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Enhancement of Echinocandin B Production by a UV- and Microwave-Induced Mutant of *Aspergillus nidulans* with Precursor- and Biotin-Supplying Strategy

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Abstract Echinocandin B belongs to lipopeptide antifungal antibiotic bearing five types of direct precursor amino acids including proline, ornithine, tyrosine, threonine, and leucine. The objective of this study is to screen over-producing mutant in order to improve echinocandin B production; a stable mutant *Aspergillus nidulans* ZJB12073, which can use fructose as optimal carbon source instead of expensive mannitol, was selected from thousand isolates after several cycles of UV and microwave irradiation in turn. The results showed that mutant strain ZJB12073 exhibited 1.9-fold improvement in echinocandin B production to 1656.3 ± 40.3 mg/L when compared with the parent strain. Furthermore, the effects of precursor amino acids and some chemicals on echinocandin B biosynthesis in *A. nidulans* were investigated, respectively. Tyrosine, leucine, and biotin were selected as key factors to optimize the medium employing uniform design method. The results showed that the optimized fermentation medium provided another 63.1 % increase to 2701.6 ± 31.7 mg/L in final echinocandin B concentration compared to that of unoptimized medium.

Keywords Echinocandin B · *Aspergillus nidulans* · Precursor · Amino acid · Biotin · Fructose · Uniform design

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Introduction

Echinocandins, a class of lipopeptide antifungal antibiotics, are cyclic hexapeptides modified with fatty acid side chains. These lipopeptide antimycotics, which can inhibit the activity of β-1,3-glucan synthase and biosynthesis of fungal cell wall, are highly effective against a broad spectrum of *Aspergillus* species without crossing resistance to other drugs [1, 2]. As the secondary metabolites produced by *Aspergillus nidulans*, the chemical structure and biosynthesis of echinocandin B have been reported, although the information is not comprehensive [3].

Echinocandin B, also known as A30912, A22082, 7810, and SL7810F, has a linoleic acid side chain and a six cyclic hexapeptides. It consists of the following six amino acids: 4R, 5R-dihydroxyl-L-Orn, L-Thr, 4R-hydroxyl-L-Pro, 3S,4S-dihydroxyl-L-homo Tyr, L-Thr, and 3S-hydroxyl,4S-methyl-L-Pro [3–6]. Given its chemical structure and previous reports, it can be assumed that the biosynthesis of echinocandin B is connected with linoleic acid side chain which is biosynthesized by fatty acid synthase, and the peptide chain, linoleic acid-Orn-Thr-Pro-Tyr-Thr-Pro [3, 7–11]. The detail information about echinocandin B biosynthesis procedure are outlined in Fig. 1 [3].

Mutant breeding and medium optimization were the main ways for improvement of echinocandin B production. In recent years, more concerns have been raised on genetic background of *Aspergillus* species which can produce echinocandin B. However, there are few literatures about mutagenesis programs for screening over-producing mutants for industrial production [12]. There are both asexual and sexual spore development in *A. nidulans*. The formation of asexual spores is the main way of asexual reproduction, and the formation of ascospore is in sexual reproductive stage. Although asexual spores are used to accept radiation mutagenesis, it cannot maintain the good stability of passage. Thus, ascospore, as a sexual spore, is the optimal material for mutagenesis process [13–15]. Microbial mutation process by physical and chemical methods is random; however, how to screen mutants with high fermentation performance and which special fermentation performance of mutant we want to screen are crucial for industrial biotechnology, although it is very time consuming work. It is

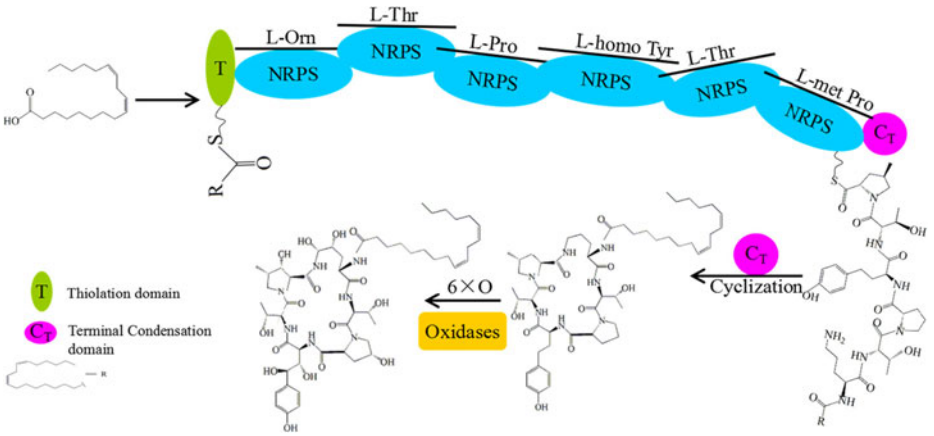

Fig. 1 Schematic representation of the echinocandin B biosynthetic pathway [3]. *T* and C_T are the domains of the NRPS. *T* thiolation domain, C_T a terminal condensation domain responsible for macrocyclization. Connection of linoleic acid and L-ornithine is the first step in echinocandin B biosynthesis. The cyclic hexapeptide scaffold is synthesized by a non-ribosomal peptide synthase (NRPS) consisting of six modules

reported that the optimal carbon source for echinocandin B production at large scale is mannitol, which is much expensive as compared to glucose, sucrose, and fructose [16–18]. In this work, we want to screen echinocandin B over-producing isolate which can use other cheap carbon source for fermentation, and sexual spores were selected as the material for UV and microwave irradiations.

Addition of some compounds, such as precursor amino acids and biotin, is a strategy of fermentation control in some industrial fermentation, especially antibiotic fermentation [19–21]. Echinocandin-type lipohexapeptides possess a serial high-complexity chemical structure, and the optimization of the fermentation process, such as supply of precursor during fermentation, is crucial to make a competitive product and to obtain target product. Interestingly, some information about the influence of precursor on echinocandin analogs (such as pneumocandin B_0) fermentation have been reported. The main control strategy for pneumocandin B_0 fermentation by adding precursors is the reduction of the formation of minor side products (e.g., pneumocandin C_0, D_0, and E_0 and the "Ser analogue pneumocandins"), thus enhancement of production. The addition of L-Pro to the medium at a concentration of 5–10 g/L can hinder the intracellular formation of 4R-hidroxyl-L-Pro, which can biosynthesize side-product pneumocandin C_0, thus increasing the yield of pneumocandin B_0 [22]. Moreover, the supply of L-Glu to the medium may increase pneumocandin B_0 production under certain fermentation conditions. However, either L-Thr or L-Ser at a concentration (5 g/L) reduces peumocandin B_0 titers [22]. Not surprisingly, few information about echinocandin B has been published in this field so far. Four components including peptone, K_2HPO_4, mannitol, and L-ornithine have significant effects on echinocandin B production in our laboratory [16]. Echinocandin B biosynthesis may be enhanced by L-Tyr and L-Phe supplement as precursor without increasing the by-product sterigmatocystin titers [23, 24]. By analogy with echinocandin B synthesis in *A. nidulans*, the synthesis of linoleic acid can be the first step of echinocandin B biosynthesis [25]. Therefore, some precursor amino acids and some chemical compounds which involve in the anabolic synthesis of linoleic acid are selected to evaluate their influence of echinocandin B fermentation by *A. nidulans*, in order to optimize the fermentation condition for enhancement of echinocandin B production.

Materials and Methods

Microorganism and Medium

A. nidulans ZJB12073 (CCTCC M 2015677) used in this study was frozen at −80 °C in our lab at Zhejiang University of Technology, China. The strain was maintained at PDA slants, stored at 4 °C, and renewed periodically.

Plate A (PDA) was composed of 200 g/L potato, 20 g/L glucose, and 20 g/L agar. Plates B and C were similar to plate A, which contained sucrose and fructose, respectively, instead of glucose.

The inoculum medium A for shake flask fermentation was composed of 10 g/L glucose,10 g/L glycerin, and 25 g/L soybean cake power, and pH was about 6.8–7.0. Inoculum medium B and C were similar to fermentation medium A, which contained sucrose and fructose, respectively, instead of glucose.

The basal fermentation medium was composed of 20 g/L of peanut oil, 10 g/L of glycerin, 8.6 g/L of peptone, 40 g/L of soybean meal powder, 15 g/L of tomato powder, 8.4 g/L of

$K_2HPO_4 \cdot 3H_2O$, and 0.5 g/L of $MgSO_4 \cdot 7H_2O$, pH 7.0. Fermentation medium A, B, and C contained 80 g/L of glucose, sucrose, and fructose based on the basal fermentation medium, respectively. Fermentation medium D contained 100 g/L fructose based on the basal fermentation medium.

All media were autoclaved for 20 min at 121 °C.

Mutagenesis and Mutant Screening

Spore suspensions were obtained by culturing the strain at 25 °C on a PDA slant for 7 days, followed by washing with 10-mL sterile saline. Spore suspension of the strain *A. nidulans* was adjusted to 10^7 cells/mL in sterile water. Of spore suspension, 5 mL was transferred to an aseptic plate. The plate, with cover removed, was exposed to ultraviolet (UV) irradiation for 0, 30, 60, 90, 120, 150, 180, and 210 s, respectively, at a distance of 20 cm from a UV lamp with wavelength of 254 nm and power of 15 W. Of spore suspension, 5 mL was transferred to an aseptic test tube. The test tube in an ice-water bath was exposed to microwave irradiation for 0, 30, 60, 90, 120, 150, 180, and 210 s, respectively, in a microwave oven with output power of 850 W and rated microwave power of 2450 MHz. The killing ratio was approximately 90 %. After appropriately diluting, the suspension of survived spores was spread on the plates A, B, and C. After incubation at 25 °C for 7 days, some single colonies were screened from plates A, B, and C, respectively, and kept in the PDA slants. Then, echinocandin B fermentation performance was checked by shake flask fermentation. The inoculum medium A and corresponding fermentation medium A were used for shake flask fermentation over mutants which were selected from plate A. The similar process was carried out for mutants, which were selected from plates B and C, respectively.

Testing the Stability of Mutant Strains

The stability of mutant strains was tested as follows: the 14-day-old slant of mutant isolate was transferred to the new slant, and the echinocandin B production was checked by shake flask fermentation described in following section. This process was repeated at least 10 times.

Shake Flask Fermentation for Echinocandin B Production

Inocula were prepared by transferring a loop of cells from 14-day-old slant into 250-mL Erlenmeyer flasks containing 50-mL inoculum medium. The flasks were kept in rotary shaker at 220 rpm for 48 h at 25 °C. The inocula were introduced into 250-mL Erlenmeyer flasks containing 50-mL fermentation medium. An inoculum ratio of 10 % (*v*/*v*) was used in all the bioprocess. Three batches were repeated for each experiment.

Analytical Methods

Samples were withdrawn from the shake flasks after 12-day fermentation, and 10 mL of fermentation broths was centrifuged (5000×*g*, 10 min); the supernatant was discarded and lower sediment was immersed in 5 mL methanol to settle overnight at 4 °C. Methanol extracts were centrifuged at 5000×*g* for 10 min, and the supernatant was used for quantification of echinocandin B. Biomass was calculated by dry cell weight (DCW,

g/L). In detail, 5-mL cultures were sampled and centrifuged at 5000×*g* for 10 min. The supernatant of culture was replaced and the sample was placed in the 80 °C electrothermal constant temperature oven drying to constant weight, then weighed DCW on the analytical balance.

The process of HPLC for checking echinocandin B concentration was as follows: methanol extracts were filtered through a 0.2-μm-pore-size cellulose acetate membrane. Analytical reverse phase HPLC was performed using a C_{18} Hypersil column (4.6 × 250 mm I.D., 5 μm) at 40 °C. Injection volume was 20 μL. The mobile system was a mixed solvent of methanol (70 %), acetonitrile (10 %), and water (20 %), at a constant flow of 1 mL/min. Peak of echinocandin B was determined at wavelength of 222 nm. Echinocandin B concentrations were calculated by comparison of peak area with a standard curve. The retention time of echinocandin B was 14.0 min.

Experiment Design for Medium Optimization

To evaluate the effects of fructose, amino acids, and other compounds on echinocandin B production, single-factor experimental method was used in this work. Based on the basal fermentation medium, different fructose concentrations were chosen in experiment while the other experimental factors were fixed. The concentrations of echinocandin B and the DCW were measured, respectively. Then, other experimental factors (such as amino acids and biotin) which based on the fermentation medium D were carried out in the similar process. In these processes, the inoculum medium C was used for liquid seed preparation. Three batches were repeated for each experiment.

Then, based on the fermentation medium D, uniform design method using a three-factor four-level experimental design with 12 experiments ($U_{12}(4^3)$) was performed. The concentrations of tyrosine, leucine, and biotin were chosen as the critical variables and designed as X_1, X_2, and X_3, respectively. The experimental range of each variable was equally divided into four levels and given in Table 1.

To determine the relationship between the concentrations of the three variables, regression analysis was performed. A second-order polynomial equation, as in Eq. (1), was obtained to correlate the variables with echinocandin B concentration.

$$Y = B_0 + \sum B_i X_i + \sum B_{ii} X_i^2 + \sum B_{ij} X_i X_j \quad (1)$$

where Y is the response variable of echinocandin B production, B is the regression coefficient, and X is the independent variable. The statistical software of Data Processing System (DPS, version 11.5) was used for regression and statistical analyses.

Each experiment was repeated at least three times.

Table 1 Experimental levels of the independent variables

Variables	Coded symbols	Levels			
		1	2	3	4
Tyrosine concentration (g/L)	X_1	2.0	4.0	6.0	8.0
Leucine concentration (g/L)	X_2	2.0	4.0	6.0	8.0
Biotin concentration (mg/L)	X_3	1.0	2.0	3.0	4.0

Results

Sequential Mutagenesis and Screening High-Yield Mutants

A. nidulans ULN-59, as a parent strain, was initially exposed to UV irradiation. Then, the suspension of spores was spread on the plates A, B, and C, respectively. After several cycles of breeding, more than 700 colonies were selected, and some over-producing isolates, such as J-1, J-8, and J-10, were found from plate C which chose fructose as carbon source (Table 2), while few mutants with good fermentation activity were selected from plates A and B (data were not shown). The maximum echinocandin B production of 1357.2 ± 15.9 mg/L was obtained from strain J-10, showing a 1.6-fold production improvement compared to the parent strain. In the next step experiment of mutagenesis, strain J-933 was further treated with microwave irradiation, and plate C was used for screening mutants. After three times of mutagenesis, some over-producing mutants, such as J-901, J-915, and J-933, were screened from more than 500 colonies (Table 2). The strain J-933 had the highest production of echinocandin B (1656.3 ± 40.3 mg/L), showing a 1.9-fold production improvement compared to the parent strain. Moreover, the mutant J-933 was stable by testing of stability and renamed as *A. nidulans* ZJB12073 which was stored at China Center for Type Culture Collection (CCTCC).

Effects of Fructose on Echinocandin B Fermentation

Mutant *A. nidulans* ZJB12073 was screened from plate C which chose fructose as only carbon source. Meanwhile, in order to recheck fructose as the optimal resource, some other economic carbon sources for production, fructose, glucose, sucrose, and glycerin were investigated, respectively, using single-factor experimental method. The results confirmed that fructose was more appropriate carbon source for echinocandin B fermentation by *A. nidulans* ZJB12073 (data were not shown). Then, based on the medium C, different fructose concentrations, 80, 90, 100, 110, and 120 g/L, were carried out for fermentation using single-factor experimental method in order to optimize the medium. The optimal fructose concentration for fermentation was 100 g/L. The maximal production was 1978.2 mg/L and a 19.4 % increase in echinocandin B production in contrast with the previous experiment (Fig. 2).

Table 2 Part of mutants screened through various mutagenic treatments

Mutagenic treatment	Strain	Echinocandin B titer (mg/L)
None	ULN-59	869.0 ± 23.6
UV irradiation treatment	J-1	1026.5 ± 38.9
UV irradiation treatment	J-8	1188.6 ± 43.8
UV irradiation treatment	J-10	1357.2 ± 15.9
Microwave irradiation treatment	J-901	1433.7 ± 40.7
Microwave irradiation treatment	J-915	1512.5 ± 19.9
Microwave irradiation treatment	J-933	1656.3 ± 40.3

Effects of Precursor Amino Acids on Echinocandin B Fermentation

According to the results of our previous experiments and some literature reported, fermentation medium optimization was continuously conducted. Based on the fermentation medium D, proline, ornithine, tyrosine, threonine, or leucine was added to the medium, respectively, as precursor for echinocandin B fermentation. As shown in Fig. 3, precursor amino acids played a key role on the echinocandin B production. According to the data of DCW, it means that ornithine and threonine could increase mycelium growth. The DCW of the ornithine-supplemented culture (73.4 g/L) was the maximum among the all experiments, which was higher than that of the tyrosine-supplemented culture (48.7 g/L) by 50.7 %. Meanwhile, the DCW of the threonine-supplemented culture (69.8 g/L) was more than that of the leucine-supplemented culture (56.0 g/L) by 24.6 %. Therefore, proline, tyrosine, and leucine could hinder the mycelium growth. However, more DCW could not correspond with higher echinocandin B production; echinocandin B production was very significantly suppressed by threonine and ornithine. Among all experiments, tyrosine and leucine showed the more favorable for echinocandin B production by *A. nidulans* ZJB12073 with the significant increase of echinocandin B production by 13.5 and 11.4 % as compared to control. So, both tyrosine and leucine were selected as key factors for medium optimization experiment in detail.

Effects of Some Compounds on Echinocandin B Fermentation

By analogy with echinocandin B synthesis in *A. nidulans*, the synthesis of linoleic acid could be the first step of echinocandin B biosynthesis. Therefore, based on the fermentation medium D, other compounds (pyruvic acid, linoleic acid, citric acid, and biotin), which involved in the anabolic synthesis of linoleic acid, were selected to evaluate their influence of echinocandin B fermentation. As shown in Fig. 4, pyruvic acid and linoleic acid could induce mycelium growth; however, they decreased echinocandin B production. On the contrary, biotin and citric acid had seldom effects on mycelium growth, and biotin played an important role in increasing echinocandin B production. With the addition of biotin (2.0 mg/L) into the medium,

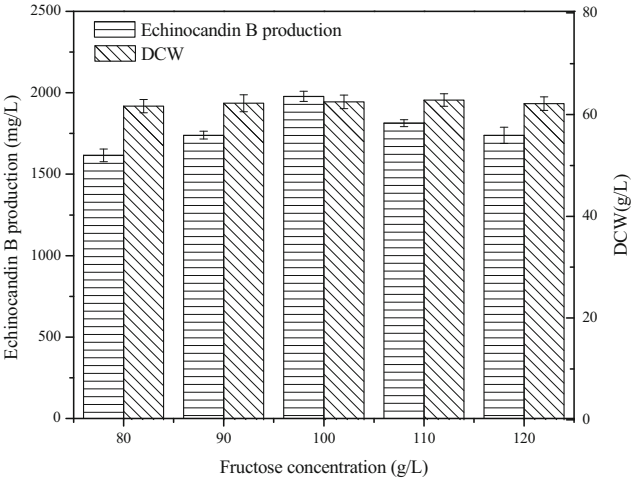

Fig. 2 Effects of fructose on echinocandin B fermentation

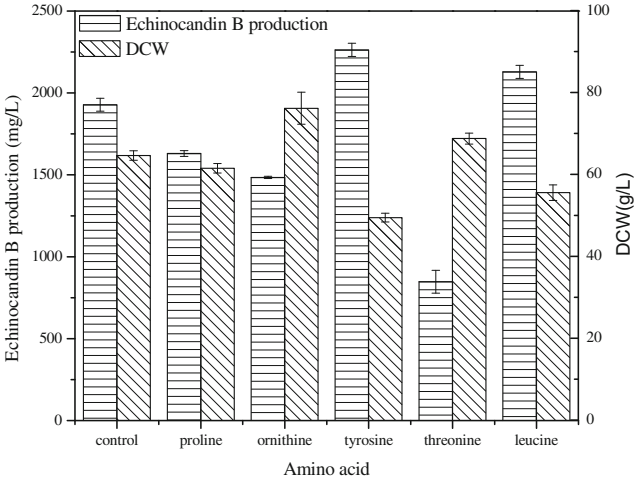

Fig. 3 Effects of precursor amino acids on echinocandin B fermentation

echinocandin B production increased 12.2 % over the control. So, biotin was chosen as a key factor for medium optimization experiment in detail.

Optimization of Tyrosine, Leucine, and Biotin for Echinocandin B Fermentation

According to the results of a preliminary study, tyrosine, leucine, and biotin, which have profound effects on echinocandin B production by shake flask fermentation, were chosen as independent factors for optimization of echinocandin B production by uniform design. The average echinocandin B concentration obtained from different experimental conditions was listed in Table 3, and the results were analyzed by ANOVA, shown in Tables 4 and 5. The uniform design model F value was 8.80, calculated as the ratio of mean square regression and mean square residual, and the model p value was 0.026, which was very low. These two values (F and p) implied that this model was significant. The crossed terms, X_1X_2 and X_1X_3, were deleted for the high correlation with variable X. The p values were also used in the analysis of the independent variables to check the significance of the coefficients, which were necessary to elucidate the pattern of the mutual interactions between them. The larger the magnitude of the t value and smaller the p value, the more significant is the corresponding (Table 5). Among the independent variables, addition of tyrosine and leucine to the culture medium had more significant effect on the echinocandin B production than that of biotin. All the quadratic terms having small p values indicated that the quadratic terms also had significant effects on the echinocandin B production, and the effects of the variables on the responses were not a simple linear relationship. The regression equation was as the following Eq. (2):

$$Y = 2161.9 + 95.8X_1 + 80.5X_2 + 201.3X_3 - 11.8X_1^2 - 8.5X_2^2 - 44.4X_3^2 - 5.4X_2X_3 \quad (2)$$

where Y is the concentration of echinocandin B and X_1, X_2, and X_3 are the concentrations of added tyrosine, leucine, and biotin, respectively.

The fit of model was also indicated by the coefficient of determination R^2, which was calculated to be 0.94, suggesting that the model was efficient and could explain 93.9 % of the experimental data. The optimization was based on taking the partial derivative with respect to

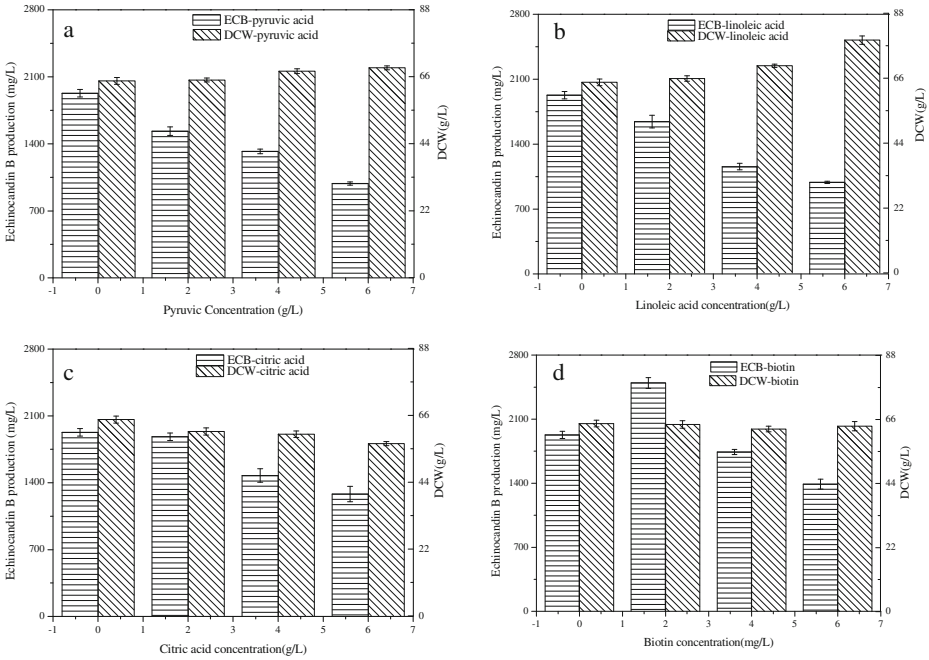

Fig. 4 Effects of some compounds on echinocandin B fermentation (**a** Pyruvic acid; **b** Linoleic acid; **c** Citric acid; **d** Biotin)

X, which were calculated as 4.1, 4.1, and 2.0 mg/L for the concentrations of added tyrosine, leucine, and biotin, respectively. This gave the maximum predicted result for echinocandin B production (*Y*) as 2725.1 mg/L.

The verification of the results was carried out by performing fermentation at optimized condition with five batch experiments. The average echinocandin B production was 2701.6 ±31.7 mg/L, which was close agreement with the model prediction. The prediction deviation was only 0.9 %, indicating that these models could be considered quite reliable for predicting the echinocandin B concentration from fermentation over *A. nidulans* ZJB12073.

Table 3 Results of the uniform design for optimizing the media for echinocandin B production

Runs	X_1 (g/L)	X_2 (g/L)	X_3 (mg/L)	Y (mg/L)
1	2	6	1	2564.7 ± 31.2
2	6	8	1	2574.3 ± 20.5
3	4	4	3	2687.2 ± 38.3
4	8	8	3	2305.9 ± 36.1
5	4	6	2	2694.5 ± 37.1
6	2	8	4	2348.1 ± 31.8
7	6	6	3	2596.0 ± 30.7
8	6	4	2	2679.2 ± 20.5
9	4	2	4	2489.8 ± 20.1
10	8	2	1	2430.3 ± 16.8
11	2	2	2	2682.5 ± 19.5
12	8	4	4	2413.6 ± 23.5

Note that *Y* is the concentration of echinocandin B and X_1, X_2, and X_3 are the concentrations of added tyrosine, leucine, and biotin, respectively

Table 4 Analysis of variance (ANOVA) for the quadratic model

Source	SS	*df*	MS	*F* value	*P* value
Regressions	199,641.47	7	28,515.78	8.80	0.026
Residual error	12,956.45	4	3,239.11		
Total	212,566.92	11			

SS sum of squares, *df* degree of freedom, *MS* mean square, $R^2 = 0.94$, adjusted $R^2 = 0.83$

Discussion

Mutation has been one of the most effective means of improving antibiotic production in fermentation. Effective selection of hyperproductive strains from thousand mutants is very important for efficient breeding and improvement of strains. There have been only few reports made concerning the development of an *Aspergillus* strain via mutagenesis for the production of echinocandin B, which suggest that physical and chemical mutations are considered to have the potential to change the echinocandin-type compound content of fungal strain [16]. Selection of cheap substrate for industry fermentation was a promising method to reduce the price of production. One objective of this work is to screen a mutant which can use cheap carbon source instead of expensive mannitol, and a breeding method was developed using plates containing cheap sugars for screening isolate after mutation process. If positive colonies appeared on the plates, these colonies might adapt to the economic carbon source. In our study, more than 1000 colonies, which chose fructose as carbon source, were screened after sequential mutation that involved the use of UV radiation and microwave radiation treatments, and a total of 10 mutants were detected with notably improved level of echinocandin B production.

Mannitol is the main carbon source for echinocandin B fermentation and its metabolism may generate the levels of NADPH required for antibiotic synthesis, while using mannitol as main substrate for echinocandin B production is uneconomic, because it is an expensive sugar. From the perspective of economy, selection of cheap sugar for echinocandin B production plays a key role in reduction of product price. Fructose is the common carbon source in industry biotechnology and about 50 % cheaper than mannitol [17, 18]. The data (Fig. 2) have shown that fructose have positive effect on echinocandin B production. It means that fructose is a good alternative substrate for echinocandin B production. Fructose and mannitol have the metabolical process for mutual transformation, and fructose can transform into mannitol via fructose-6-P (Fig. 5a) [26]. The accumulated mannitol is degraded via the same pathway in the opposite direction to produce fructose-6-P by M2DH and hexokinase. Interestingly, some

Table 5 Model coefficients estimated by multiple linear regression (significance of regression coefficients)

Factor	Coefficient	*t* value	*P* value
X_1	95.8	1.647	0.175
X_2	80.5	1.687	0.169
X_3	201.3	1.580	0.189
X_1^2	−11.8	2.044	0.110
X_2^2	−8.5	1.863	0.136
X_3^2	−44.4	2.170	0.095
X_2X_3	−5.4	0.662	0.544

fungi also possess an alternative pathway called a mannitol cycle (Fig. 5b), wherein mannitol is synthesized from fructose-6-P via mannitol-1-P by mannitol-1-phosphate dehydrogenase (M1PDH) and mannitol-1-phosphatase (M1Pase); when degraded, the accumulated mannitol is decomposed to fructose-6-P via fructose by M2DH and hexokinase. This pathway is found in various fungi, such as *Aspergillus niger* [27]. Thus, mannitol and fructose may have the similar metabolic pathway, and fructose also can provide enough NADPH for the production of secondary metabolites during the stable fermentation period.

The chemical structure of echinocandin B contains six cyclic peptide β-amino acid peptide chain and the linoleic acid side chain. According to its biosynthesis pathway, we want to use metabolic regulation fermentation strategy that the biosynthesis of six cyclic peptide β-amino acid peptide chain was enhanced by supplying of precursor amino acid and the biosynthesis of linoleic acid chain was induced by adding of biotin. In this work, firstly, five amino acids selected as precursors for echinocandin B fermentation, such as proline, ornithine, tyrosine, threonine, and leucine, may participate in the formation of the six cyclic peptide chain and are relevant to intracellular metabolism and secondary metabolites (Fig. 6). Threonine, leucine, and tyrosine can form into acetyl-CoA after their carbon skeleton is decomposed. Moreover, proline and tyrosine form into α-ketoglutarate and fumaric acids, respectively. Acetyl-CoA, α-ketoglutarate, and fumaric acids all join the tricarboxylic acid cycle [28]. The experiments results showed that tyrosine and leucine honestly increased the value of echinocandin B production by *A. nidulans* ZJB12073. As to tyrosine, echinocandin compounds contained a non-protein amino acid L-high tyrosine, which was biosynthesized by tyrosine and acetate. Tyrosine could participate in the citric acid cycle through two ways, turning to fumaric acid or acetyl-CoA. While, as to leucine, on one hand, (2 s,3 s,4 s)-4-methyl-3-hydroxy-proline in the six cyclic peptide chain was formed due to the cyclic effects of leucine, not proline. On the other hand, leucine firstly formed into α-ketonic acid by transamination, then formed to isovaleryl

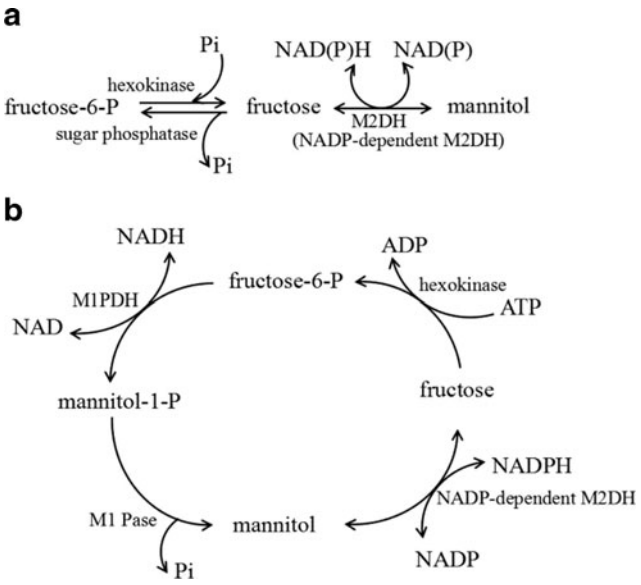

Fig. 5 Proposed mannitol metabolism in fungi (**a** Transformation way of mannitol and fructose; **b** Mannitol cycle) [26, 27]

CoA by oxidative decarboxylation. Moreover, isovaleryl CoA formed to β-hydroxy-β-mothyl glutaryl CoA, which was the precursor of fatty acid, by dehydrogenation and carboxylation.

Secondly, for the linoleic acid side chain of echinocandin B, fatty acid was the first step of ehinocandin B biosynthesis. Because linoleic acid could not be assimilated directly by *A. nidulans*, some compounds which could facilitate fatty acid biosynthesis were added to the fermentation medium in order to enhance the biosynthesis of linoleic acid side chain, resulting in improvement of echinocandin B production. The accumulation of fatty acids mainly occurred during cell growth phase, and then, the increased fatty acids were degraded into acetyl-CoA via beta oxidation during the main phase of echinocandin B biosynthesis [29]. Acetyl-coenzyme A is the only source of all carbon atoms for fatty acid molecules, and it is from oxidation and decomposed of glucose or decomposition of amino acid. These processes are conducted in the mitochondria; however, the fatty acid synthesis enzymes exist in cytosol. Acetyl-CoA goes into the cytosol from mitochondria by citric acid-pyruvate cycle. Thus, citric acid goes into the cytosol, then cleaves to form acetyl-CoA and oxaloacetate. The procedures of glycolysis and tricarboxylic acid (TCA) cycle are shown in Fig. 6.

Meanwhile, the level of acetyl-CoA in the main phase of antibiotic biosynthesis displayed downward trends. Thus, according to the analysis of CoA-ester profile, we inferred that the reduced acetyl-CoA might be converted into malonyl-CoA or succinyl-CoA. As a result, we tried increasing fatty acids in the cells through adding pyruvic acid, linoleic acid, citric acid, and biotin, and the experiment results (Fig. 4) showed that addition of biotin can induce the echinocandin B biosynthesis. Actually, biotin was a coenzyme for a variety of carboxylase and as CO_2 carrier in carboxylase reactions and participated in pyruvate carboxylation and transformed into oxaloacetic acid. Moreover, it was also involved in the carbonylation that acetyl-CoA transformed into malonyl-CoA and played a key role in the biochemical reaction such as fat synthesis and glycolysis [19, 20]. Consequently, biotin joined into TCA cycle by

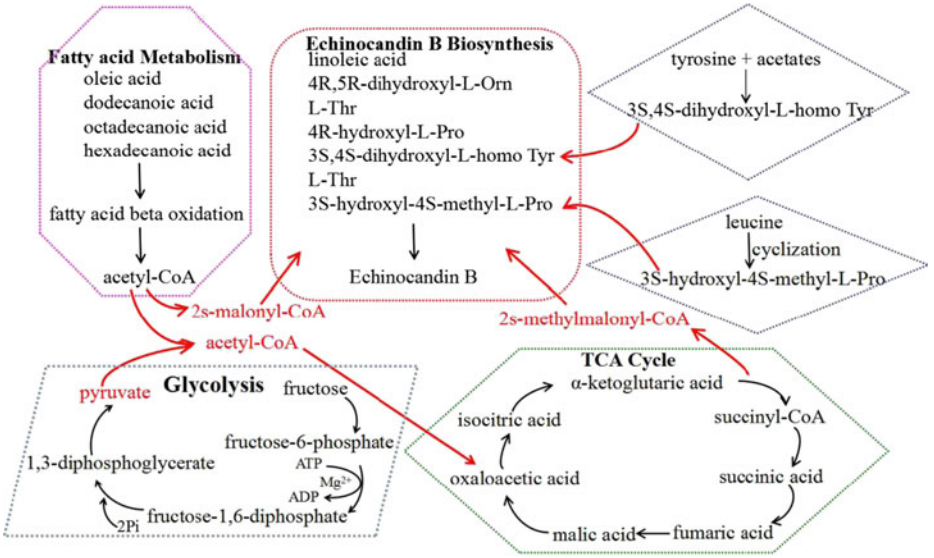

Fig. 6 The proposed relationships of echinocandin B biosynthesis with TCA cycle, glycolysis, fatty acid metabolism, and amino acid metabolism in *A. nidulans*. The *thin arrows* represent the directions of metabolites reactions; *thick arrows* represent the directions of metabolic flux [6, 28, 29]

transforming into oxaloacetate and provided enough malonyl-CoA for echinocandin compound biosynthesis, so it stimulated the synthesis of echinocandin B.

Overall, metabolic profiling analysis provided important insights into the potential factors of improving echinocandin B production by *A. nidulans* ZJB12073. To investigate the effect of precursor amino acids on echinocandin B production and its biosynthesis was very helpful to understand the regulation mechanism for echinocandin B biosynthesis during fermentation. The successful application of precursor supplying fermentation in this work indicates that the strategies can be applied to the rational guidance of other antibiotic production improvement, as well as the mechanism analysis of secondary metabolite biosynthesis.

Conclusion

In the current work, a stable echinocandin B over-producing mutant, *A. nidulans* ZJB12073, was screened after several cycles of UV and microwave mutagenesis using cheap carbon source fructose instead of mannitol. The results of the fermentation for echinocandin B production by *A. nidulans* ZJB12073 showed that the mutant performed excellently in echinocandin B production. Moreover, the maximum echinocandin B concentration of 2701.6 ± 31.7 mg/L was achieved at 12 days of fermentation using precursor-adding strategy at 25 °C with the medium composition of 20 g/L of peanut oil, 10 g/L of glycerin, 8.6 g/L of peptone, 100 g/L of fructose, 40 g/L of soybean meal powder, 15 g/L of tomato powder, 8.4 g/L of $K_2HPO_4 \cdot 3H_2O$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 4.1 g/L of leucine, 4.1 g/L tyrosine, and 2.0 mg/L biotin, pH 7.0. Continuously, mutation breeding was a good suggestion for improvement of echinocandin B production, meanwhile, by finely tuning of amino acid-feeding strategy, there might be a scope for further enhancement of the echinocandin B concentration.

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References

1. Denning, D. W. (2002). Echinocandins: a new class of antifungal. *The Journal of Antimicrobial Chemotherapy, 49*, 889–891.
2. Scott, L. J. (2012). Micafungin a review of its use in the prophylaxis and treatment of invasive *Candida* infections. *Drugs, 72*, 2141–2165.
3. Emri, T., Majoros, L., Toth, V., & Pocsi, I. (2013). Echinocandins: production and applications. *Applied Microbiology and Biotechnology, 97*, 3267–3284.
4. Hensens, O. D., Liesch, J. M., Zink, D. L., Smith, J. L., Wichmann, C. F., & Schwartz, R. E. (1992). Pneumocandins from *Zalerion arboricola*. III. Structure elucidation. *The Journal of Antibiotics, 45*, 1875–1885.
5. Iwamoto, T., Fujie, A., Sakamoto, K., Tsurumi, Y., Shigematsu, N., Yamashita, M., Hashimoto, S., Okuhara, M., & Kohsaka, M. (1994). WF11899A, B and C, novel antifungal lipopeptides. I. Taxonomy, fermentation, isolation and physico-chemical properties. *The Journal of Antibiotics, 47*, 1084–1091.
6. Kanasaki, R., Sakamoto, K., Hashimoto, M., Takase, S., Tsurumi, Y., Fujie, A., Hino, M., Hashimoto, S., & Hori, Y. (2006). FR209602 and related compounds, novel antifungal lipopeptides from *Coleophoma crateriformis* no.738—I. taxonomy, fermentation, isolation and physico-chemical properties. *The Journal of Antibiotics, 59*, 137–144.
7. Adefarati, A. A., Giacobbe, R. A., Hensens, O. D., & Tkacz, J. S. (1991). Biosynthesis of L-671,329, an echinocandin-type antibiotic produced by *Zalerion arboricola*—origins of some of the unusual amino acids and the dimethylmyristic acid side chain. *Journal of the American Chemical Society, 113*, 3542–3545.

8. Schwarzer, D., Finking, R., & Marahiel, M. A. (2003). Nonribosomal peptides: from genes to products. *Natural Product Reports, 20*, 275–287.
9. Youssar, L., Gruning, B. A., Erxleben, A., Gunther, S., & Huttel, W. (2012). Genome sequence of the fungus *Glarea lozoyensis*: the first genome sequence of a species from the *Helotiaceae* family. *Eukaryotic Cell, 11*, 250.
10. Cacho, R. A., Jiang, W., Chooi, Y. H., Walsh, C. T., & Tang, Y. (2012). Identification and characterization of the echinocandin B biosynthetic gene cluster from *Emericella rugulosa* NRRL 11440. *Journal of the American Chemical Society, 134*, 16781–16790.
11. Chen, L., Yue, Q., Zhang, X. Y., Xiang, M. C., Wang, C. S., Li, S. J., Che, Y. S., Ortiz-Lopez, F. J., Bills, G. F., Liu, X. Z., & An, Z. Q. (2013). Genomics-driven discovery of the pneumocandin biosynthetic gene cluster in the fungus *Glarea lozoyensis*. *BMC Genomics, 14*, 339.
12. Baltz, R. H. (2011). Strain improvement in actinomycetes in the postgenomic era. *Journal of Industrial Microbiology and Biotechnology, 38*, 657–666.
13. Brown, D. W., & Salvo, J. J. (1994). Isolation and characterization of sexual spore pigments from *Aspergillu nidulans*. *Applied and Environmental Microbiology, 60*, 979–983.
14. Kaur, B., Sharma, M., Soni, R., Oberoi, H. S., & Chadha, B. S. (2013). Proteome-based profiling of hypercellulase-producing strains developed through interspecific protoplast fusion between *Aspergillus nidulans* and *Aspergillus tubingensis*. *Applied Biochemistry and Biotechnology, 169*, 393–407.
15. Rohrig, J., Kastner, C., & Fischer, R. (2013). Light inhibits spore germination through phytochrome in *Aspergillus nidulans*. *Current Genetics, 59*, 55–62.
16. Zou, S. P., Zhong, W., Xia, C. J., Gu, Y. N., Niu, K., Zheng, Y. G., & Shen, Y. C. (2015). Mutagenesis breeding of high echinocandin B producing strain and further titer improvement with culture medium optimization. *Bioprocess and Biosystems Engineering, 38*, 1845–1854.
17. Li, T. L., Fan, Y. X., Nambou, K., Hu, F. X., Imanaka, T., Wei, L. J., & Hua, Q. (2015). Improvement of ansamitocin P-3 production by *Actinosynnema mirum* with fructose as the sole carbon source. *Applied Biochemistry and Biotechnology, 175*, 2845–2856.
18. Pandit, N. T., & Pandit, A. B. (2014). Exploration of a cheaper carbon source for extracellular beta-glucosidase synthesis from *Debaryomyces pseudopolymorphus* NRRL YB-4229. *Applied Biochemistry and Biotechnology, 172*, 3606–3620.
19. Qi, K., Xia, X. X., & Zhong, J. J. (2015). Enhanced anti-oxidative activity and lignocellulosic ethanol production by biotin addition to medium in *Pichia guilliermondii* fermentation. *Bioresource Technology, 189*, 36–43.
20. Xi, Y. L., Chen, K. Q., Xu, R., Zhang, J. H., Bai, X. F., Jiang, M., Wei, P., & Chen, J. Y. (2012). Effect of biotin and a similar compound on succinic acid fermentation by *Actinobacillus succinogenes* in a chemically defined medium. *Biochemical Engineering Journal, 69*, 87–92.
21. Ng, I. S., Ye, C. M., Zhang, Z. X., Lu, Y. H., & Jing, K. J. (2014). Daptomycin antibiotic production processes in fed-batch fermentation by *Streptomyces roseosporus* NRRL11379 with precursor effect and medium optimization. *Bioprocess and Biosystems Engineering, 37*, 415–423.
22. Connors, N., & Pollard, D. (2004). *Pneumocandin B_0 production by fermentation of the fungus Glarea lozoyensis: physiological and engineering factors affecting titer and structural analogue formation* (pp. 515–538). New York: Hand book of Industrial Mycology, Marcel Dekker.
23. Chen, S. C. A. (2011). Echinocandin antifungal drugs in fungal infections: a comparison (vol 71, pg 11, 2011). *Drugs, 71*, 253.
24. Toth, V., Nagy, C. T., Miskei, M., Pocsi, I., & Emri, T. (2011). Polyphasic characterization of "*Aspergillus nidulans* var. *roseu*s" ATCC 58397. *Folia Microbiologica, 56*, 381–388.
25. Kraas, F. I., Helmetag, V., Wittmann, M., Strieker, M., & Marahiel, M. A. (2010). Functional dissection of surfactin synthetase initiation module reveals insights into the mechanism of lipoinitiation. *Chemistry & Biology, 17*, 872–880.
26. Iwamoto, K., & Shiraiwa, Y. (2005). Salt-regulated mannitol metabolism in algae. *Marine Biotechnology, 7*, 407–415.
27. Jennings, D. H. (1984). Polyol metabolism in fungi. *Advances in Microbial Physiology, 25*, 149–193.
28. Imai, K., Fukushima, T., Santa, T., Homma, H., Hamase, K., Sakai, K., & Kato, M. (1996). Analytical chemistry and biochemistry of D-amino acids. *Biomedical Chromatography, 10*, 303–312.
29. Mouslim, J., David, L., Petel, G., & Gendraud, M. (1993). Effect of exogenous methyl oleate on the time-course of some parameters of *Streptomyces hygroscopicus* NRRL B-1865 culture. *Applied Microbiology and Biotechnology, 39*, 585–588.