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Enhancement of ascomycin production in *Streptomyces hygroscopicus* var. *ascomyceticus* by combining resin HP20 addition and metabolic profiling analysis

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Abstract Combinatorial approach of adsorbent resin HP20 addition and metabolic profiling analysis were carried out to enhance ascomycin production. Under the optimal condition of 5 % m/v HP20 added at 24 h, ascomycin production was increased to 380 from 300 mg/L. To further rationally guide the improvement of ascomycin production, metabolic profiling analysis was employed to investigate the intracellular metabolite changes of *Streptomyces hygroscopicus* var. *ascomyceticus* FS35 in response to HP20 addition. A correlation between the metabolic profiles and ascomycin accumulation was revealed by partial least-squares to latent structures discriminant analysis, and 11 key metabolites that most contributed to metabolism differences and ascomycin biosynthesis were identified. Based on the analysis of metabolite changes together with their pathways, the potential key factors associated with ascomycin overproduction were determined. Finally, rationally designed fermentation strategies based on HP20 addition were performed as follows: 2 % v/v *n*-hexadecane was added at 24 h; 1.0 g/L valine was supplemented at 48 h; 1.0 g/L lysine was added at 72 h. The ascomycin production was ultimately improved to 460 mg/L, a 53.3 % enhancement compared with that obtained in initial condition. These results demonstrated that the combination of HP20 addition and metabolic profiling analysis could be successfully applied to the rational guidance of production improvement of ascomycin, as well as other clinically important compounds.

Keywords Ascomycin · *Streptomyces hygroscopicus* var. *ascomyceticus* · Adsorbent resin HP20 · Metabolic profiling analysis · Fermentation optimization

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Introduction

Ascomycin is a 23-membered macrocyclic natural product that was biosynthesized by *Streptomyces hygroscopicus* var. *ascomyceticus* ATCC 14891 and *S. hygroscopicus* subsp. *yakushimaensis* No. 7238. Ascomycin and its analogues displayed biological and pharmacological activities, such as antifungal, immunosuppressive [9], antimalarial [23], antispasmodics [31], nerve regeneration and functional recovery [28]. Ascomycin is also an interesting precursor of the chemical synthesis of pimecrolimus which has been proven to be effective in curing various inflammatory skin diseases [4, 11, 13]. Due to the pharmaceutical potential and clinical importance described above, ascomycin and its analogues have aroused great interest of researchers.

In order to meet the demand for ascomycin, effort has been made to increase ascomycin yield through strain

improvement and fermentation development. For example, the production of ascomycin was increased to 400 mg/L by *S. hygroscopicus* var. *ascomyceticus* ATCC 14891 through optimizing the fermentation parameters [22]. The titer of 13-desmethoxy-13-methyl-ascomycin was tripled by selecting an excellent fermentation medium and decreasing the dissolved oxygen [27]. A high-yield ascomycin-producing strain *S. hygroscopicus* var. *ascomyceticus* FS35 was isolated, achieving ascomycin production up to 300 mg/L after medium optimization [25]. A shikimic acid-resistant strain *S. hygroscopicus* var. *ascomyceticus* SA68 was obtained with ascomycin production of 450 mg/L after shikimic acid addition [26]. One simple and effective method to optimize the fermentation process is in situ product adsorption, which could be used to enhance the natural product fermentation titers in a relatively short span of time, and many examples had been presented and summarized by Phillips et al. [24]. In our preliminary experiments, we found the positive effect of adsorbent resin HP20 addition on ascomycin production by *S. hygroscopicus* var. *ascomyceticus* FS35. However, adsorbent resin addition for the enhancement of target product reported previously mainly focused on the adsorption capacity or the apparent adsorption effect [17, 32, 40], and little was known about the intracellular metabolic change. The potential mechanism of adsorbent resin HP20 addition in improving ascomycin production is ambiguous, which limits the further improvement of ascomycin production. Thus, it is of vital importance to investigate the metabolic change for the higher ascomycin titer.

So far, the genome sequences of *S. hygroscopicus* ATCC14891 and the regulatory mechanism of ascomycin biosynthesis have not been revealed. As a secondary metabolite, the direct biosynthetic precursors of ascomycin are composed of (4*R*,5*R*)-4,5-dihydroxycyclohex-1-enecarboxylic acid, pipecolic acid, malonyl-CoA, methylmalonyl-CoA, methoxymalonyl-ACP, and ethylmalonyl-CoA. These precursors involve multiple primary metabolic pathways, such as glycolysis (EMP), pentose-phosphate pathway, tricarboxylic acid cycle (TCA), fatty acids, shikimic acid and amino acids metabolism [2, 35]. Thus, the change profiles of intracellular metabolites related to the above pathways are crucial for revealing the potential key factors of higher ascomycin biosynthesis. Metabolomics, focusing on comprehensive metabolites analysis in a biological system, can provide unique insights into the metabolism of cell [30]. Many researchers have employed it to identify metabolites which were depleted or accumulated during cultivation, thus developing strategies to optimize process and increase product titers [8]. For example, metabolomics was carried out to investigate the underlying metabolic mechanisms of the limited production performance of recombinant protein in *Bacillus megaterium* under large-scale conditions [15]. Metabolic profiling analysis revealed that light intensity was a vital limiting factor of lipid accumulation in high initial cell density culture by *Chlorella sorokiniana* [18]. Metabolic profiling analysis was employed to rationally guide culture medium optimization for target production improvement [34, 36, 39]. To our knowledge, there is no report of the metabolic profiling-based investigation on identifying the potential key factors influencing ascomycin overproduction under the condition of adsorbent resin HP20 addition.

In the present study, the adsorbent resin HP20 addition was used to improve ascomycin titer. Furthermore, a metabolic profiling approach based on GC–MS and LC–MS/MS was employed to characterize the intracellular metabolite changes in response to HP20 addition. The changes of these metabolites combined with relevant pathways were evaluated and the potential key factors associated with ascomycin overproduction were determined. Finally, rational fermentation optimization strategies were proposed towards efficient enhancement of ascomycin production.

Materials and methods

Chemical reagent

Except for those noted, reagents and solvents were purchased from Sigma Chemical Company (St. Louis, MO), Fluka Chemie (Buchs, Switzerland) or Junsei Chemicals (Tokyo, Japan) at the highest grade commercially available.

Strain, medium, and culture conditions

Streptomyces hygroscopicus var. *ascomyceticus* FS35 isolated after femtosecond laser irradiation [25] was used throughout this work. The slant, plate and seed medium were prepared as described before as well as the culture conditions [26]. The fermentation medium contained 24 g/L soluble starch, 40 g/L dextrin, 5.0 g/L peptone, 7.0 g/L yeast powder, 2.0 g/L corn steep liquor, 11 mL/L soybean oil, 1.5 g/L shikimic acid, 0.5 g/L $K_2HPO_4{\cdot}3H_2O$, 1.5 g/L $(NH_4)_2SO_4$, 1.0 g/L $MgSO_4{\cdot}7H_2O$, and 1.0 g/L $CaCO_3$. In addition, the above reagents were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China) or Tianjin GuangFu Fine Chemicals (Tianjin, China).

In this study, 5 % (w/v) wet HP20 was added after 24 h of initial cultivation, which was the optimal condition for HP20 supplementation (the optimization process were not shown). Diaion HP20 (Mitsubishi Chemical Industries Limited, Tokyo, Japan) was purchased through a vendor. Prior to use, the adsorbent resin was soaked and swelled in 100 % (v/v) methanol for 12 h to remove impurities and

the solvent was removed by washing with distilled water sufficiently. Following the washes, activated resin was immersed in fresh distilled water and stored at 4 °C until needed. Slurry of the activate resin was filtered onto a filter, then the wet resin was weighed and sterilized at 121 °C for 30 min and added to each flask at the selected time.

Analytical methods

For determining the dried cell weight (DCW), the culture sample was passed through a 0.3 mm stainless steel sieve to separate resin, and then centrifuged at 5,000×*g* for 5 min. The mycelium was washed once with 0.1 M HCl solution and twice with sterile saline water, centrifuged again and dried to constant weight at 80 °C. To detect the concentration of ascomycin with resin HP20 addition, the broth was pretreated according to the previous report with minor changes [26]. In brief, 10 mL of the whole broth from each flask was collected into a 50-mL polypropylene tube and centrifuged at 10,000 rpm for 10 min. The supernatant was poured off, and 10 ml of ethyl acetate was added to the tube containing the pellet (cells and resin). Extraction was performed by vortexing vigorously for 30 min followed by centrifugation, a portion (100 μL) of the extract was transferred to a small microfuge tube, chilled at −80 °C for 30 min, and the solvent was evaporated under vacuum for 30 min. Dried extract was dissolved into 100 μL of methanol and analyzed using high-performance liquid chromatography as described by Qi et al. [25, 26]. The total residual sugar was detected as described before [26].

Determination of intracellular metabolites

To profile the intracellular metabolite pools of *S. hygroscopicus* var. *ascomyceticus* FS35 in response to HP20 addition, samples for metabolic analysis were drawn at 60 h (the late exponential phase, exhibiting noticeable difference of ascomycin yield) and 120 h (the late stationary phase, displaying rapid biosynthesis of ascomycin). Sample quenching and extraction of intracellular metabolites for GC–MS and LC–MS/MS analysis were carried out according to the methods described in detail before [36]. Parameter settings of GC–MS system and LC–MS/MS were also consistent with Xia et al. [36].

Data processing and statistical analysis

Each experiment data was obtained from five biological repeats for each treatment. The error bars represented the standard deviations.

The area of each metabolite peak generated by GC–MS and LC–MS/MS was normalized with the internal standard and cell biomass, and after being handled by mean-centering and scaling the obtained data (variables) were imported into SIMCA package (Ver 11.5; Umetrics AB, Sweden) for partial least-squares to latent structures discriminant analysis (PLS-DA). In PLS-DA model, *X* matrix meant the metabolites and *Y* matrix represented ascomycin productivity. The parameters $R^2(X)$, $R^2(Y)$, and $Q^2(Y)$ with the range of 0–1 evaluate the quality of PLS-DA model, and the higher the value of three parameters, the more explanation and reliable predictive ability the model has. The variable importance in the projection (VIP) index was also introduced in order to quantitatively estimate the contribution of each metabolite variable to the pattern recognition in PLS-DA model.

Results and discussion

Fermentation performance comparison with and without HP20 addition

To improve the production of ascomycin, in situ adsorption optimization with adsorbent resin HP20 addition was performed, and the optimal conditions were obtained as 5 % (m/v) HP20 added at 24 h. Ascomycin concentration, biomass and residual sugar concentration with and without HP20 addition were detected and showed in Fig. 1. Strain FS35 displayed the same fermentation periods divided into lag phase (Phase I, 0–24 h), exponential phase (Phase II, 24–72 h), and stationary phase (Phase III, 72–144 h) based on strain growth (Fig. 1). The optimal addition time (24 h) for HP20 was the start time of exponential phase. To investigate the effect of resin HP20 on the fermentation properties, the fermentation parameters with and without HP20 addition in Phase II and Phase III were comparatively analyzed. The samples without HP20 addition were regarded as the control.

In the exponential phase (Phase II, 24–72 h) (Fig. 1), the biomass both increased rapidly and reached the peak of 23.5 g/L (with HP20 addition) and 21 g/L (the control) at 72 h, respectively. The sugar consumption rate increased in this period with the average sugar consumption rates of 0.85 g/L/h (with HP20 addition) and 0.72 g/L/h (the control), respectively. Ascomycin was all biosynthesized at the mid-exponential phase (48 h) in both conditions, but the synthetic rate of ascomycin with HP20 addition was significantly higher than that in the control, and the production of ascomycin was accumulated up to 65 mg/L (with HP20 addition) and 43.4 mg/L (the control), respectively, at 72 h. During the stationary phase (Fig. 1), the biomass in the control gradually declined from 21 g/L at 72 h to 17 g/L at 144 h, while with HP20 addition the biomass displayed no obvious change (from 23.5 g/L at 72 h to 21.6 g/L at 144 h). The sugar consumption rates were

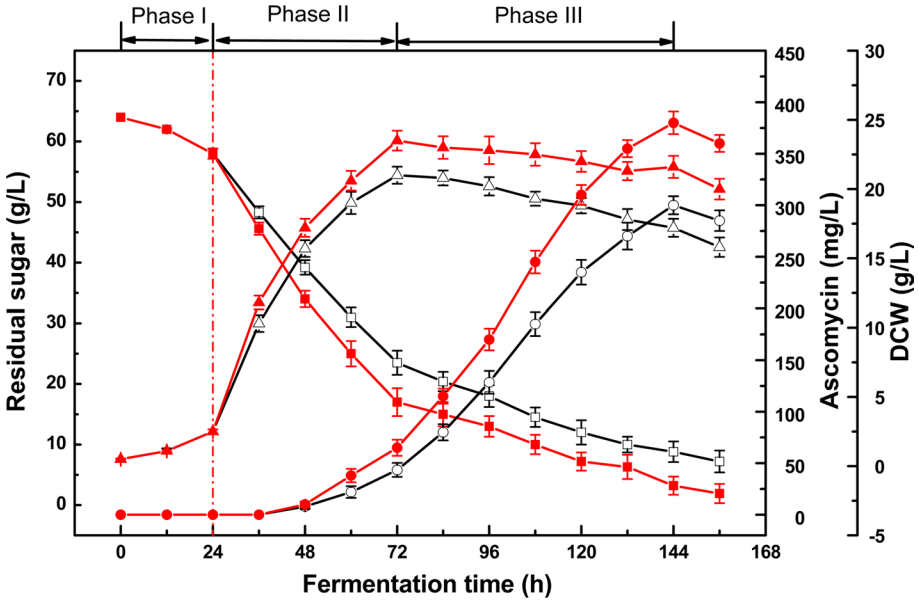

Fig. 1 Fermentation properties of FS35 supplemented with and without resin HP20. *Solid*, *red* symbols and *open*, *black* symbols represent the samples with HP20 and without HP20 (the control), respectively. *Square* total sugar, *triangle* DCW, *circle* ascomycin production. Phase I, lag phase; Phase II, exponential phase; Phase III, stationary phase. The *red dashed line* indicates the adding time point of resin HP20 (color figure online)

reduced compared with that in the exponential phase, both about 25 % of that in the exponential phase. Most importantly, ascomycin was mainly biosynthesized in this phase, with the average production rates of 4.4 mg/L/h with HP20 addition, up to 380 mg/L at 144 h, a 26.7 % improvement compared with that of the control (300 mg/L).

Taking the above results, strain FS35 exhibited positive fermentation behaviors for ascomycin biosynthesis with HP20 addition. However, it is difficult to determine the underlying factors that influence ascomycin production based on the above analysis. Hence, it is necessary to fully investigate the changes of intracellular metabolites as well as the strain metabolism discrimination between the two conditions. For this purpose, multivariate statistical analysis using PLS-DA on the intracellular metabolite profile was conducted.

Identification of key metabolites and pathways influencing ascomycin biosynthesis

To get deep insights into the different ascomycin production capabilities observed above, intracellular metabolites alterations caused by HP20 addition were analyzed by GC–MS and LC–MS/MS. A total of 83 compounds were identified, including organic acids, amino acids, sugars, phosphate compounds and other compounds (Supplementary Table S1). These metabolites were involved in EMP, TCA cycle, energy and amino acids metabolism, etc., effectively reflecting the intracellular metabolic characterization of the strain FS35. PLS-DA was carried out evaluate the correlation between metabolism and ascomycin productivity, and identify the potential key metabolites responsible for the accumulation of ascomycin. The R^2X (cum), R^2Y (cum) and Q^2 (cum) of acquired PLS-DA model were 0.862, 0.934 and 0.864, respectively, indicating the reliability of PLS-DA analysis.

In the PLS-DA score plot t [1]/u [1] (Fig. 2a), the samples under different conditions as well as in different fermentation phases displayed a clear clustering. The samples from resin HP20 addition group at 120 h were located in the upper right quadrant due to the relatively higher ascomycin yield, while the other samples were located in the lower left quadrant of the plot. The significant discrimination on the PLS-DA score plot profiling revealed the strong relationships between metabolism and ascomycin accumulation, and also reflected the metabolic discrimination of the strain FS35 cultured with and without HP20 addition. The VIP plot demonstrated the contribution of each metabolite identified to sample clustering and ascomycin accumulation (Fig. 2b). Generally, a metabolite with VIP value greater than one indicates a significant contribution to sample clustering within PLS-DA model, and the higher the VIP value of a metabolite, the more contribution it has [14]. The analysis of PLS-DA revealed 11 key metabolites with VIP values greater than one (Fig. 2b). These metabolites (VIP >1) were mainly involved in EMP, TCA cycle, energy and amino acids metabolism. The schematic representation of the metabolic network consisting of the primary metabolism pathways mentioned above is presented in Fig. S1. To investigate the factors of higher ascomycin production with HP20 addition, the metabolites with VIP value greater than one and the related metabolic pathways would be discussed in detail as follows.

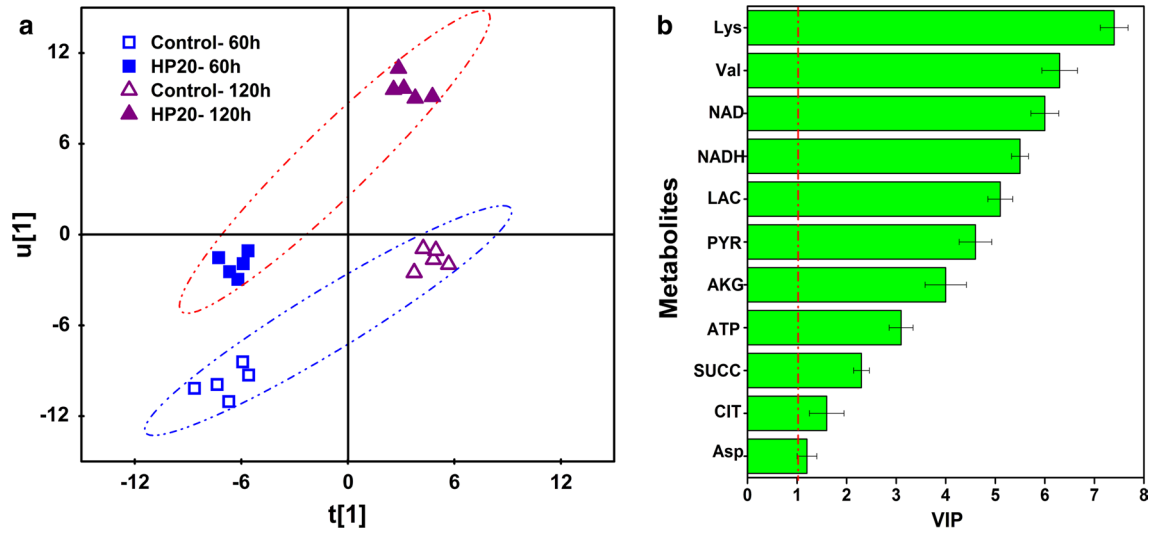

Fig. 2 PLS-DA analysis of intracellular metabolites. **a** The score plots of PLS-DA. **b** The VIP plot of PLS-DA. *AKG* α-ketoglutarate, *Asp* aspartate, *ATP* adenosine-5-triphosphate, *CIT* citrate, *LAC* lactate, *Lys* lysine, *NAD* nicotinamide adenine dinucleotide, *NADH* reduced nicotinamide adenine dinucleotide, *PYR* pyruvate, *SUCC* succinate, *Val* valine

Central carbon metabolism and energy metabolism

The change of metabolites in central carbon metabolism pathways for strain FS35 was shown in Fig. 3a. The accumulated concentrations of pyruvate, citrate, α-ketoglutarate and succinate under HP20 addition condition were all higher, displaying 32, 30, 28, and 25 % improvement at 60 h (the late exponential phase), respectively. Subsequently, in the late stationary phase, the relative abundance of these metabolites reduced sharply, especially α-ketoglutarate and succinate declined by 70 and 58 % under HP20 addition condition and 40 and 60 % in the control, respectively. The reduction of these metabolites above at the end of fermentation may be due to the strain metabolic activity becoming weak, and the more significant decrease of α-ketoglutarate and succinate may be also caused by the fact that they could be converted into more precursors of secondary metabolites in the stationary phases [36, 39]. However, compared with the control, the relative abundance of above metabolites was still higher under HP20 addition condition, which indicated that the activities of EMP and TCA cycle for strain FS35 were enhanced. The enhanced central carbon metabolism could provide more cell materials and energy like ATP and NADH for growth, which also could be demonstrated by the change of biomass (Fig. 1). However, contrary to the above phenomenon, the relative abundance of lactate declined with the addition of HP20, presenting 80 % reduction in the late exponential phase and 50 % in the late stationary phase, respectively, compared with that of the control. Lactate is a by-product produced by the strain under the oxygen limitation condition. The accumulation of lactate in cell could lead to the acidification of cytoplasm and the aggregation of intracellular acid radical ion, inhibiting enzyme activity related to glycolysis [19] and impairing cell growth and product formation.

Besides, the relative abundance of NADH and ATP was significantly higher with HP20 addition, showing a 24 and 50 % increment at 120 h, respectively (Fig. 3b), which directly reflected the higher activity of strain in the late stationary phase. Moreover, NAD, playing a central role in cellular metabolism by functioning as a cofactor in over 300 red-ox reactions [12], was improved by 60 % in the later fermentation with resin HP20 addition, which was consistent with the previous report about the positive correlation between intracellular nucleotide phosphate concentration and strain growth rate [21]. Notably, the ratio of NADH/NAD^+ was enhanced to 1.2 at 120 h from 0.8 at 60 h in the control, while with the HP20 addition, it was improved to 0.92 from 0.75 and the maximal ratio was reduced by 30 %. NADH was the main source of ATP, and the ratio of NADH/NAD^+ was lower one in the normal metabolism of strain. Moreover, the high ratio of NADH/NAD^+ would repress the activity of citrate synthase [3] and pyruvate dehydrogenase complex [7], resulting in the reduction of TCA cycle activity in the late stationary phase (Fig. 3a). Under aerobic conditions, NADH was oxidized to NAD^+ through electron transport chain with molecular oxygen as the terminal electron acceptor in microorganism, while under oxygen-limited conditions the oxidation of NADH is mainly through the fermentation pathway. Compared with electron transport chain, the oxidation efficiency of NADH through the fermentation pathway is lower, leading to the increment of intracellular NADH/NAD^+ ratio [38].

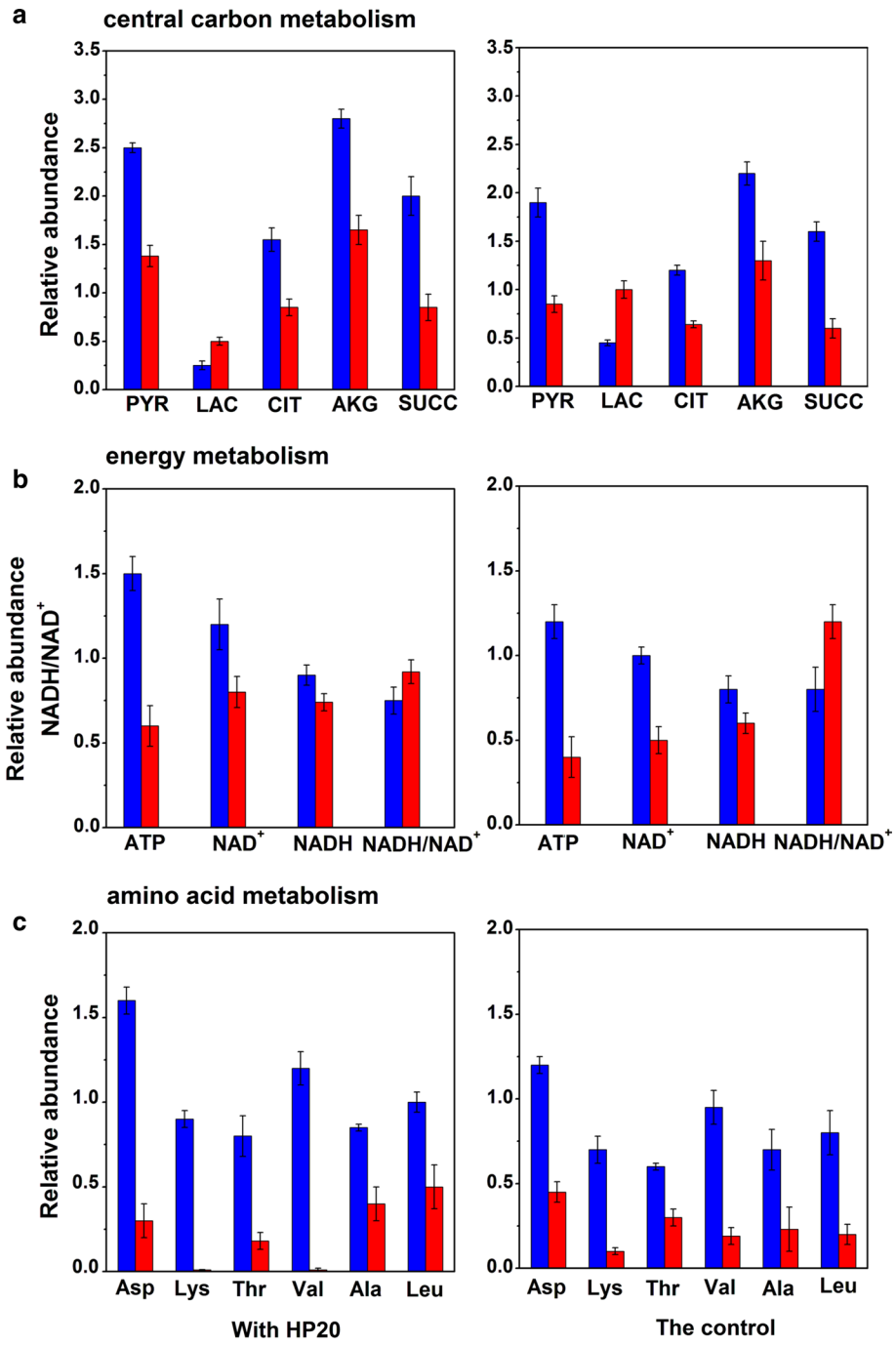

Fig. 3 The relative abundances of intracellular metabolites from central carbon metabolism (**a**), energy metabolism (**b**) as well as amino acid metabolism (**c**) in different fermentation phases. The *blue* and *red* bar charts represent the samples drawn at 60 and 120 h, respectively. *Ala* alanine, *Leu* leucine, *Thr* threonine (color figure online)

Combined with the analysis of the central carbon metabolism and energy metabolism, it was hypothesized that the metabolic differences with and without HP20 addition might be related to the dissolved oxygen in fermentation broth. In the aerobic fermentation, oxygen is essential for cell growth and maintenance [10], and the availability of oxygen strongly affected cell metabolism pathways and metabolic flux [5]. Moreover, it was reported that the morphology was affected by the change of dissolved oxygen [10, 37]. When a pellet reaches a certain critical size, oxygen limitation within the core of pellet occurs, resulting in cell lysis and finally influencing cell growth and target product formation. To verify this speculation, the effect of dissolved oxygen change in fermentation broth on the product formation would be evaluated next.

Amino acid metabolism

As shown in Fig. 3c, after the addition of resin HP20 the levels of amino acids were significantly higher than that

in the control at 60 h, displaying a 33, 29, 26, 34, 28 and 22 % improvement for aspartate (Asp), lysine (Lys), valine (Val), threonine (Thr), alanine (Ala) and leucine (Leu), respectively, but comparable or lower than that in the control at 120 h. These amino acids above were generated from EMP or TCA cycle (Fig. S1), the activities of which were enhanced with HP20 addition (Fig. 3a), thus their level was higher after resin HP20 addition in the exponential phase. Thereinto, Asp, Lys and Val were considered as the key metabolites closely associated with ascomycin biosynthesis (Fig. 2b), and their levels dropped rapidly during the phase of ascomycin biosynthesized quickly, showing a 63, 86 and 80 % reduction in the control, respectively. Under HP20 addition condition, the level of Asp was decreased by 80 % and the levels of Lys and Val were all reduced to about 0.

Besides being used for strain growth and protein synthesis, these amino acids could be used to synthesize precursors of many polyketide compounds, such as CoA-esters [33]. The Val from EMP could be converted into methylmalonyl-CoA, one of important precursors of ascomycin [35] (Fig. S1). Under the addition of HP20 condition, the level of Val was rapidly reduced to 0 at 120 h, while levels of Ala and Leu were improved by 74 and 150 %, respectively, compared with the control. This phenomenon indicated that the lack of Val which was transformed into methylmalonyl-CoA may be a potential rate-limiting factor for ascomycin biosynthesis with HP20 addition. Besides, Asp from TCA cycle could be converted into Thr and Lys (Fig. S1), and the reduction of Asp, Thr and Lys levels in the late fermentation phase may be related to the reduced activity of TCA cycle in both conditions. Although the activity of TCA cycle was higher with the HP20 addition, the levels of three amino acids above were lower than the control, especially the level of Lys rapidly reducing to about 0, which may be associated with the rapid synthesis of ascomycin during this phase. Because the heterocyclic ring of ascomycin derive from pipecolic acid, the catabolite of Lys [2], and the rapid synthesis of ascomycin need to consume plentiful pipecolic acid, resulting to quick consumption of Lys. Under the addition of HP20 condition, more Lys in this phase was transformed into heterocyclic ring unit for ascomycin biosynthesis, leading to its level being reduced to 0, which may be another key factor for higher ascomycin biosynthesis.

Taken together, it was speculated that under the addition of HP20 condition the poor intracellular levels of Val and Lys were the potential rate-limiting factors for higher ascomycin biosynthesis. Therefore, the rational supplement of Val and Lys in the fermentation of ascomycin would be performed to promote the production of ascomycin.

Improved strategies based on metabolic profiling analysis

According to the metabolic profiling analysis involved in central carbon metabolism, energy metabolism and amino acids metabolism, the following rational strategies on the basis of resin HP20 addition were carried out to improve ascomycin production and evaluate the above speculations.

Oxygen supply strategies were carried out based on the above analysis of central carbon metabolism and energy metabolism. Oxygen is only sparingly soluble in culture medium and in liquids in general, ensuring a sufficient oxygen transfer to *Streptomyces* appears critical [20]. Compared with the conventional techniques to improve oxygen supply such as increasing agitation or aeration rate, raising the oxygen partial pressure in the gas phase, another simple and environmentally friendly way is the addition of oxygen vectors in fermentation medium [16]. The mechanism of enhanced oxygen transfer rate from the gas phase to the microorganisms by oxygen vectors has been elucidated [1, 29]. Here, *n*-hexadecane was used as oxygen carrier to increase the oxygen concentration in fermentation medium and as a consequence, enhances cell growth rates and the yields of ascomycin products. 1 % (v/v, similarly hereinafter), 2, 3 and 4 % *n*-hexadecane was supplemented, respectively, on the basis of HP20 addition, and the addition time (0, 24, 48, 72 and 9 6 h) was also optimized, the results are shown in Fig. 4a. 2 % *n*-hexadecane added at 24 h presented the optimal effect on ascomycin production which was improved to 435 from 380 mg/L, displaying a 14.5 % improvement. Meanwhile, the *S. hygroscopicus* var. *ascomyceticus* FS35 showed a 16 % increase in ascomycin production with 2 % *n*-hexadecane added at 24 h only compared to the control (Fig. 4d), which also proved the correctness of the above analysis.

Taking consideration of the above amino acids analysis, the addition of Val and Lys was performed on the basis of HP20 addition. The addition concentration and time for Val was optimized, as well as Lys, and results were illustrated in Fig. 4b, c, respectively. 1.0 g/L Val added at 48 h displayed the best effect, and ascomycin production was enhanced to 415 from 380 mg/L, a 9.2 % enhancement (Fig. 4b). When 1.0 g/L Lys was added at 72 h, the production of ascomycin was increased to 430 from 380 mg/L, approximately a 13.2 % increment. These results further demonstrated that the insufficiency of Val and Lys was the limiting factors for higher ascomycin biosynthesis by *S. hygroscopicus* var. *ascomyceticus* FS35 with HP20 addition. Furthermore, the results obtained here were in accordance with the previous reports [36, 39], in which the exogenous Val and Lys were used to promote the production of the ascomycin analogues tacrolimus and rapamycin, respectively. However, the *S. hygroscopicus* var. *ascomyceticus* FS35 exhibited similar ascomycin production

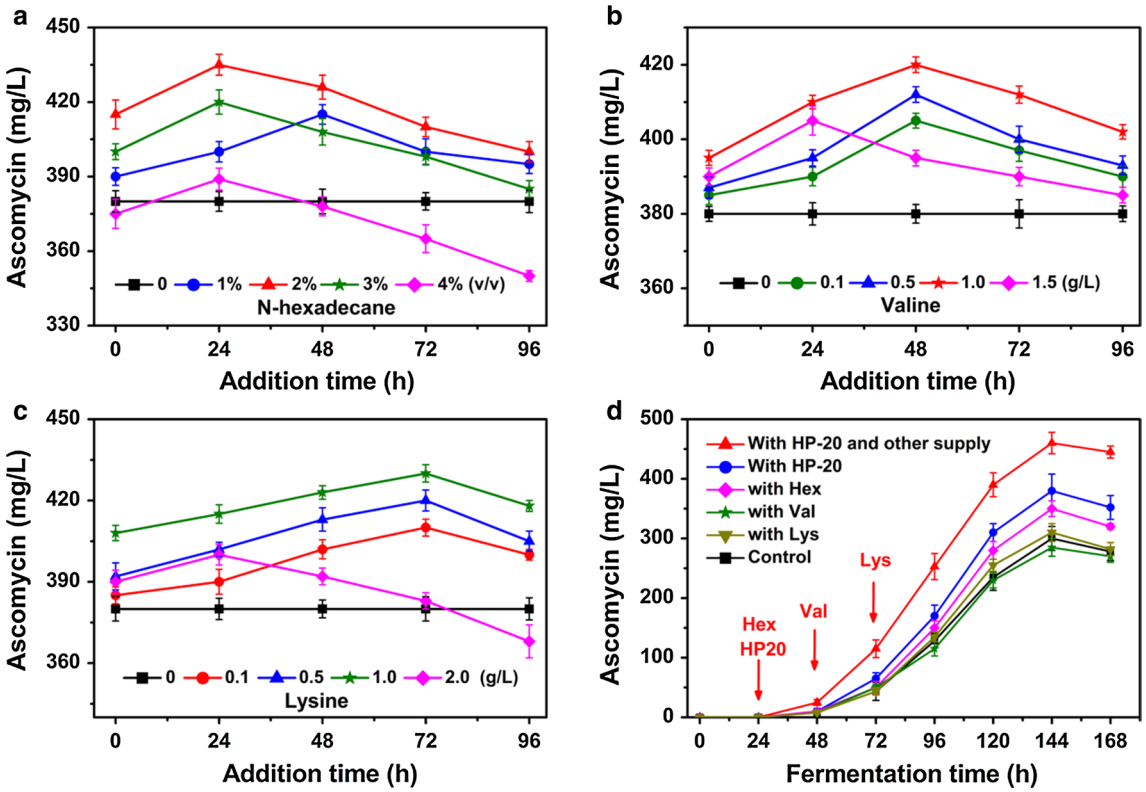

Fig. 4 The effects of rational designed fermentation strategies on the ascomycin production. The strategies contain different concentrations of *n*-hexadecane (**a**), valine (**b**) and lysine (**c**) at various feeding time, and the combinatorial strategies (**d**). *Arrows* indicate the feeding time point of each target feeding nutrient. *Hex n*-hexadecane

with Val or Lys supplementation only compared to the control (Fig. 4d). This may be due to the content of Val and Lys, which were not lacking (Fig. 3) and might not be the limited factors for higher ascomycin biosynthesis in the control.

The presumed bottlenecks for higher ascomycin biosynthesis have been confirmed based on the HP20 addition, and a combinatorial feeding strategy was further achieved: 2 % *n*-hexadecane added at 24 h, 1.0 g/L Val added at 48 h and 1.0 g/L Lys was added at 72 h. As shown in Fig. 4d, the impact of the combinatorial feeding on ascomycin production was a positively synergistic effect. The production of ascomycin by *S. hygroscopicus* var. *ascomyceticus* FS35 peaked at 144 h, up to 460 mg/L, a 21 % improvement compared with that obtained with only HP20 addition and a 53.3 % increment relative to that obtained under the initial fermentation condition. Besides, a high-yield ascomycin-producing strain *S. hygroscopicus* var. *ascomyceticus* SA68 with shikimic acid resistance was obtained in our group [26]. Though the ascomycin production of SA68 reached 450 mg/L after shikimic acid addition, comparable with current optimized production, the acquisition of SA68 was time-consuming and laborious using femtosecond laser irradiation while the proposed strategies here were relatively easy to follow. In future the ascomycin titer may be further enhanced combining the method developed here and the strain SA68, which would have a great significance for ascomycin production in large-scale fermentation.

Conclusions

Ascomycin production enhancement was achieved by the combination of resin HP20 addition and metabolites profiling analysis based on GC–MS and LC–MS/MS. Metabolic profiling analysis provided new insights into the potential factors of improving ascomycin production under the condition of HP20 addition. The production of ascomycin by *S. hygroscopicus* var. *ascomyceticus* FS35 based on a rational fermentation optimization was ultimately improved to 460 mg/L, a 53.3 % enhancement compared with that obtained under the initial fermentation condition. The strategies developed here could be extended to titer improvement of other important natural products and process optimization.

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