Boosting Ascomycin Production through n-Butanol Addition in the Fermentation Process of *Streptomyces hygroscopicus var. ascomyceticus* ATCC 14891

Jinyu Meng[a], Qi Chen[a], Shuhong Gao[a,*] and Daojing Zhang[a,*]

Abstract

Ascomycin (FK520) is a macrolide antibiotic known for its immunosuppressive activities. In this study, we screened several short-chain alcohols to enhance FK520 production in *Streptomyces hygroscopicus var. ascomyceticus* ATCC 14891, with a particular focus on n-butanol addition. After optimizing the n-butanol addition process, we achieved a FK520 yield of 569.37 mg/L with 0.8% n-butanol addition at 27 h, representing a 1.72-fold increase compared with the control group. We subsequently found that ROS levels of n-butanol addition group reached 1.49×10^5 RFU/g biomass at 29 h, which was 3.02 times higher than the control group. The 0.8% n-butanol addition also promote the accumulation of biosynthesis precursors for FK520 production. The highest ethylmalonyl-CoA content surged to 93.1 nmol/g DCW at 48 h, marking a 5.3-fold increase. Likewise, the highest methylmalonyl-CoA and malonyl-CoA levels increased remarkable 4.33-fold and 3.33-fold compared with the control group at 72 h and at 120 h, respectively. Then, we explored the effects of oxygen supply on FK520 production with n-butanol addition, and improving oxygen supply caused a significant increase of FK520. Our research has revealed that addition of short-chain alcohols can regulate carbon flux toward FK520 biosynthesis by supplementing various CoA-esters, including ethylmalonyl-CoA, methylmalonyl-CoA, and malonyl-CoA.

Keywords: *Streptomyces hygroscopicus var. Ascomyceticus*; Ascomycin; n-Butanol Addition; Reactive Oxygen Species; FK520 biosynthesis precursors

[a] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China

1. Introduction

Ascomycin (FK520) is a pharmacologically significant 23-membered macrocyclic polyketide antibiotic with immunosuppressive properties [1], and effectiveness against malaria [2]. Furthermore, it displays antifungal and antispasmodic activities [3]. Notably, FK520 has a structural analog known as tacrolimus (FK506), which is a widely recognized immunosuppressive agent used in the prevention of xenograft rejection after organ transplants in humans [4]. FK520 is highly regarded as a versatile and valuable drug [5]. Most importantly, it can be used as an important chemical intermediates for the production of Pimecrolimus approved by FDA in 2001.

Pimecrolimus being semi-synthetic products of the Streptomyces-produced FK520, many researchers utilized *S. hygroscopicus var. Ascomyceticus* ATCC 14891 and its mutant strains to produce FK520. How to improve FK520 titer has become hotspots in the world. Qi employed *S. hygroscopicus var. ascomyceticus* FS35 as the initial strain to derive a mutant strain, *S. hygroscopicus* SA68, through a combination of femtosecond laser irradiation and shikimic acid endurance screening, improving the FK520 production to 270 mg/L [6]. Then, they identified and engineered two potential gene manipulation targets (*pyc* and *fkbO*), resulting in an enhanced FK520 production of 610 mg/L [7]. Most of the studies about the enhancement of FK520 titer are associated with primary metabolic pathways inside of secondary metabolites for FK520 biosynthesis, which is regulated by precursor synthetic pathways and regulatory networks in *S. hygroscopicus var. ascomyceticus*. In nature, many antibiotics are only produced in response to specific signals, such as environmental stress or signals from surrounding microbes (symbionts or competitors) [8]. Novel molecular methods can help us understand and identify the triggers that activate antibiotic production in nature, which has garnered significant interest among multiple research groups. Presently, substantial efforts are dedicated to stimulating the accumulation of secondary metabolites and activating the expression of silent antibiotic biosynthetic gene clusters using chemical elicitors, nonionic surfactants, and extracellular disturbances [9,10]. For instance, scandium and/or lanthanum significantly enhanced the production of actinomycin, actinorhodin, and streptomycin by two- to as much as 25-fold at concentrations of 10–100 μM in *Streptomyces antibioticus, Streptomyces coelicolor*, and *Streptomyces griseus*, respectively [11]. Triton X-100 could increase the production of hypocrellin A and stimulate its release out in *Shiraia bambusicola* [12]. DMSO could enhance the production of FK520 by 2.32-fold at 0.6% concentrations in *S. hygroscopicus var. ascomyceticus* H16 [13].

As extracellular disturbances, short-chain alcohols could stimulate the overproduction of secondary metabolites in Streptomyces by causing environmental stress and altering the metabolic states of bacteria due to their toxicity [14,15], which has been reported by many research groups. Isobutanol could promote the biosynthesis of AP-3 in *Actinosynnema pretiosum* [16]. n-propantol could improve the biosynthesis of erythromycin production in *Streptomyces erythreus* [17]. Ethanol could promote the biosynthesis of validamycin A production in *Streptomyces hygroscopicus* 5008 [18]. In these reports, short-chain alcohols could not only stimulate biosynthesis of secondary metabolites, but also could participate in the metabolic reactions of the bacteria by supplying the biosynthesis precursor of

secondary metabolites. However, there have been no reports so far of promoting FK520 accumulation in *S. hygroscopicus var. ascomyceticus* ATCC 14891 by adding short-chain alcohols.

In this study, we assessed the impact of various short-chain alcohols including n-propanol, isopropanol, n-butanol, isobutanol, 1, 3-propanediol on FK520 accumulation in *S. hygroscopicus var. ascomyceticus* ATCC 14891. We further explored the change of fermentation process parameters and CoA-esters to identify potential mechanisms that could regulate carbon flux towards FK520 production. Simultaneously, we used the Genome-Scale Metabolic Model (GSMM) to stimulate the impact of oxygen limitation on FK520 biosynthesis under the n-butanol addition conditions. Subsequently, based on the simulation and analysis results from GSMM, we further optimized the oxygen supply conditions by increasing the rotational speed during flask fermentation, thereby increasing the yield of FK520 with and without n-butanol addition. The insights gained from this research not only serve as a valuable reference but also provide guidance for future investigations into FK520 biosynthesis. Furthermore, these findings offer a rational metabolic engineering strategy that can be applied to increase the production of other secondary metabolites.

2. Materials and methods

2.1. Strain and media

S. hygroscopicus var. ascomyceticus ATCC 14891 served as the fermentation strain throughout this study. The seed medium consisted of the following components: 15g/L starch, 10g/L glucose, 10g/L soybean meal, 6g/L yeast extract, 2g/L NaCl, 1g/L $(NH_4)_2SO_4$, 2g/L $CaCO_3$, with a pH of 7.2. The fermentation medium in the shake flask included: 9g/L glycerol, 5g/L tryptone, 7g/L yeast extract, 2g/L corn steep liquor, 0.5g/L $MnSO_4$, 1.5g/L $(NH_4)_2SO_4$, 0.5g/L KH_2PO_4, 1g/L $MgSO_4 \cdot 7H_2O$, 12g/L soybean oil, 1g/L $CaCO_3$, with a pH of 6.7.

Spore culture of *S. hygroscopicus var. ascomyceticus* ATCC 14891 was conducted on Yeast Malt Extract Agar ATCC Medium 196 for 14 days at 28 °C. The spores were then inoculated into 250 mL flasks containing 25 mL of seed culture medium and cultivated at 30 °C with agitation at 220 r/min for 44 h. Subsequently, 2.5 mL of the seed culture was transferred to a 250 mL flask containing 25 mL of fermentation medium, and this culture was incubated at 30 °C with agitation at 220 r/min and 250 r/min for 5 d. All the short-chain alcohols including n-propanol, isopropanol, n-butanol, isobutanol,1,3-propanediol were obtained through filtration using a sterile 0.22 μm filter and were added individually to the fermentation medium. The percentage (1.2%) short-chain alcohols (Fig. 1) were introduced into the batch medium after 24 h. Each group underwent three replicates.

2.2. Product, impurity, Glycerol, dry cell weight (DCW) and K_La determination

Dry cell weight was determined by washing the cells twice with phosphate-buffered saline after centrifuging at 8000 r/min for 10 min and then drying them at 105 °C until a constant weight was achieved. The final biomass was expressed as grams per liter (g/L).

FK520 extraction and yield determination were performed using high-performance liquid chromatography (HPLC) (1260 Infinity II, Agilent Technologies, USA) equipped with a Zorbax SB-C18 analytical column (250 mm × 4.6 mm, Agilent Technologies, USA), following the method described by Wang[19]. In brief, 1 mL of culture was mixed with an equal volume of methanol and then sonicated in water for 30 min. After centrifugation at 12000 r/min (Centrifuge 5415 R, Eppendorf) for 5 min, the supernatant was analyzed by HPLC. The mobile phase consisted of acetonitrile and 0.1% phosphoric acid in water (65:35, v/v) with a flow rate of 1 mL/min. The sample injection volume was 10 μL. The column temperature was set at 55 ℃, and the UV detector was set at 210 nm. The glycerol content was determined using the titrimetric method [20]. K_La of different Rotational speed were assessed following the method outlined by Linek V[21].

The FK523 extraction determination was carried out in the same manner as for the product. Subsequently, samples were analyzed using ultra-high-performance liquid chromatography (UPLC, Ultimate 3000, Thermo Scientific) - electrospray ionization (ESI) - tandem mass spectrometry (MS, Q Exactive Plus, Thermo Scientific). The mobile phase consisted of solvent A (50 mM ammonium formate solution) and solvent D (acetonitrile) at a flow rate of 300 μL/min, programmed as follows: 0.00 - 10.00 min, 35% A/65% D. The structure of impurity was identified using Full SCAN mode in positive mode.

2.3. Evaluation of intracellular antioxidant activities

Intracellular levels of reactive oxygen species (ROS) were assessed following the method outlined by Miranda, R. U[22]. ROS-induced oxidation of the fluorogenic probe H2DCF-DA was monitored at various time during culture. 100 mg of mycelium was combined with 5 mL of a 10 mM H2DCF-DA solution in cold PBS buffer. The reaction was carried out in the dark at 37 °C for 30 min. Subsequently, each sample underwent three freeze-thaw cycles using liquid nitrogen and was then centrifuged at 12000 r/min at 4 °C for 5 min. Next, 200 μL of the supernatant was transferred to a microplate reader (SYNERGY H1, BioTek, USA) with an excitation filter set at 488 nm and an emission filter set at 525 nm. The obtained signal was normalized based on biomass content. Triplicate analysis was conducted for each sample.

2.4. Extraction and analysis of intracellular CoA-esters

Each sample was centrifuged at 12,000 r/min for 5 min to isolate mycelium, which was then washed twice with PBS buffer. Cell extracts were prepared using a modified method based on the procedure described by Park, J. W [23]. The mycelium was resuspended

in 1 mL of 10% TCA. Subsequently, each sample underwent three freeze-thaw cycles using liquid nitrogen and was then centrifuged at 12,000 r/min at 4 °C for 5 min. Each TCA extract was passed through an OASIS HLB SPE cartridge under vacuum following this protocol: The cartridge was initially conditioned with 3 mL of methanol, followed by 3 mL of 0.15% TCA solution. The cell extract was applied to the cartridge, followed by a 1 mL water wash step. The elution of CoA-esters was accomplished using 1 mL of methanol, and the eluate was evaporated to dryness at room temperature using the Termovap Sample Concentrator. The dried samples were stored in a freezer until analysis. For analysis, samples were reconstituted in 80 µL of a 20 mmol/L ammonium acetate solution, and a portion of this solution was subjected to LC-ESI-MS/MS analysis. The analysis was conducted using ultra-high-performance liquid chromatography (UPLC, Ultimate 3000, Thermo Scientific) - electrospray ionization (ESI) - tandem mass spectrometry (MS, Q Exactive Plus, Thermo Scientific), following a previously described method with some modifications [24]. The column employed was the Hypersil Gold C18 3µm, 10 mm x 2.1 mm. The mobile phase, composed of solvent C (50 mM ammonium formate solution) and solvent D (acetonitrile), was delivered at a flow rate of 250 µL/min and programmed as follows: 0–1.00 min, 95% C/5% D; 1.00–1.10 min, a linear gradient from 95% C/5% D to 75% C/25% D; 1.10–7.00 min, a linear gradient from 75% C/25% D to 60% C/40% D; 7.00 - 8 min, 60% C/40% D. Quantification of CoA-esters were performed using the selected ion monitor mode in positive mode. All authentic CoA-ester standards were procured from Cayman Chemical (USA).

2.5. Model iMK1208 reconstruction, simulation, and analysis

The reconstruction process strictly adhered to the protocol recommended by [25]. In this study, we incorporated the n-butanol metabolic pathway into the model, which is detailed in supplement.

Constraint-based FBA was conducted using the COBRA toolbox and Gurobi 9.5.2 as the linear programming optimization solver, with the stoichiometric matrix of the metabolic network providing the constraints. Linear programming algorithms were employed to compute the optimal solution within the space of feasible flux solutions. In this study, our primary focus was on maximizing FK520 product synthesis rates as the biological phenotype of the optimal solution. This problem is formulated using matrix notation and is presented as Equations (1)–(3) [26,27].

$$\text{maximize}: c^T \cdot v \tag{1}$$

$$\text{Constraints}: S \cdot v = 0 \tag{2}$$

$$v_{min} \leq v \leq v_{max} \tag{3}$$

S represents a stoichiometric matrix that contains the stoichiometric coefficients of metabolites involved in metabolic reactions, while v represents a vector containing the fluxes of these metabolic reactions. The parameters v_{min} and v_{max} correspond to the minimum and maximum constraints, signifying the maximum enzymatic reaction rate and the reversibility of metabolic reactions, respectively. The vector C^T signifies a weight vector that indicates the contribution of each reaction to the objective function. Glucose was selected as the sole carbon source for the simulations, all of which were executed using the MATLAB 2023a platform. Specifically, the glucose uptake rate was set at -0.9 mmol/gDW/h, and the specific growth rate was fixed at 0.0616 mmol/gDW/h [28]. The principle of robustness analysis was first proposed by [29]. The complete code of robustness analysis can be accessed in Supplementary Material (S1).

2.6 | Statistical analysis

All results presented in this paper represent the averages of three replicates and are expressed as the mean ± standard deviation (SD). To assess differences among various treatments, we performed analysis of variance (ANOVA) using Prism 10.0 software.

3. Results and discussion

3.1. short-chain alcohols facilitate FK520 accumulation

Firstly, we attempted to supply five short-chain alcohols (n-propanol, isopropanol, n-butanol, isobutanol, 1,3-propanediol) during the fermentation in *S. hygroscopicus* ATCC 14891 and explore their influence for the production of FK520. As shown in Fig. 1a, the supplement of 1.2% n-propanol, 1.2% n-butanol, and 1.2% isobutanol could stimulate the production of FK520 significantly. The highest yield was 375.47 ± 50.35 mg/L with 1.2% n-butanol addition at 24 h, a 2.08-fold enhancement over the control group. And the supplement of 1.2% n-propanol at 24 h also increase the production of FK520 by 1.73-fold. Likewise, 1.2% isobutanol addition at 24 h increase the FK520 production by 1.59-fold. To investigate the effects of short-chain alcohols on FK520 accumulation, different exogenous feeding concentration of n-propanol and n-butanol were further evaluated during FK520 fermentation.

As shown in Fig. 1b, FK520 yield was improved to some extent at n-propanol concentrations of (0.8%, 1.0%, 1.2%, and 1.4%). All different concentration of n-propanol can obviously stimulate the overproduction of FK520, but there is no significant difference between the experimental groups. The highest FK520 yield was 364.98 ± 41.39 mg/L with 0.8% n-propanol addition. However, the addition of n-propanol also leads to a significant increase in the content of an impurity with a peak very close to that of FK520 (Fig. 2a). As we know, one difference between FK520 and its structural analog such as FK506 lies in the different biosynthesis precursors of C21.

The former uses ethylmalonyl-CoA as precursors to build FK520. In previous report, n-propanol could be used to synthesis methylmalonyl-CoA in *Streptomyces erythreus* [17]. Methylmalonyl-CoA competes with ethylmalonyl-CoA for the carbon skeleton at position 21 (Fig. 2c), resulting in the strain producing a structurally similar compound, FK523, alongside the synthesis of FK520 [30]. To confirm whether the impurity produced during fermentation under the conditions of adding n-propanol is FK523, we conducted UPLC-MS qualitative analysis on the fermentation extract. The molecular formula of FK523 is $C_{42}H_{67}NO_{12}$, and the electrospray mass spectrum (Fig. 2d) of fermentation extract with n-proponal revealed the presence of sodium adducts at m/z 800, fragment ions were observed at m/z 782[(M − H_2O)Na]$^+$, 689 [(M − 111)Na]$^+$, 671[(M − 111 − H_2O)Na]$^+$, 627[(M − 111 − H_2O − CO_2)Na]$^+$, 590 [(M − 210)Na]$^+$, 479 [(M − 210 − 111)Na]$^+$, based on the analysis of fragment characteristics of FK506 and structurally similar compounds as reported in Lhoest, G's work [31], we have confirmed that the impurity produced in large quantities upon adding n-propanol to *S. hygroscopicus* ATCC 14891 is a structural analogue of FK520, namely FK523. Therefore, while the addition of n-propanol increases the yield of FK520, the large amounts of close structural resemblance of FK523 (the peak area of FK523 reached 40% of FK520) undoubtedly complicates the later stages of FK520 isolation and extraction.

To further enhance the yield of FK520 with addition of n-butanol, we first optimized the oxygen supply conditions in shake flask fermentation and the relevant results and analysis are described in section 3.4. Then, we conducted experiments with the different concentrations of n-butanol (0.4%, 0.8%, 1.2%, 1.6%) as the experimental groups (Fig. 1c). The data revealed that 0.8% n-butanol addition yielded the best results, with a production of 523.57 ± 10.08 mg/L, which was 1.77 times higher than the control. Slightly less favorable results were obtained with 1.2% n-butanol (486.77 ± 23 mg/L), while the addition of 1.6% n-butanol had a pronounced inhibitory effect on FK520 production. Next, to explore the influence of the timing of n-butanol addition on FK520 accumulation, we added 0.8% n-butanol at five different time points during the fermentation process (Fig. 1d). Results indicated that the addition of 0.8% n-butanol at 27 h yielded the best results with a production of 569.37 ± 24.07 mg/L. Early addition of n-butanol (at 18 h or 21 h) during fermentation significantly inhibited FK520 production. Similar findings have been reported in Li, M's work[32], where the early addition of n-propanol in *Streptomyces natalensis* has also led to suppression in mycelia growth and a decrease in Natamycin production. To further investigate the reasons behind the enhanced FK520 production due to n-butanol addition, we conducted additional monitoring and analysis of fermentation process parameters with 0.8% n-butanol supplementation in subsequent experiments.

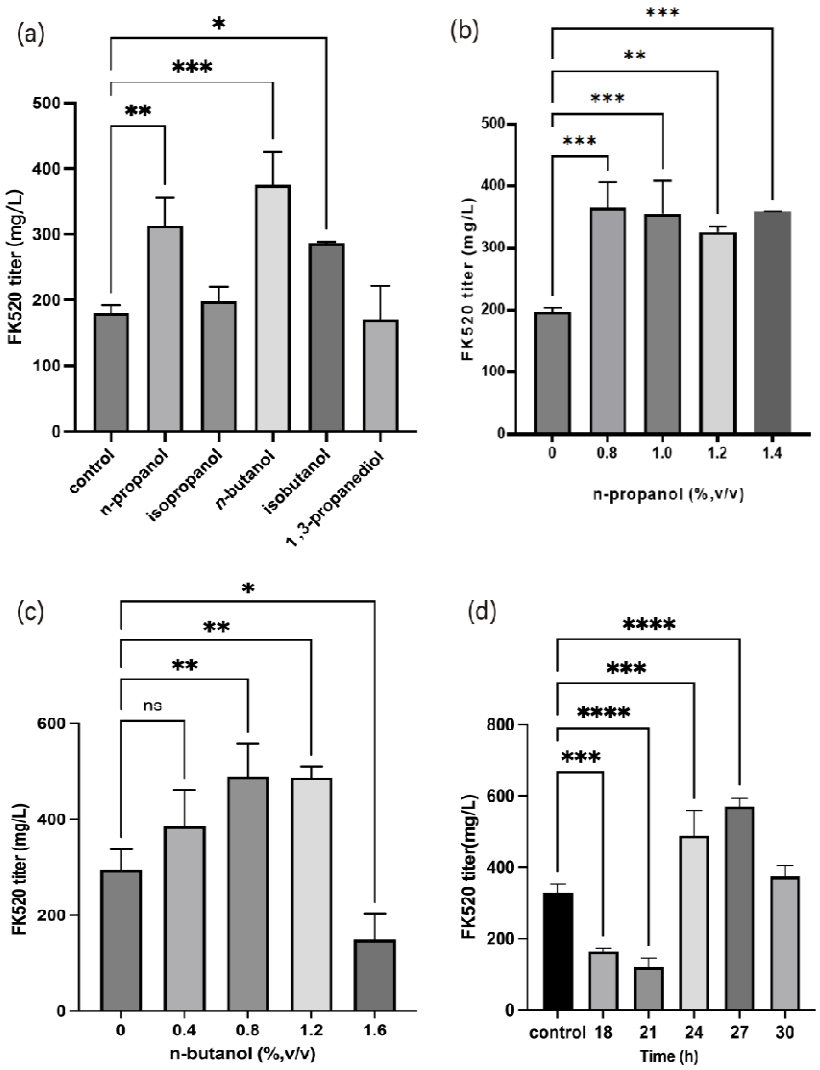

Fig. 1 Effects of various short-alcohols on FK520 titer in *S. hygroscopicus var. Ascomyceticus* ATCC 14891. (a) Effects of supplementation with n-propanol, isopropanol, n-butanol, isobutanol, and 1,3-propanediol on FK520 production. (b)Effects of supplementation with the different concentrations of n-propanol on FK520 production. (c)Effects of supplementation with the different concentrations of n-butanol on FK520 production. (d)Effects of different feeding time on the final FK520 production under 0.8% n-butanol treatment. Values are means ± standard deviations. n = 3. p-values are calculated using one-way ANOVA on respective means (*p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001)

Note: the fermentation of (a) and (b) were done under the rotational speed at 220 r/min, and the fermentation of (c) and (d) were done under the rotational speed at 250r/min.

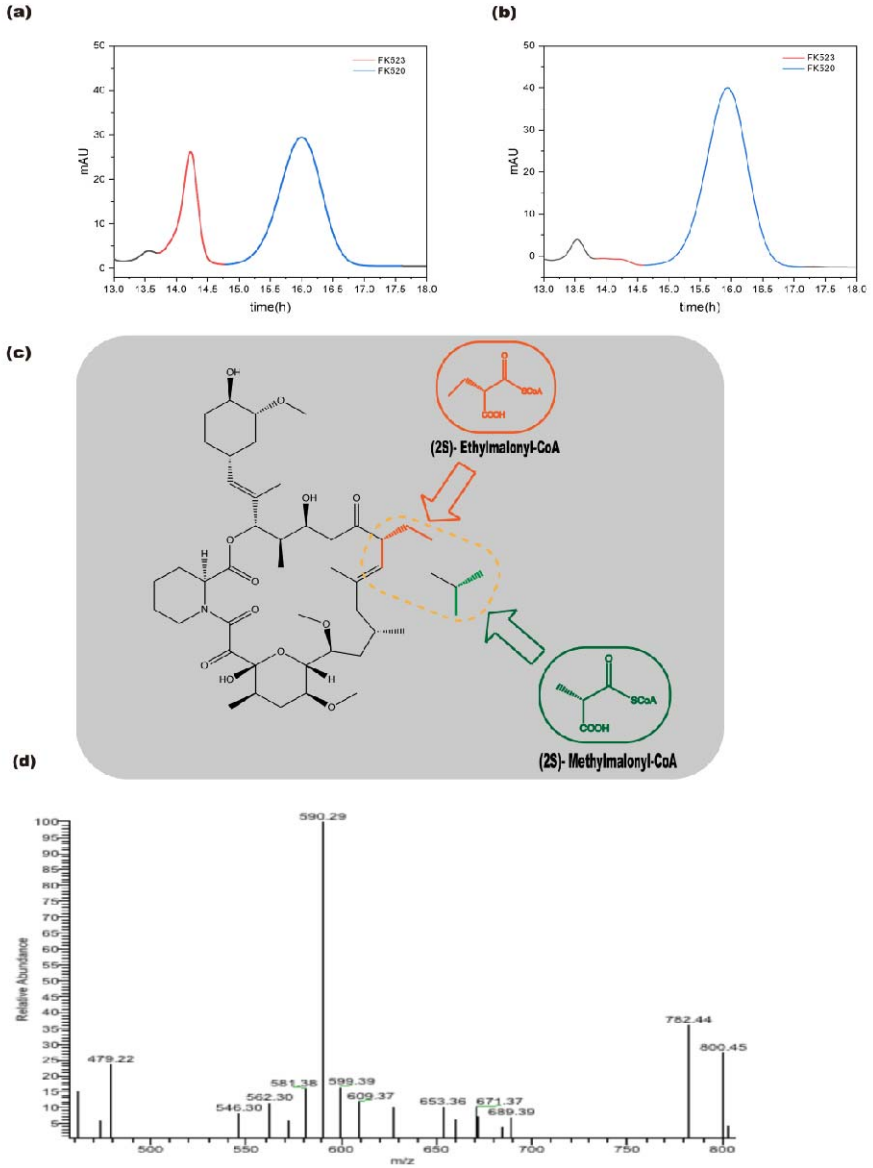

Fig. 2 (a)The HPLC plots of strains with concentration of 0.8% n-propanol which was added at 24h. (b)The HPLC plots of strains with concentration of 0.8% n-butanol which was added at 27h. (c)Proposed biosynthetic pathway of CoA-esters involved in FK520 biosynthesis in the structure of FK520, the C21 part composed of ethylmalonyl-CoA was marked in orange. Compared with FK520, the C21 part of FK523 was synthesized by methylmalonyl-CoA and labeled in green. (d)Electrospray mass spectrum of compound FK523

3.2. Effects of n-butanol addition on fermentation process parameters

3.2.1 Influence on the pH, total Glycerol, FK520 titer and DCW

As shown in Fig. 3a, glycerol consumption exhibited significant changes between the groups with and without n-butanol. For the strains fermentation with n-butanol supplementation, the glycerol content at 96 h and 120 h were significantly higher than those of the control group. This indicates that the addition of n-butanol altered the strain's utilization of carbon sources especially at the late stage of FK520 fermentation. One reason for the decreased glycerol utilization capacity of *S. hygroscopicus var. ascomyceticus* ATCC 14891 under n-butanol addition conditions may be that GAPDH activity was inhibited by the addition of short-chain alcohols [18]. The addition of short-chain alcohols may also lead to an increase in Triacylglycerols (TAGs) content [33], and the accumulation of TAGs plays a crucial role in regulating carbon flux towards the synthesis of secondary metabolites, reducing carbon flux into the TCA cycle [34].

In the early stages of fermentation (0-24 h), the pH slowly increased, followed by a rapid decrease over a short period (24-48 h), and then stabilized. When 0.8% n-butanol was added at 27 h, we observed a significant slowdown in pH reduction, and throughout the subsequent fermentation process, the pH remained higher than the control group, which suggested that 0.8% n-butanol addition inhibited the glycolytic pathway and led to a decrease acid-producing capacity in *S. hygroscopicus var. ascomyceticus* ATCC 14891 and caused a higher pH compared with the control group after 0.8% n-butanol addition. Additionally, in the group with the addition of 0.8% n-butanol, the pH was consistently maintained at approximately 5.6 on the 3rd, 4th, and 5th days of fermentation. Previous research by Liu has demonstrated that maintaining a pH of around 5.6 in a 5L bioreactor is highly conducive to the accumulation of FK520 [35]. We suppose that the appropriate environmental pH could promote the biosynthesis of FK520, leading to the observed significant accumulation of FK520 in the late stage of fermentation. Current perspectives suggest that antibiotics may function as 'collective regulators of microbial community homeostasis' acting as signals or cues rather than weapons [36,37]. Therefore, when cells perceive the pressure from the addition of 0.8% n-butanol, they regulate the environment pH to enhance FK520 biosynthesis and use FK520 as a signaling molecule to promote symbiotic relationships among different organisms, benefiting each organism through nutrition or protection.

Despite the reduced glycerol utilization rate, it was evident from the Dry Cell Weight (DCW) data that the addition of n-butanol did not lead to a significant decrease in biomass. This indicated that *S. hygroscopicus var. ascomyceticus* ATCC 14891 exhibited a certain degree of tolerance to n-butanol and could ease toxicity by altering its metabolism. According to the literature by Halan B[14], under conditions with a high concentration of n-butanol, medium supplementation with yeast extracts and other substances can alleviate toxicity by reducing the cost of sustaining biomass synthesis through the direct supply of amino acids and other precursors. Therefore, we speculate that the appropriate addition of n-butanol promotes the utilization of amino acids and other precursors and then redirects more carbon flux towards the synthesis of secondary metabolites in *S. hygroscopicus var. ascomyceticus* ATCC 14891. As the fermentation progresses from 0 to 120 h, the production of FK520 gradually increases. However, we observed that the synthesis rate of FK520 was lower than that of the control group at 48 h. We supposed that this could be attributed to the addition of 0.8% n-butanol at

27 h, which imposed a certain level of stress on the strain's growth and metabolism, disrupting its normal growth and metabolic activities. Consequently, this led to a temporary reduction in the synthesis rate of FK520. Subsequently, as time progressed, the strain adapted to the metabolic stress caused by the addition of n-butanol and began to produce FK520 in larger quantities.

In summary, we conclude that the addition of 0.8% n-butanol at 27 alters the internal metabolism of the strain and promotes the biosynthesis and accumulation of FK520. To explore the reasons behind the metabolic changes induced by 0.8% n-butanol addition, we conducted further exploration and analysis of other possible changes during the fermentation process.

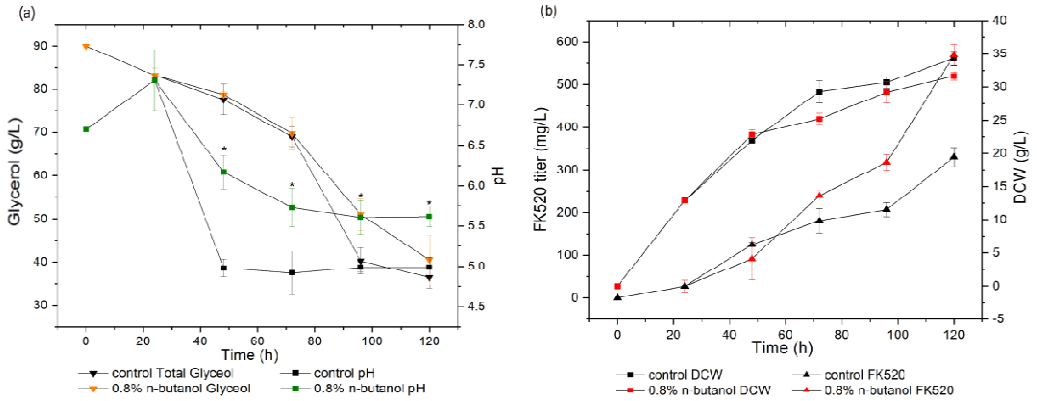

Fig. 3 Effects of n-butanol addition on FK520 production in *S. hygroscopicus var. Ascomyceticus* ATCC 14891. (a)The biosynthetic profiles of pH and glycerol supplementation with 0.8% (v/v) n-butanol at 27 h. (b)The biosynthetic profiles of biomass and FK520 supplementation with 0.8% (v/v) n-butanol at 27 h, p-values are calculated using one-way ANOVA on respective means (*p <0.05).

3.2.2 Effects of exogenous n-butanol stress to metabolism

n-butanol has high solubility in cellular membranes, reaching up to 1.59 M, and it intercalates within the membrane. This intercalation disrupts hydrogen bonds between lipid tails, resulting in changes of the cell membrane [38].

Furthermore, according to literature reports in Rutherford, BJ's work [39], oxidative stress is one of the most potent stress responses induced by the addition of n-butanol in *Escherichia coli*. In this experiment, we sampled at 2 h and 9 h after the addition of n-butanol to assess intracellular ROS changes. The results showed that after adding 0.8% n-butanol at 27 h of fermentation, intracellular ROS levels rapidly increased within 2 h, reaching 1.49×10^5 RFU/g biomass at 29 h, which is 3.02 times higher than the control. When ROS levels increased, regulatory factors involved in oxidative stress responses such as OxyR, SoxRS, RpoS, and PerR would regulate a series of reactions to eliminate the excessive ROS within the bacteria [40,41,42,43]. Therefore, we observed that the ROS levels at 36 h, while still higher than the control group, had significantly decreased compared to 29 h, indicating continuous elimination of ROS through bacterial decomposition and metabolism.

n-butanol-induced ROS also serves as a signaling molecule and plays an important role in the regulation of cell growth [44] and secondary metabolism [45]. it has been reported that ROS could stimulate the expression of key genes encoding enzymes involved in substrates such as acetyl-CoA and malonyl-CoA via the oxidative stress response [46,47], and the accumulation of acetyl-CoA and malonyl-CoA during the fermentation process was also been observed. We suppose that this accumulation may be related to the accumulation of ROS. Furthermore, Al Rowaihi IS's work suppose that ROS-induced stress may stimulate PHB production [48], which could degraded by FkbU to 3-hydroxybutyryl-CoA, and then further degraded by FkbE to ethylmalonyl-CoA in *Streptomyces hygroscopicus* ATCC 14891[49,50]. Additionally, ROS can oxidize unsaturated fatty acids, leading to the formation of aldehydes such as acrolein, which can damage proteins and DNA [51] and then cause cellular damage or even cell lysis and death. This may also be one of the reasons for the decrease in FK520 production with a higher n-butanol concentration (1.6%) (Fig. 1c).

Therefore, as n-butanol supplement during the fermentation, the n-butanol or the n-butanol-induced ROS could damage cell membranes. A measure that bacteria respond to environmental stress and membrane damage is altering membrane composition, such as synthesizing more saturated or unsaturated fatty acids [52,53]. As a precursor of intracellular fatty acids, in our opinion, the accumulation of malonyl-CoA (Fig. 5d) after the addition of n-butanol were related to changes in intracellular fatty acid composition aroused by the n-butanol or the n-butanol-induced ROS.

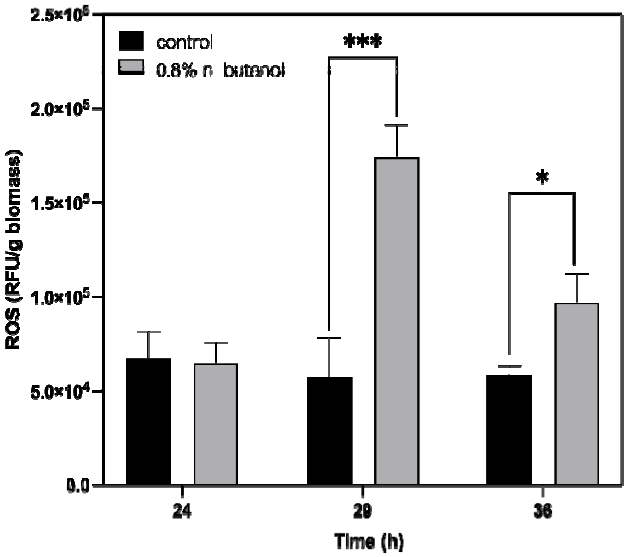

Fig. 4 Time course of ROS changing when 0.8% n-butanol were added at 27h. Values are means ± standard deviations. n = 3. p-values are calculated using one-way ANOVA on respective means (*p < 0.05, ***p < 0.001).

3.3 Effects of intracellular CoA-esters change aroused by n-butanol

Apart from its regulatory role, we hypothesize that n-butanol may also participate in the metabolic reactions of the bacteria by

promoting the accumulation of direct precursors of FK520 biosynthesis. To investigate this, we monitored the levels of several important FK520 biosynthesis precursors (methylmalonyl-CoA, ethylmalonyl-CoA, malonyl-CoA, and acetyl-CoA) within the *Streptomyces hygroscopicus* ATCC 14891 at different time during fermentation using UPLC-MS. As shown in Fig. 5, when 0.8% n-butanol was added at 27h, the content of ethylmalonyl-CoA rapidly increased, and reached the maximum level at 48h. The result suggests that n-butanol is metabolized within the bacterium to generate butyryl-CoA, which is subsequently converted into the FK520 synthesis precursor ethylmalonyl-CoA, thus facilitating FK520 biosynthesis. Then the ethylmalonyl-CoA was decrease in the 3rd day and we consider that as bacterial metabolism progresses, the supplemented ethylmalonyl-CoA was participating in bacterial metabolic reactions and caused the decrease of itself. Beside of participating in biosynthesis of FK520, ethylmalonyl-CoA also can be converted into methylmalonyl-CoA through a series of reactions(Fig. 6). Methylmalonyl-CoA serves as both a precursor for FK520 synthesis and can be further converted to succinyl-CoA, entering the TCA cycle, thereby providing energy for bacterial growth. The fluctuation in the content of ethylmalonyl-CoA, with a decrease on the 3rd day followed by an increase on the 4th day, suggested that the intracellular content of ethylmalonyl-CoA is a dynamic and changing process. In contrast to the n-butanol supplementation group, the control group exhibited stable levels of ethylmalonyl-CoA and methylmalonyl-CoA. Notably, the average content of methylmalonyl-CoA was approximately five times higher than that of ethylmalonyl-CoA in control group. It is known that FK520 biosynthesis necessitates 1mol of the precursor ethylmalonyl-CoA and 5mol of methylmalonyl-CoA. The consistent levels of ethylmalonyl-CoA and methylmalonyl-CoA in the control group suggested that the bacteria actively regulated the proportions of FK520 biosynthesis precursors. As the precursors of FK520 biosynthesis, the higher accumulation of ethylmalonyl-CoA, methylmalonyl-CoA, and malonyl-CoA in addition of n-butanol group led to significant increment of FK520 production. Furthermore, the increment of ethylmalonyl-CoA could effectively reduce impurity FK523 content [30]. Therefore, we consider n-butanol addition being exogenous precursor can better promote FK520 biosynthesis than n-propanol addition.

In comparison to increasing the flux of ethylmalonyl-CoA synthesis pathway through genetic engineering [30], the addition of n-butanol also promoted the accumulation of methylmalonyl-CoA and malonyl-CoA , which are not observed in Yu's research. This suggests that, apart from serving as a precursor supplement for ethylmalonyl-CoA, the addition of n-butanol also regulates the intracellular carbon flux towards the accumulation of other precursors in the FK520 biosynthesis. After the addition of isobutanol in *Actinosynnema pretiosum*, a significant increase in malonyl-CoA content was also been detected [16], so we suppose that short-chain alcohols supplement may promote the accumulation of malonyl-CoA in Actinomycetes. As an extension unit for the carbon skeleton, malonyl-CoA plays an important role in the biosynthesis of FK520. The changing trend of malonyl-CoA content closely mirrors the intracellular content of FK520 (Fig. 3b). Furthermore, there is a rapid increase in malonyl-CoA content from the 4th day to the 5th day, coinciding with the period of significant FK520 accumulation. Yu's work demonstrates that through genetic engineering means, increasing the malonyl-CoA content can enhance FK520 production by 73.3% [30], which means that malonyl-CoA also play important roles in biosynthesis of FK520. Acetyl-CoA is a crucial precursor metabolite involved in the biosynthesis of lipids, polyketides, isoprenoids,

amino acids, and various other bioproducts. As a central node molecule, its levels are tightly regulated by the cell's central metabolism [54]. Despite its high robustness compared to other CoA-esters, we did not observe significant changes in response to n-butanol addition. However, starting from the 3rd day, the average acetyl-CoA content was consistently higher than that of the control group, suggesting that the addition of butanol may have a discernible impact on acetyl-CoA accumulation.

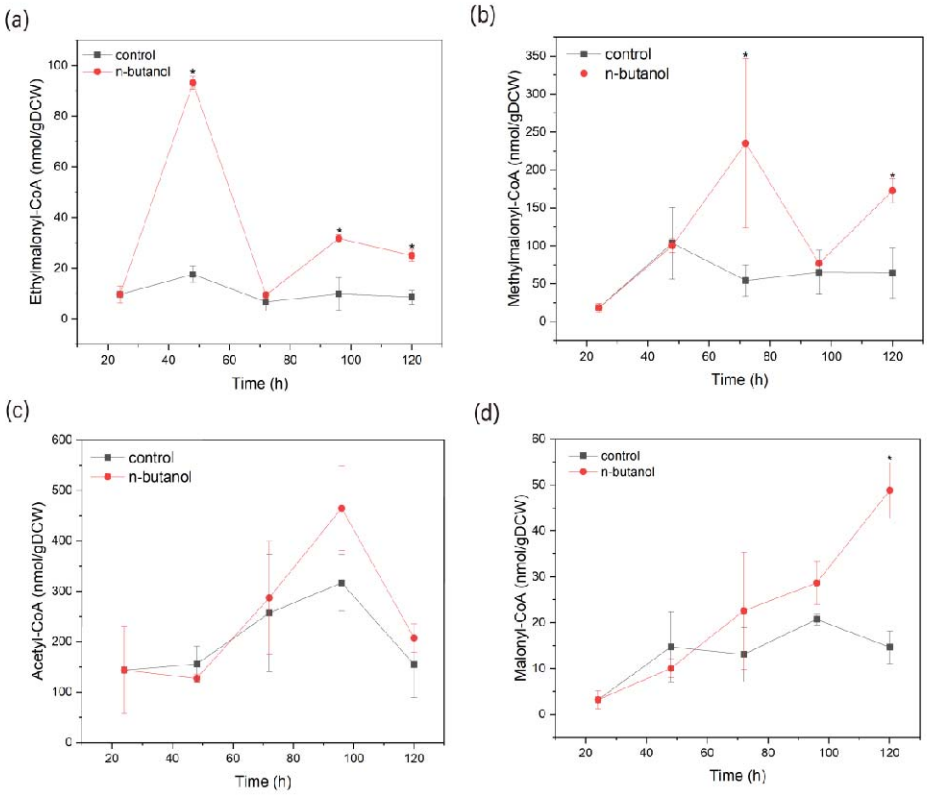

Fig. 5 Time course of concentrations of intracellular CoA-esters of in *S. hygroscopicus var. ascomyceticus* ATCC 14891 supplemented with 0.8%(v/v) n-butanol (a)Ethylmalonyl-CoA. (b)Methylmalonyl-CoA. (c)Acetyl-CoA. (d)Malonyl-CoA. Values are means ± standard deviations. n = 3. p-values are calculated using one-way ANOVA on respective means *p <0.05.

3.4 Identifying limitation factors in FK520 biosynthesis with n-butanol using Genome-Scale Metabolic Modeling(GSMM)

3.4.1 Analysis of n-butanol addition on FK520 metabolism

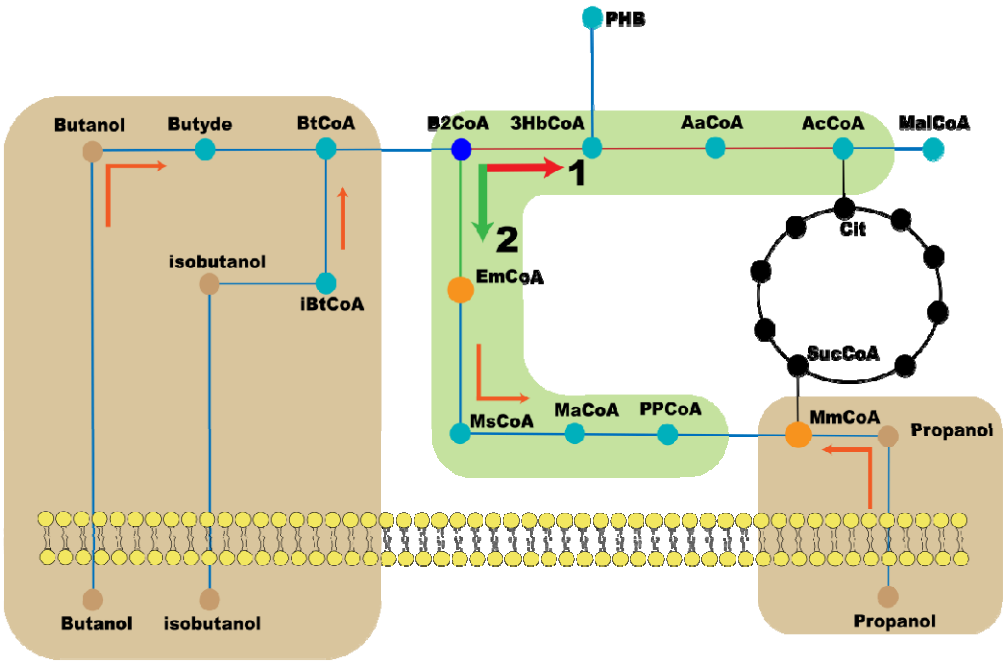

Fig. 6 Main alterations in *S. hygroscopicus var. ascomyceticus* response pathways with short-chain alcohols treatment adding n-proponal, n-butanol, and isobutanol to the cultivation medium (brown); catabolic pathway of crotonoyl-CoA (green). Butyde, butyraldehyde; btCoA, butyryl-CoA; B2CoA, Crotnnoyl-CoA; 3HbCoA, 3-hydroxybutyryl-CoA; AaCoA, acetoacetyl-CoA; cit, citric acid; EmCoA, (2S)-ethylmalonyl-CoA; MsCoA, methylsuccinyl-CoA; MaCoA, (2R,3S)-β-methylmalyl-CoA; PPCoA, propionyl-CoA; MmCoA, (2S)-methylmalonyl-CoA; SucCoA, succinylCoA; MalCoA, malonyl-CoA;

Due to the complexity of intracellular metabolism, which is regulated by various factors, the mere supplementation of precursor substances does not result in an unlimited increase in FK520 production. To further analyze the impact of n-butanol addition on the metabolic network of *S. hygroscopicus var. ascomyceticus* ATCC 14891, we utilized a previously reported Genome-Scale Metabolic Model (GSMM) IMK1208 [28] to analyze the metabolic changes with n-butanol addition. Through *in silico* experiments, we aimed to find the limitation factors in FK520 biosynthesis with n-butanol, providing insights for the optimization and scale-up of FK520 fermentation processes.

Because of the high conservation of enzymes in different Streptomyces species [28], we could refer to the metabolic pathway of n-butanol in *Streptomyces azureus* A3. According to report in Rauter, M's work[55], alcohol dehydrogenase (Aadh2p) from *Arxula*

adeninivorans, identified as a Zn^{2+}-binding NAD-dependent alcohol dehydrogenase, exhibited the highest oxidation activity with 1-butanol as a substrate.

Using NCBI Blast comparison, we aligned the sequence of this enzyme with the Zn^{2+}-binding alcohol dehydrogenase in *Streptomyces coelicolor* A3(2), with an identity of 27% and a score of 96.7 (Fig. 7a). Therefore, we suppose that in *Streptomyces coelicolor* A3(2), n-butanol may be converted to n-butanal through alcohol dehydrogenase and then further transformed into butanoyl-CoA under the action of acetaldehyde dehydrogenase(Bentley, S[56]). The reactions were been incorporated into the GSMM model.

The robustness of n-butanol supplementation was analyzed on the specific FK520 synthesis rate using GSMM. The results indicate that when the n-butanol addition rate is 0 mmol/gDCW/h, the shadow price of n-butanol is 0.0181 (Fig. 7b). This suggests that an increase of 1 mmol/gDCW/h in the n-butanol uptake rate will lead to an increase of 0.018 mmol/gDCW/h in FK520 synthesis rate. As the n-butanol uptake rate increases, the relative FK520 synthesis rate continuously rises (Fig. 7b). Notably, the shadow price of n-butanol are changing with n-butanol uptake rate change, and when n-butanol uptake rate is 2 mmol/gDCW/h and 10 mmol/gDCW/h, the shadow price of n-butanol is 0.0159 and 0.0136, respectively. This demonstrates that n-butanol supplementation, as a precursor for FK520 synthesis, effectively promotes FK520 production. However, in actual situations, n-butanol supplementation does not result in unlimited FK520 synthesis. Yu's work indicates that the fermentation of FK520 in *S. hygroscopicus* is severely constrained by oxygen level and integrating the Vitreoscilla hemoglobin gene (*vgb*) into the production strain *S. hygroscopicus* can alleviate oxygen limitations during fermentation and increase FK520 yield[57]. Therefore, it is necessary to restrict the simulated oxygen uptake rate to mimic real conditions. Simultaneously, we analyze the impact of oxygen uptake rate and n-butanol uptake rate on FK520 synthesis. We employed phenotype phase plane analysis to investigate the interaction between these two parameters and their effect on FK520 specific synthesis rate [58]. For detailed execution code, please refer to Supplementary Material (S2). From Fig. 7c, it can be observed that this surface plot contains two distinct regions, each representing a different phenotype. The characteristic of plane I is that when oxygen supply is sufficient, FK520 specific synthesis rate is limited by the n-butanol uptake rate. As the n-butanol uptake rate increases, FK520 specific synthesis rate also increases. Plane II, on the other hand, exhibits a different behavior. When oxygen is limited, the FK520 specific synthesis rate initially increases and then decreases with increasing n-butanol uptake rate. Analysis of the flux distribution simulated by GSMM reveals that when oxygen content is constant, the excessive n-butanol addition will lead to insufficient carbon sources to maintain the desired biomass. At this point, a large amount of n-butanol is converted into actyl-CoA and enters the TCA cycle (Pathway 1 in Fig. 6) as the second carbon source to support cell growth. This reduces the flux toward the synthesis of ethylmalonyl-CoA. According to the analysis of plane I and plane II, we found that a higher oxygen uptake rate facilitates greater utilization of n-butanol in cellular metabolism for ethylmalonyl-CoA synthesis, and improving oxygen supply is a crucial factor in promoting cell metabolism for FK520 biosynthesis.

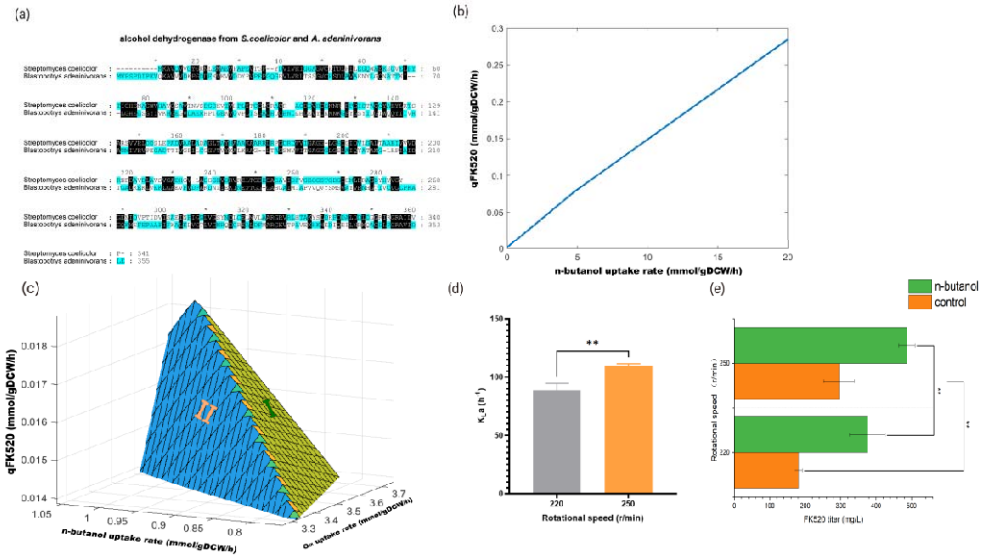

Fig. 7 (a) Homology analysis of alcohol dehydrogenase from *S.coelicolor* and *A. adeninivorans*. (b)Robustness analysis of the n-butanol uptake rate to the specific FK520 synthesis rate with infinity oxygen. (c)Phenotypic phase planes analysis for growth with varying n-butanol and oxygen uptake rates. (d)K_La value during different rotational speed. (e)FK520 titer in different rotational speed with and without 1.2% n-butanol. Values are means ± standard deviations. n=3. p-values are calculated using Student's t-test on respective means **p <0.01.

3.4.2 Effects of different rotational speed on FK520 in shake flask fermentation

In flask fermentation, common methods to enhance oxygen supply include using baffled shake flasks and increasing the shaking speed. However, we found that the use of baffled flasks could lead to splashing of the fermentation liquid and greater evaporation. Therefore, we opted to increase the rotational speed to improve oxygen supply capacity in flask fermentation. We raised the rotational speed from 220 r/min to 250 r/min and gauged the K_La of the flask to assess oxygen supplement of flasks [21]. As shown in Fig. 7c and Fig. 7d, when we increased the rotational speed to 250 r/min, the K_La value of the shake flask reached 109.5 h^{-1}, representing a 24% increase compared to the control. Under this condition, we observed significant increases in FK520 production both with and without the addition of n-butanol, which confirm the results of stimulation. In the group without n-butanol addition, FK520 production reached 295.2 mg/L, a 63.2% increase compared with the oxygen-limited group. In the group with 1.2% butanol addition, FK520 production reached 486.77 mg/L, a 29.6% increase compared to the oxygen-limited group. Furthermore, our simulations suggest that, besides improving the oxygen supply capacity during the fermentation process, controlling the feed rate of n-butanol is another method to regulate its metabolic flow towards ethylmalonyl-CoA synthesis, thereby promoting FK520 biosynthesis. However, controlling the feed rate of n-butanol in flask fermentation presents challenges, so this method requires further investigation in subsequent bioreactor

fermentation.

4. Conclusion

The addition of 0.8% n-butanol regulates the carbon flux within the *S. hygroscopicus var. ascomyceticus* ATCC 14891 towards secondary metabolic reactions, significantly promoting the accumulation of FK520. Besides that, it also directly contributes to the significant supplementation of three key precursor, including ethylmalonyl-CoA, methylmalonyl-CoA, and malonyl-CoA, for FK520 biosynthesis. Through our experiments *in silico*, we found that the insufficient oxygen supply in shake flask fermentation is one of the limiting factors to improve the FK520 production and it's essential to provide sufficient oxygen conditions so that the bacteria can convert more n-butanol into the FK520 biosynthesis precursor, ethylmalonyl-CoA. Our work provides valuable insights for optimizing the fermentation process of FK520 in the future.

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CRediT authorship contribution statement

JM: Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Data Curation Software, Writing - Original Draft. QC: Investigation ; DZ and SG: Supervision, Fund acquisition, Writing - Review & Editing. All authors read and approved the final manuscript.

Declaration of competing Interest

The authors declare no conflict of interest. Neither ethical approval nor informed consent was required for this study.

Data availability

Data will be made available on request.

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