Ziheng An: Methodology, Investigation, Writing – original draft. Hui Tao: Methodology, Investigation. Yong Wang: Investigation. Bingqing Xia: Investigation. Yang Zou: Investigation. Shuai Fu: Investigation. Fang Fang: Investigation. Xiao Sun: Investigation. Renqiong Huang: Investigation. Yao Xia: Investigation. Xiang Liu: Conceptualization, Supervision. Ran Liu: Conceptualization, Investigation, Writing – review & editing, Supervision. Tiangang Liu: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

**Declaration of competing interest**

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Wuhan University have applied patents based on this work.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2021.09.008.

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