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Evaluation and optimization of analytical procedure and sample preparation for polar Streptomyces albus J1074 metabolome profiling

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ABSTRACT

Metabolomics is an essential discipline in omics technology that promotes research on the biology of microbial systems. Streptomyces albus J1074 is a model organism used in fundamental research and industrial microbiology. Nevertheless, a comprehensive and standardized method for analyzing the metabolome of S. albus J1074 is yet to be developed. Thus, we comprehensively evaluated and optimized the analytical procedure and sample preparation for profiling polar metabolites using hydrophilic interaction liquid chromatography (HILIC) coupled with high-resolution mass spectrometry (HRMS). We systematically examined the HILIC columns, quenching solutions, sample-to-quenching ratios, and extraction methods. Then, the optimal protocol was used to investigate the dynamic intracellular polar metabolite profile of the engineered S. albus J1074 strains during spinosad (spinosyn A and spinosyn D) fermentation. A total of 3648 compounds were detected, and 83 metabolites were matched to the standards. The intracellular metabolomic profiles of engineered S. albus J1074 strains (ADE-AP and OE3) were detected; furthermore, their metabolomes in different stages were analyzed to reveal the reasons for their differences in their spinosad production, as well as the current metabolic limitation of heterologous spinosad production in S. albus J1074. The HILIC-HRMS method is a valuable tool for investigating polar metabolomes, and provides a reference methodology to study other Streptomyces metabolomes.

1. Introduction

Metabolomics aims to characterize changes in the metabolites that participate in the biochemical reactions of an organism. Metabolite molecules were defined as those below 1500 Da. With the development of analytical techniques, metabolomics has been applied in the field of industrial microbiology [1]. Microbial metabolomics has been applied for discovering unknown gene functions [2], characterizing the metabolic profiles of microbial cultures [3], guiding microbial metabolic engineering [4], and revealing the mechanism of high yield of natural products [5]. Microbial metabolomics has focused on the study of Saccharomyces cerevisiae [6–8], Escherichia coli [9,10], Corynebacterium glutamicum [11], and fungi [12,13]. Actinobacteria, especially Streptomyces, have been used to produce important marketed antibiotics, insecticides, and medicines [14], such as spinosyns, tylosin, avermectin, and chlorotetracycline. Spinosyns are bioactive secondary metabolites obtained through the fermentation of Saccharopolyspora spinosa [15]. Spinosyn A and spinosyn D, which were developed as commercial forms of spinosad insecticide, are the most efficient spinosyn factors. Streptomyces albus J1074 has been used to produce heterogenous products because of its clear genetic background and relatively simple genetic manipulation. The heterologous biosynthesis of spinosad was successfully performed in S. albus J1074 to construct strain OE3 in our previous work [4], after which we increased the heterologous production of spinosad by enhancing the biosynthesis of polyketide skeletons to...
construct strain ADE-AP [16]. Metabolomic analyses of Streptomyces have been previously studied [17–19]. A comprehensive metabolome preparation and analysis method for S. albus J1074 has not yet been developed. Therefore, developing a method for the metabolomic profiling of S. albus J1074 is important to guide the metabolomic engineering of heterologous spinosad production.

Liquid chromatography (LC) coupled with high-resolution mass spectrometry (HRMS) is a dominant and popular platform for metabolomics because of its high sensitivity, high mass accuracy, and high resolution [20]. Water-soluble metabolites, such as central carbon, amino acids, purines, pyrimidines, organic acids, nucleotides, and vitamins are polar, and a reverse-phase LC column is not appropriate for analysis of these metabolites due to their poor retention time on the reverse-phase LC column. Hydrophilic interaction liquid chromatography (HILIC) columns are good solution for this. HILIC conditions are conductive to MS, and water-soluble metabolites can be detected by HILIC-HRMS [21].

A standardized sample preparation method is vital for metabolomics analysis, and cell quenching to arrest intracellular enzyme activity is unavoidable when obtaining the metabolome at the time of sampling [22,23]. Cell quenching with cold methanol is the most widely used method in microbial metabolomics. This method enables the separation of intracellular and extracellular metabolites while inhibiting cell metabolism [7]. Quenching cold methanol concentrations have been reported to be different owing to microbial diversity, metabolome complexity, and differences in experimental techniques [22,24]. The sample-to-quenching solvent ratio should also be evaluated using a step aims to induce the production of intracellular metabolites in the sample preparation protocol [24]. Extracting intracellular metabolites is the second step in the metabolism sample preparation. The extraction step aims to induce the production of intracellular metabolites in the extraction solvent with minimal loss. Mixtures of organic reagents and H2O were used to extract intracellular metabolites. The extraction of intracellular metabolites of Streptomyces have been reported using only a single solvent, such as methanol: water = 1:1 (v/v) [18], and chloroform: ethanol: water = 2:2:1 (v/v/v) [17].

In this study, we evaluated and optimized the metabolome analytical procedure and sample preparation for S. albus J1074 based on the HILIC-HRMS platform. There were 118 metabolite standards included in the in-house library, and the retention times of the metabolites within a 10 months test were stable. The results showed that the cold pure methanol was more suitable for metabolomic quenching of S. albus J1074. The best sample-to-quenching solvent ratio was 1:4 (v/v). The mixture of methanol-acetonitrile-H2O was more suitable for metabolite extraction. This method was used to profile the different growth stage metabolites of the engineered strains ADE-AP and OE3 during spinosad fermentation. A total of 3648 compounds were detected. Then, we compared the changes in the central carbon metabolism, amino acid metabolism, and nucleotide metabolism, and the results showed that the metabolisms of strains ADE-AP and OE3 were different during spinosad fermentation.

### 2. Material and methods

#### 2.1. Reagents and standards

The 118 authentic standards were purchased from various companies (Supplementary material 2-S1). Ammonium bicarbonate and ammonium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Watsons-distilled water was purchased from the Jingdong Mall (Beijing, China). Analytical grade chloroform was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

#### 2.2. Microorganism, media and incubation conditions

The engineered strains ADE-AP and OE3 were cultivated as described previously [16]. In general, strains were cultured on soybean flour-mannitol agar plates (2% (w/v) soybean flour, 2% (w/v) mannitol, and 2% (w/v) agar). The spores were collected, suspended in 20% (v/v) glycerol, and stored at −80 °C. For fermentation, spores were grown in trypticase soy broth and the fermentation medium was 4% (w/v) glucose, 1% (w/v) glycerol, 3% (w/v) soluble starch, 1.5% (w/v) Difco soytone, 1% (w/v) beef extract, 0.65% (w/v) peptone, 0.05% (w/v) Difco yeast extract, 0.2% (w/v) MgSO4, 0.2% (w/v) NaCl, and 0.24% (w/v) CaCO3. All strains were cultured in 250 mL Erlenmeyer flasks at 30 °C. In the sample pretreatment optimization process, the fermentation broth of S. albus J1074 was harvested. Then, biological replicates of the fermentation broth were pooled together, and aliquoted into tubes for the following experiments. For methodological application and verification experiments, the engineered strains OE3 and ADE-AP broths were harvested on the 3rd, 5th, and 8th days of fermentation. The peak area of each metabolite was normalized by the accurate weight of cell debris.

#### 2.3. Sample quenching

The cold methanol quenching method was adopted for S. albus J1074. Briefly, 2 mL of fermentation broth was quenched quickly by adding a cold (−40 °C) methanol solution with different concentrations (60% or 100% methanol, (v/v)) and the pellet was collected by centrifugation at 4 °C and 4000 rpm for 5 min. Sample to quenching solution ratios of 1:3 (v/v), 1:4 (v/v), and 1:5 (v/v) were evaluated to select a suitable quenching solution for S. albus J1074.

#### 2.4. Metabolites extraction

Five extraction solvents were used for the extraction of intracellular metabolites from S. albus J1074. The solvent mixtures used were as follows: pure methanol, methanol: H2O = 1:1 (v/v), acetonitrile: H2O = 1:1 (v/v), methanol: acetonitrile: H2O = 2:2:1 (v/v/v), and chloroform:
methanol: H₂O = 2:1:1 (v/v/v). These solvents were pre-chilled at -40 °C prior to extraction. Biomass pellets were transferred into a pre-chilled centrifuge tube (10 mL), and then 1 mL of extraction solvent and zirconia beads were sequentially added to the centrifuge tube. The exact process was performed under mechanical disruption for three cycles (run for 60 s, stop for 10 s as one cycle) using a vibration mill. The supernatant was collected and stored at -40 °C for 1 h to precipitate the proteins and was collected by centrifugation (12,000 rpm, 20 min, 4 °C) and freeze-dried. The freeze-dried powders were constituted using methanol: H₂O = 1:1 (v/v) and the supernatant was used for intracellular metabolite analysis. The cell debris from the extraction process was dried in an oven until the constituent weights were obtained.

2.5. Exaction and analysis of spinosad

Spinosad were extracted and analyzed as described previously [16]. In general, fermentation cultures (1 mL) were mixed with acetonitrile (2 mL), then the mixture was vortexed for 20 min and incubated for 30 min at room temperature. After this, the mixture was centrifuged at 4000 rpm for 10 min. The supernatant was filtered using a 0.22 µm filter and injected into a C18 column (250 × 4.6 mm, 5 µm, Waters, Milford, USA) for spinosad analysis. The analysis was performed using an UltiMate 3000 HPLC system (Thermo Fisher Scientific, MA, USA). The column was kept at 25 °C and isocratically eluted with an acetonitrile: methanol: 0.05% ammonium acetate buffer (4.5:4.5:1, v/v/v). The flow rate was 1 mL/min, and UV detection wavelength was 250 nm. The spinosad titer of strains ADE-AP and OE3 was determined by comparison with standard spinosyn A and spinosyn D.

2.6. Determination of intracellular metabolites by HILIC-HRMS

The metabolite samples were analyzed by HILIC-HRMS using an Ultimate 3000 UPLC (Dionex) coupled with Q Exactive MS (Thermo Fisher Scientific, MA, USA). Q Exactive was equipped with a HESI probe and operated in the negative mode. The detailed mass spectrometer are as follows: spray voltage, 3.2 kV (-); capillary temperature, 320 °C; auxiliary gas temperature, 300 °C; sheath gas, 40 arb; auxiliary, 10 arb; sweep gas, 0 arb; mass range (m/z), 70–1050; full MS resolution, 70,000; MS/MS resolution, 17,500; TopN, 5; NCE, 20/40/60; isolation window, 1.2 Da; apex trigger, 5–15 s; dynamic exclusion, 5 s.

A SeQuant ZIC-pHILIC (150 mm × 2.1 mm, 5 µm, MERCK, Darmstadt, Germany) was used at 25 °C for chromatography. The mobile phase consisted of 15 mM ammonium bicarbonate in 5/95 acetonitrile/water (pH = 8.5, buffer A) and acetonitrile (buffer B) at a flow rate of 0.2 mL/min. The gradient was as below: 0 min, 10% A; 1 min, 10% A; 13 min, 70% A; 16 min, 70% A; 17 min, 10% A; 25 min 10% A. The total run time was 25 min and the sample injection volume was 1 µL.

2.7. Metabolite identification and statistical analysis

The raw format files were imported into the Compound Discoverer (CD) 3.0 software (Thermo Fisher Scientific, MA, USA) for compounds detection. Then, the raw format files were imported into the Xcalibur software (Thermo Fisher Scientific, MA, USA) for targeted compounds analysis, and the parameter setting for mass accuracy was ±10 ppm. The mzCloud, KEGG, YMDB, Metabolika, and ChemSpider databases were used for metabolite annotation. In general, we annotated metabolites at five levels based on the metabolomics standards initiative (MSI) [24]. Retention time, precise mass, and MS/MS spectra were used for a metabolite annotation. The detailed requirements for different levels are listed in supplementary material 2- S2. Principal component analysis (PCA) was performed using the web-based platform Metaboanalyst 5.0 (http://www.metaboanalyst.ca). Hierarchical clustering analysis (HCA) was performed using TIBtools.

3. Results and discussion

3.1. Evaluation of the HILIC-HRMS analytical procedure

HILIC methodologies have been optimized for the global metabolomic profiling of water-soluble metabolites [26]. BEH amide (100 mm × 2.1 mm, 1.7 µm, Waters, USA) and zwitterionic SeQuant ZIC-pHILIC columns were evaluated for their selectivity toward a wide variety of water-soluble compounds. The mixture of 118 authentic standards was analyzed using a BEH amide column and ZIC-pHILIC column. The number of these metabolites retained on the columns and the repeatability of three injections were evaluated. Repeatability was defined as the distribution of the relative standard deviation (RSD) for all of the detected metabolites. Seventy metabolites were retained and had an acceptable peak shape on the BEH amide column, whereas 83 metabolites were retained for ZIC-pHILIC (Fig. 1A). For an RSD less than 10%, the BEH amide column had 67.14% of the total responses, and the ZIC-pHILIC column had 90.36% of the total responses (Fig. 1B). These results showed that the ZIC-pHILIC column had the better compatibility and repeatability. Amide and zwitterionic columns are two different stationary phases in HILIC columns. The stationary phase of the amide column was uncharged, and the zwitterionic columns has a chemical group that has a positive and negative charge. These columns are commonly used for metabolomic analysis, particularly for water-soluble metabolites, including amino acids, organic acids, purines, and pyrimidines. A previous study reported that zwitterionic HILIC column was more suitable for phosphate metabolites analysis [27], such as AMP, UMP, NADPH, and Coenzyme A. Our data are in accordance with those of ref. [27]. Finally, when the compatibility, repeatability, and peak shape (Fig. S1) were considered, ZIC-pHILIC was recommended for the analysis of water-soluble metabolites.

Reproducible results are important for metabolic profiling studies. In particular, retention time must be stable for accurate metabolite identification. However, the retention time drifts in HILIC columns. After establishing the optimal chromatographic solution, we evaluated the long-term (10 months) inter-batch reproducibility of the retention time of the ZIC-pHILIC column by using standard mixtures and microbial metabolome samples. The results showed that all the retention time deviation values were less than 60 s, except for pyruvate. The 81.08% retention time deviation was less than 30 s (Fig. 2A). All the coefficient of variation were less than 5% (Fig. 2B), except for pyruvate, phenylpyruvate, and 2,3-dihydroxybenzoic acid. A previous study reported retention time deviations of less than 12 s (1% variation) for the retention time reproducibility of the ZIC-HILIC column in a 42 days long-term test [27]. Altogether, the drift in retention time was sufficiently small for microbial samples with the ZIC-pHILIC column in this study.

Retention time, precursor mass, and MS/MS spectra are important parameters for metabolite identification on a HILIC-HRMS platform. For MSI level 1, we developed a HILIC-HRMS library of 118 authentic standards (Supplementary material 2-S1), including retention time, precursor mass, and MS/MS spectra. The separation and identification of isomers are challenging in metabolomics. In this study, sugar phosphate isomers were not distinguished.

3.2. Optimization of sample quenching

Three main criteria were used for each optimization experiment. The RSD values of the peak intensities of the detected metabolites were used to evaluate the repeatability of the sample preparation method [13]. HCA and PCA were used to evaluate all the detected metabolite profile differences [6].

In the process of microbial metabolome preparation, quenching is important for preventing metabolic activity to avoid unwanted changes in intracellular metabolite levels. Cold methanol has been used extensively to quench microbial metabolomes. After quenching with cold
methanol, the cells and supernatant were separated using centrifugation or fast filtration. Intracellular metabolites were then be extracted from the cell using cold solvents. First, we tested fast filtration using a 0.45 \( \mu \)m pore size filter. The cells did not pass through the filter completely because of the viscosity of the sample, even when in a vacuum. Therefore, we evaluated 60% methanol (\(-40^\circ\text{C}, \text{quenching method 1}\) \[28\]) and 100% methanol (\(-40^\circ\text{C}, \text{quenching method 2}\) \[24\]) quenching combined with centrifugation for \( S. \text{albus J1074} \). For RSD values less than 30%, quenching method 2 had 100% of the detected metabolites, whereas quenching method 1 had 89.8% of the detected metabolites (Fig. 3 A). The HCA results showed the levels of recovered metabolites for the different methods (Fig. 3 B). There were higher levels of recovered metabolites for quenching method 2 for most detected metabolites, which is consistent with \( \text{yeast metabolomics quenching}\) \[24\], in contrast to \( \text{Aspergillus niger}\) \[3\]. The PCA score plot showed a difference between quenching methods 1 and 2 (Fig. S2). Cold methanol quenching could arrest enzyme activity because of the low temperature combined with the organic solvent. Cold shock and organic solvents may result in metabolite leakage owing to reduced cell integrity. The quenching solution supernatants were freeze-dried and analyzed in this study. Only a few amino acids and vitamins were detected in the supernatants. The results indicated that intracellular amino acids and vitamins were underestimated. In general, 100% methanol was better for \( S. \text{albus J1074 metabolomics quenching}\) in this study.

The ratio of sample to quenching solution resulted in different final methanol concentrations, which played an important role in metabolome sample preparation. Sample to quenching ratios of 1:3 (v/v), 1:4 (v/v), and 1:5 (v/v) were studied. For RSD values less than 30%, they had 88.8% (1:3), 90% (1:4) and 85% (1:5) of all detected metabolites respectively (Fig. S3A). The levels of representative detected metabolites showed no obvious differences among the three sample preparation methods (Fig. S3B). In general, 100% methanol at a ratio of 1:4 has been suggested for \( S. \text{albus J1074 metabolomics quenching}\).

3.3. Optimization of extraction solvent

Cold mixtures of methanol, acetonitrile, and \( \text{H}_2\text{O} \) are commonly used for water-soluble metabolite extraction, and these extraction solvents are compatible with MS-based analytical platforms. Because of the diversity of microorganisms, culture conditions, and physicochemical properties of metabolites, there is no unified sample preparation method for the microbial metabolome. In this study, five extraction solvents were evaluated, including pure methanol, methanol/\( \text{H}_2\text{O} \) (1:1, v/v), acetonitrile/\( \text{H}_2\text{O} \) (1:1, v/v), methanol/acetonitrile/\( \text{H}_2\text{O} \) (2:2:1, v/v/v), and chloroform/methanol/\( \text{H}_2\text{O} \) (2:1:1, v/v/v).

We used RSD values, HCA, and PCA to evaluate different extraction efficiency of different mixtures. For RSD values less than 30%, the detected metabolites for the chloroform/methanol/\( \text{H}_2\text{O} \), pure methanol, methanol/\( \text{H}_2\text{O} \), methanol/acetonitrile/\( \text{H}_2\text{O} \), and acetonitrile/\( \text{H}_2\text{O} \) extraction methods were 51.7%, 94.3%, 93%, 96.5%, and 97.7%, respectively (Fig. 4A). The repeatability of the chloroform/methanol/\( \text{H}_2\text{O} \) extraction method was poor, and those of the other four methods were similar. As shown in Fig. 4B, the methanol/acetonitrile/\( \text{H}_2\text{O} \) method had the highest amount for most detected metabolites, and
chloroform/methanol/H₂O had the worst performance. The metabolite profiles of the five extraction methods were clearly distinguished by PC1 and PC2 in the PCA score plots (Fig. S4), accounting for 31.5% and 22.3% for the total variance, respectively. When PCA, the RSD value, and HCA results of the metabolite profiles were considered, cold methanol/acetonitrile/H₂O (2:2:1, v/v/v) was the best solvent for metabolite extraction of S. albus J1074. This mixture is also used to extract mid non-polar metabolites. For this reason, combining HILIC analysis with C18 analysis is a common method in untargeted metabolomic analysis, and this combined method can provide more information regarding metabolites.

Apart from cold solvent extraction, boiling-ethanol extraction has been developed for microbes [29]. A cold solvent combined bead mill has been reported for microbial metabolome extraction owing to its good repeatability and high throughput [10]. Therefore, we considered five cold extraction solvents combined with a bead mill for S. albus J1074, and boiling-ethanol was excluded because of its incompatibility with the bead mill. The cycle numbers of mechanical disruption using a bead mill were optimized (Fig. S5), and mechanical disruption with 3 cycles (run for 60 s, stop for 10 s as one cycle) was determined to be suitable for S. albus J1074.

3.4. Metabolic profiling of engineered strains ADE-AP and OE3

To validate the established method, we assessed intracellular polar metabolite profiles during spinosad shake flask fermentation of the engineered strains ADE-AP and OE3. OE3 is the first generation engineered strain for the heterologous production of spinosad in our laboratory [4], and ADE-AP is the second generation engineered strain after overexpressing polyketide synthase (PKS) genes and the genes for overproduction of polyketide’s precursors (acyl-CoAs overproduction genes) based on OE3 [16]. The titer of spinosad in ADE-AP was 50 times higher than that in OE3. ADE-AP yielded the highest spinosad titer in heterologous Streptomyces reported in the literature until now. However, spinosad did not further accumulate in strain ADE-AP after 8 days of fermentation [16]. Therefore, the following questions are raised: 1) How did the metabolome changes in ADE-AP result in a much higher titer of spinosad than that in OE3? 2) Why did the spinosad in ADE-AP not continue to be produced after 8 days?

To unveil the possible reasons, we first investigated the stationary phase in the engineered S. albus J1074, the residual glucose decreased from days 1–7, and there was no available glucose in the culture medium starting from day 7 [16]. Furthermore, the growth curve of S. albus showed that the strain was in the exponential phase on days 1–3 of fermentation and reached the stationary phase after the 3rd fermentation (Fig. S6). Therefore, to determine the intracellular polar metabolite changes in different stationary phases, fermentation broths on the 3rd, 5th, and 8th days were extracted and determined. Using the established method, 3648 compounds were detected (Supplementary material 2-S3), including 83 metabolites annotated at level 1 and 48 annotated at level 2. The dynamic changes in metabolites related to central carbon, amino acids, nucleotides, energy, reducing equivalent, and intermediate metabolites for spinosad synthesis were compared. The repeatability of these metabolite levels is shown in Fig. S7. The metabolite levels of strains OE3 and ADE-AP were different on different days of fermentation.
Fermentation reached the end of exponential phase on the 3rd day, and there was sufficient glucose in the culture medium at that time [16]. The spinosad titer of strain ADE-AP was 8.5 times that of strain OE3 (Fig. 5A). Fig. 5B and C shows a comparison of the metabolic intensity of strain ADE-AP and that of OE3 on day 3. The intracellular levels of glucose, ATP, acetyl-CoA, and pyruvate in strain ADE-AP were 1.1, 1.2, 1.6, and 65.9 times greater, respectively, than those in strain OE3 (Fig. 5B and C). The levels of sixteen amino acids in strain ADE-AP were upregulated to 1.1–2.9 times those in strain OE3 (Fig. 5B and C). Furthermore, the levels of intermediate metabolites including TDP-\(L\)-rhamnose (TDP-Rha) and TDP-\(D\)-forosamine (TDP-Foro) were 1.58 and 3.55 times greater, respectively, than those of strain OE3 (Fig. 5B and C). From these results, we speculated that overexpression of PKS genes and acyl-CoAs overproduction genes in strain ADE-AP may strengthen intracellular metabolism levels and lead to the excessive accumulation of pyruvate at the end of exponential phase. At the same time, more ATP, acetyl-CoA, and intermediate metabolites were accumulated for efficient spinosad synthesis in strain ADE-AP.

From the 3rd day to 5th days of fermentation, fermentation proceeded from the exponential phase to the stationary phase. The levels of intracellular glucose and ATP in strain OE3 decreased by 72.9% and 62.5%, respectively, on day 5 compared with those on day 3 (Fig. 5B). However, the levels of NADPH and acetyl-CoA in strain OE3 increased by factors of 1.58 and 1.25, respectively (Fig. 5B). The levels of fourteen amino acids in strain OE3 were upregulated by factors of 1.1–2.03. However, in strain ADE-AP, the levels of intracellular glucose, ATP, acetyl-CoA, and NADPH decreased by 81%, 96%, 64%, and 46%, respectively, on day 5 compared with those on day 3, and the levels of amino acids also decreased (Fig. 5B). Comparing OE3 with ADE-AP (Fig. 5B and D), the levels of NADPH, ATP, and acetyl-CoA in strain ADE-AP were reduced to 16%, 15% and 44% of those of strain OE3, respectively. The levels of succinate, fumarate, (s)-malate, and citrate were reduced to 59%, 62%, 32%, and 45% of those of strain OE3, respectively. The levels of TDP-Rha and TDP-Foro were reduced to 58% and 52% of those of strain OE3, respectively. However, the titer of spinosad in strain ADE-AP was 18.5 times that in strain OE3 on day 5 (Fig. 5A). From these results, we speculated that the overexpression of PKS genes and acyl-CoAs overproduction genes promoted the efficient synthesis of spinosad, resulting in rapid consumption of substances related to the synthesis of spinosad, such as acetyl-CoA, ATP, and NADPH, from the 3rd to 5th days of fermentation. Overexpression of these genes may pull the metabolic flow towards spinosad synthesis and weaken primary metabolism.

From the 5th day to the 8th days of fermentation, the amount of glucose in the culture medium was severely inadequate [16]. Intracellular glucose in strains OE3 and ADE-AP decreased on day 8 compared with that on day 5, resulting in no signal detection in the strains (Fig. 5B). The levels of NADPH and pyruvate were reduced by 48% and 74%, respectively, on day 8 compared with those on day 5 in strain OE3, and TDP-Foro was reduced by 83% (Fig. 5B). In strain ADE-AP, the levels of acetyl-CoA, pyruvate, and TDP-Foro were reduced by 69%, 99%, and 62%, respectively, on day 8 compared with those on day 5 (Fig. 5B). The titer of spinosad in strain ADE-AP was 24.4 times that in strain OE3 on day 8 (Fig. 5A). The metabolic intensity of strain ADE-AP compared with that of OE3 on day 5 is shown in Fig. 5B and E. The levels of succinate, fumarate, (s)-malate, and citrate in strain ADE-AP were reduced to 15%–51% of those in strain OE3. Furthermore, the level of TDP-Rha was reduced to 60% of that in strain OE3. From these results, we speculated that a small amount of residual sugar maintained spinosad synthesis, but it could not provide sufficient precursors, energy, or reduce power for...
spinosad synthesis at this stage. The titer of spinosad could not continue to increase when the residual sugar was completely consumed. Taken together, we speculated that the overexpression of PKS genes and acetyl-CoA overproduction genes in strain ADE-AP may strengthen intracellular metabolism levels in the logarithmic phase, which can lead to the accumulation of more substances related to the synthesis of spinosad for spinosad overproduction, as well as the weakening of primary metabolism in the stationary phase, which can pull the metabolic flow towards spinosad synthesis. However, in the late stage of fermentation, the substrate for the synthesis of spinosad could no longer be produced intracellularly owing to the depletion of glucose, so the synthesis of spinosad stopped, which explains why the increase in glucose was able to increase the titer of spinosad in our previous work [16].

4. Conclusions

We evaluated and optimized analytical procedures and metabolome sample preparation protocols for S. albus J1074. The HILIC-HRMS analytical method exhibited good stability for 10 months. We recommend that S. albus be quenched with pure methanol at a sample/ quenching ratio of 1:4 (v/v). Water-soluble metabolites extracted using methanol/acetonitrile/H2O (2:2:1, v/v/v) were found to be more efficient for S. albus. We also applied this method to reveal the differences in the water-soluble metabolite profiles of the engineered strains OE3 and ADE-AP in different growth phases during spinosad fermentation. A total of 3648 metabolites were detected. Furthermore, we found that the metabolic profiles of strains OE3 and ADE-AP were different on different days of fermentation. The overexpression of PKS genes and acetyl-CoAs overproduction genes could stimulate metabolism, whereas the insufficient carbon source, precursors, energy, and reducing power may have resulted in the cessation of spinosad accumulation on day 8 of fermentation. Our results show that the synthesis of spinosad is highly dependent on the available levels of glucose, pyruvate, acetyl-CoA, and energy. Therefore, when we perform further engineering work to enhance the production of spinosad, we should consider the rational use of these substances in the cell and reduce the unnecessary consumption of these substances. For example, this could be done by supplying cells with more acetyl-CoA through pathway (e.g. fatty acid degradation pathway) and using inducible promoters to control high expression of genes to avoid excessive energy wastage in protein synthesis. We established a comprehensive method for metabolite extraction and analysis of S. albus J1074 in this study. At the same time, this method provides a reference for profiling polar metabolites of other actinomycetes.

CRediT authorship contribution statement

Shuai Fu: Methodology, Investigation, Writing – original draft. Ziheng An: Investigation. Liangliang Wu: Investigation. Zilei Xiang: Investigation. Zixin Deng: Conceptualization, Supervision. Ran Liu: Formal analysis, Writing – review & editing. Tiangang Liu: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

We declare that there are no conflicts of interest. We certify that this manuscript is original, has not been previously published, and will not be submitted elsewhere for publication while under consideration by Synthetic and Systems Biotechnology. This study is not split into several parts and submitted to various journals. Results are presented clearly, honestly, and without fabrication. No data, text, or theories by others are presented as our own.

Explicit permission to submit has been received from all co-authors. All the authors whose name appear on the submission have contributed sufficiently to this study.

This manuscript does not contain any studies involving humans or animals.

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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.2022.05.004.

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