

# Mutagenesis breeding of high echinocandin B producing strain and further titer improvement with culture medium optimization

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**Abstract** A combination of microbial strain improvement and statistical optimization is investigated to maximize echinocandin B (ECB) production from *Aspergillus nidulans* ZJB-0817. A classical sequential mutagenesis was studied first by using physical (ultraviolet irradiation at 254 nm) and chemical mutagens (lithium chloride and sodium nitrite). Mutant strain ULN-59 exhibited 2.1-fold increase in ECB production to  $1583.1 \pm 40.9$  mg/L when compared with the parent strain ( $750.8 \pm 32.0$  mg/L). This is the first report where mutagenesis is applied in *Aspergillus* to improve ECB production. Further, fractional factorial design and central composite design were adopted to optimize the culture medium for increasing ECB production by the mutant ULN-59. Results indicated that four culture media including peptone,  $K_2HPO_4$ , mannitol and L-ornithine had significant effects on ECB production. The optimized medium provided another 1.4-fold increase in final ECB concentration to  $2285.6 \pm 35.6$  mg/L compared to the original medium. The results of this study indicated the combined application of a classical mutation and medium optimization can improve effectively ECB production from *A. nidulans* and could be a promising tool to improve other secondary metabolites production by fungal strains.

**Keywords** *Aspergillus nidulans* · Echinocandin B · Sequential mutagenesis · Medium optimization · Response surface methodology

## Introduction

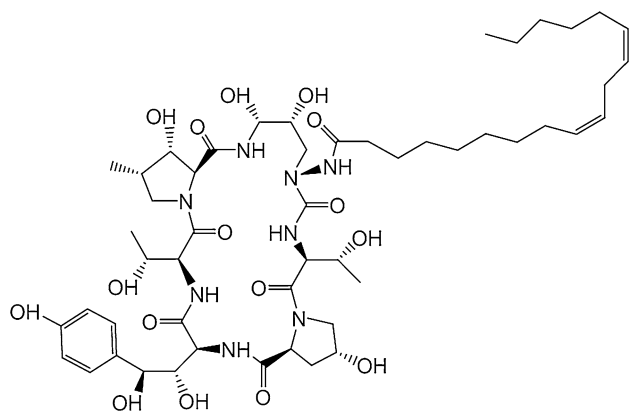
In the past several decades, both the incidence and the types of fungal infection severely detrimental to human health continuously increased, especially for the immunosuppressed patients [12]. At the same time, the clinical application of certain commonly used clinical antifungal agents, such as amphotericin, imidazoles and triazoles, was restricted due to significant neurotoxicity and drug resistance [3]. Echinocandins are a novel class of antifungal agents with antifungal activity against *Candida* and *Aspergillus species* and *Pneumocystis carinii* [9]. These compounds interrupt biosynthesis of  $\beta$ -1,3-glucan linkages, an essential component of the fungal cell wall [3]. There is little human toxicity attributed to the mechanism of action, because glucan polymers are not components of mammalian cells. In recent years, more concerns have been raised on echinocandin antifungal drug due to its low toxicity and rapid and broader-spectrum antifungal activity [1].

Echinocandin B (ECB) (see the chemical structure of ECB, Fig. 1), a novel linoleoyl-containing cyclic hexapeptide echinocandin-type compound, was isolated from a culture broth of the fungus *Aspergillus nidulans* [3]. Anidulafungin is derived from the modification of ECB with deacylase to replace linoleoyl groups and introduce newly synthesized side chains [7]. Increasing production of ECB and improvement of fermentation condition for ECB was necessary for the industrial manufacturing of anidulafungin. Furthermore, a steady supply of producing strains

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**Fig. 1** Chemical structure of echinocandin B

for each manufacturing lot was needed to maintain the high production of ECB. However, no scientific report has been published on mutant screening for improved ECB production to the best of our knowledge.

Owing to their inherent metabolic control systems, microorganisms usually produce secondary metabolites in very low concentrations. Strain improvement plays a central role in the commercial development of microbial fermentation processes [4, 10, 29, 31]. Although the modern techniques of gene engineering and metabolic engineering are considered to be effective methods in the microorganism breeding [11, 16, 27], the application of these modern techniques frequently encounters serious impediments due to limited insight into the genetics, physiology, and biochemistry of organisms [21]. Thus, the classic strain improvement technology is still an effective and practical approach to obtain improved strains for production of both primary and secondary metabolites [13, 18, 26]. Sequential mutation is methodologically simple, it is an extremely effective way to enhance productivity of strains [2], and many scientists have used this methodology to generate industrial strains [2, 15, 23]. The improvement of microbial strains using different mutagenic agents, such as ultraviolet (UV), X-rays and plasma, as well as various chemical mutagens, for increased production of industrial products has been considered in the commercial fermentation process [25].

Medium optimization is essential to enhance performance of the production system and yield of the process. Conventionally, the fermentation medium was optimized using a one-factor-at-a-time strategy, which was relatively simple and did not require statistical analysis. Major disadvantage of this approach is that it does not include interactive effects among the variable parameters, and it is highly time consuming as well [4, 22, 24]. To overcome these limitations, optimization of the variable parameters has been carried out using a statistical factorial design called as response surface methodology (RSM). RSM is a

combination of mathematical and statistical techniques used for optimizing the process. It can also be used to determine and evaluate several factors affecting the process and their relative significance, even in presence of complex interactions, in a limited number of experiments [22, 28].

The present work was carried out to find out a mutant with high level of ECB production by mutating *A. nidulans* ZJB-0817 with sequential mutagenesis. Further attempt was made to optimize the basic culture medium for achieving high level of ECB production by the mutant using RSM.

## Materials and methods

### Chemicals

Echinocandin B was purchased from BioAustralis (Smithfield, AUS). Acetonitrile and methanol were HPLC grade and purchased from Honeywell Burdick & Jackson Chemical Ltd., USA. All other chemicals and reagents were of analytical grade and purchased from Mike Chemical Reagents Co., Ltd. (Hangzhou, China).

### Microorganism mutagenesis and mutant screening

*A. nidulans* ZJB-0817, used in the present study, was preserved at the China Center for Type Culture Collection (CCTCC M 2010275, Wuhan, China). The strain was maintained on 200 g potato, 20 g dextrose, 20 g agar, and 1000 mL water (PDA) slants, stored at 4 °C, and renewed periodically. 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 wild strain was adjusted to  $10^7$  cells/mL in sterile water. A 20 mL sample of spore suspension was irradiated with ultraviolet light (wavelength, 254 nm; power, 30 W; distance, 30 cm) for 80 s, and then treated with lithium chloride (0.6 %, v/v) for 30 min, and finally treated with sodium nitrite (0.1 M, 1 M pH 4.5 acetate buffer) for 50 s. Subsequently, the treated spore suspension was plated in PDA plates and incubated at 25 °C for 7 days. After clones were formed, 460 clones were randomly selected and inoculated on PDA slants.

### Cultivation of wild strain and mutants for ECB production

Spore suspension of a 7-day-old culture grown on PDA slants was prepared by dispensing 20 mL of sterilized water and adjusted to  $10^7$  cells/mL. A 1.2 mL spore suspension was inoculated to a 500 mL Erlenmeyer flask containing 100 mL of basic medium (10 g glycerol, 10 g

peptone, 40 g soybean meal, 20 g peanut oil, 5 g L-proline, 5 g CaCl<sub>2</sub>, 8 g K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 0.05 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g MnSO<sub>4</sub>·H<sub>2</sub>O, 0.6 g CuSO<sub>4</sub>·5H<sub>2</sub>O, and 1000 mL water, initial pH 6.6) and cultured in a rotary shaker at 200 rpm and 25 °C for 12 days.

### Analysis of ECB production

The concentrations of ECB were determined by HPLC. The chromatographic conditions were as follows: ODS column (Shim-pack, VP-ODS, 4.6 mm × 250 mm, Shimadzu Corporation, Kyoto, Japan); UV detector; detection wavelength 222 nm; flow rate 1.0 mL/min; mobile phase, methanol: acetonitrile: water in the ratio of 70:10:20 (v/v); injection volume 10 µL. The retention times for ECB were around 13.5 min. Moreover, all samples were filtered through a 0.22 µm one-off filtration membrane prior to injection into HPLC system to get accurate results. The ECB was confirmed by liquid chromatography/mass spectrometry (Suppl. Fig. S1) and NMR spectroscopy (Suppl. Fig. S2), the concentration of ECB was determined using standard plots obtained with pure ECB.

### Fractional factorial design and data analysis

Fractional factorial design (FFD) was used to screen significant factors affecting ECB production. The first-order model was suggested, as follows Eq. (1):

$$Y = \alpha_0 + \sum \alpha_i x_i \quad (1)$$

where  $Y$  is the response of ECB production;  $\alpha_0$  is the model intercept and  $\alpha_i$  is the coefficient of the linear equation, and  $x_i$  is the dimensionless coded level of the independent variable. Each coded variable represents two levels, low (−1) and high (+1), according to the following Eq. (2):

$$x_i = \frac{X_{i1} - X_{i0}}{\Delta X_i} \quad (i = 1, 2, 3, \dots, k) \quad (2)$$

where  $x_i$  is the dimensionless coded value of the real test variable  $X_i$ ;  $X_{i1}$  is the real value of the variable  $X_i$ ;  $X_{i0}$  is the value of  $X_i$  using the central point of the investigated ranged, and  $\Delta X_i$  is the change in size of the step. Eight variables (peptone, MnSO<sub>4</sub>, soybean, CaCl<sub>2</sub>, K<sub>2</sub>HPO<sub>4</sub>, tomato powder, mannitol, L-ornithine) were selected and evaluated using FFD. 2<sup>8-3</sup> fractional factorial design (32 experimental runs) using four central point replications are carried out. All experiments were carried out in triplicates. The statistical software package “Minitab Release 16”, Minitab Inc., State College, PA, USA, was used for analyzing the experimental data. The fit of the first-order

equation model was checked using the coefficient of determination  $R^2$  and the adjusted  $R^2$ . The significant variables were screened on the basis of the  $F$  test and the  $p$  value at a confidence interval of 99 %.

### Central composite design and data analysis

Optimization of the four most significant variables (peptone, mannitol, L-ornithine and K<sub>2</sub>HPO<sub>4</sub>), identified by FFD was carried out by central composite design (CCD) for enhancing ECB production. The four independent variables were studied at five different levels (−2, −1, 0, +1, +2), in a set of 31 experiments that including eight axial points, sixteen factorial points and seven center points. The same software package as used for FFD was used for analyzing the experimental data. All experiments were conducted in triplicates and the mean production of ECB (mg/L) was taken as the response ( $Y$ ). Quadratic regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. The behavior of the system was explained by the following second-order polynomial Eq. (3):

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{i,j=1}^4 \beta_{ij} X_{ij} \quad (3)$$

where  $Y$  is the predicted response,  $\beta_0$  is offset term,  $\beta_i$  are coefficients for the linear terms,  $\beta_{ii}$  are for the square terms,  $\beta_{ij}$  are the coefficients of interactive terms. Optimum levels of all four independent variables were determined by solving the regression equation and analyzing the response surface contour plots.

## Results

### Sequential mutagenesis and screening high-yield mutants

The production of ECB after sequential mutagenesis by various mutants is indicated in Table 1. *A. nidulans* ZJB-0817 was initially exposed to UV irradiation. The best UV-mutant strain, UV-30, produced 1060.3 ± 43.8 mg ECB/L, showing a 1.4-fold production improvement compared to the parent strain (750.8 ± 32.0 mg/L). The UV-treated mutant strain was further treated with lithium chloride and sodium nitrite, and one of the resulting mutants, ULN-59, had the highest production of ECB (1583.1 ± 40.9 mg/L), showing a 2.1-fold production improvement compared to the parent strain (Suppl. Fig. S3). The stability of mutant A.

**Table 1** The production of echinocandin B by the mutant *Aspergillus nidulans* produced through various mutagenic treatments

Mutagenic treatment	Strain	Echinocandin B titre (mg/L)	Relative production (%) <sup>a</sup>
None	ZJB0817	750.8 ± 32	100
UV irradiation	UV-30	1060.3 ± 43.8	141.3
Lithium chloride treatment			
1st round	UL-1	1202.1 ± 40.3	153.5
2nd round	UL-6	1242.5 ± 15.5	158.7
3rd round	UL-8	1576.4 ± 19.4	209.9
Sodium nitrite treatment	ULN-59	1583.1 ± 40.9	211.1

<sup>a</sup> Percentage of echinocandin B production compared with the wild type strain

**Table 2** Effect estimates of eight component variables for the echinocandin B production from the results of Plackett–Burman design

Variable	Coefficient estimate	<i>F</i> value	<i>p</i> value Prob. > <i>F</i>
Model	936.6	35.25	0.000
$x_1$ (peptone)	−158.0	−5.94	0.002*
$x_2$ (MnSO <sub>4</sub> )	23.2	0.87	0.423
$x_3$ (soybean)	87.4	3.29	0.022
$x_4$ (CaCl <sub>2</sub> )	44.9	1.69	0.152
$x_5$ (K <sub>2</sub> HPO <sub>4</sub> )	−210.4	−7.92	0.001**
$x_6$ (tomato powder)	53.8	2.02	0.099
$x_7$ (mannitol)	110.3	4.15	0.009*
$x_8$ (L-ornithine)	106.1	3.99	0.010*

Values of probability of failure (“Prob. > *F*”) less than 0.0010 and 0.0100 indicate that model terms are very significant (\*\*) and significant (\*), respectively

*nidulans* ULN-59 for ECB production was determined by successive subculturing on ECB production agar for over 1 year. The mutant was subcultured every month and evaluated for its ability to stably produce ECB. The mutant ULN-59 maintained the same production yield after being subcultured ten times (Suppl. Fig. S4), indicating that the mutant has heritable character.

### Screening significant culture medium for enhancing ECB production

According to the results of single-factor research, peptone, MnSO<sub>4</sub>, soybean, CaCl<sub>2</sub>, K<sub>2</sub>HPO<sub>4</sub>, tomato powder, mannitol, L-ornithine were taken as the main medium component. The fractional factorial design-based experiments with eight variables were performed to select the significant culture medium affecting ECB production by mutant ULN-59 (Suppl. Table S1). The FFD was utilized because it is accurate for estimating the main effects and interaction effects with a reduced number of experimental runs compared to a complete factorial design [8]. Table 2 presents the statistical analysis of the FFD with the estimated coefficients and significant levels for the variables in the ECB production. The *R*<sup>2</sup> value was 0.963, indicating that 96.3 % of the variability in the response could be explained by the model. The *F* value and *p* value were selected for

analysis of variance (ANOVA) of the model. According to an ANOVA of FFD results with replicated center points, the curvature in the tested region was significant with a *p* value of less than 0.01, which suggests that a response surface study with a quadratic model is required to optimize ECB production with the selected significant variables.

Based upon the statistical analysis data, four variables (peptone, mannitol, K<sub>2</sub>HPO<sub>4</sub> and L-ornithine) were significant model terms at the 99 % level with *p* values of less than 0.01 (Table 2). A response surface study with a quadratic model can be applied with the significant variables determined from the FFD study. The common significant variables (peptone, mannitol, K<sub>2</sub>HPO<sub>4</sub> and L-ornithine) in the production of ECB were chosen for response surface studies.

### Optimization of culture medium for ECB production

Before optimizing the culture medium, the steepest ascent experiment was carried out, and the results (Suppl. Table S2) showed that the center point of the four significant components was chosen as 10 g/L peptone, 10 g/L K<sub>2</sub>HPO<sub>4</sub>, 100 g/L mannitol, and 6 g/L L-ornithine. To have an accurate optimization of the four significant

**Table 3** Experiential design and results of the central composite design

Runs	Coded values and real values (g/L)				Echinocandin B (mg/L)	
	$x_1$ (peptone)	$x_5$ (K <sub>2</sub> HPO <sub>4</sub> )	$x_7$ (mannitol)	$x_8$ (L-ornithine)	Experimental	Predicted
Central composite design						
1	8.6 (-1)	8.2 (-1)	95 (-1)	5 (-1)	2272.8 ± 31.1	2254.2
2	11.4 (1)	8.2 (-1)	95 (-1)	5 (-1)	1387.8 ± 18.3	1396.2
3	8.6 (-1)	11.8 (1)	95 (-1)	5 (-1)	1424.5 ± 58.7	1607.0
4	11.4 (1)	11.8 (1)	95 (-1)	5 (-1)	1116.0 ± 33.3	1170.1
5	8.6 (-1)	8.2 (-1)	105 (1)	5 (-1)	1778.8 ± 41.2	1832.1
6	11.4 (1)	8.2 (-1)	105 (1)	5 (-1)	1481.0 ± 45.2	1395.9
7	8.6 (-1)	11.8 (1)	105 (1)	5 (-1)	1300.0 ± 35.7	1184.8
8	11.4 (1)	11.8 (1)	105 (1)	5 (-1)	1083.2 ± 42.0	1169.9
9	8.6 (-1)	8.2 (-1)	95 (-1)	7 (1)	1627.5 ± 25.8	1591.2
10	11.4 (1)	8.2 (-1)	95 (-1)	7 (1)	990.0 ± 11.0	1119.0
11	8.6 (-1)	11.8 (1)	95 (-1)	7 (1)	1131.0 ± 23.1	1158.8
12	11.4 (1)	11.8 (1)	95 (-1)	7 (1)	1182.1 ± 33.3	1107.9
13	8.6 (-1)	8.2 (-1)	105 (1)	7 (1)	1497.4 ± 31.6	1503.2
14	11.4 (1)	8.2 (-1)	105 (1)	7 (1)	1656.3 ± 59.0	1452.9
15	8.6 (-1)	11.8 (1)	105 (1)	7 (1)	1029.0 ± 20.1	1070.8
16	11.4 (1)	11.8 (1)	105 (1)	7 (1)	1303.1 ± 54.2	1441.8
17	7.2 (-2)	10 (0)	100 (0)	6 (0)	1940.1 ± 20.2	1919.0
18	12.8 (2)	10 (0)	100 (0)	6 (0)	1404.0 ± 15.0	1431.9
19	10 (0)	6.4 (-2)	100 (0)	6 (0)	1535.2 ± 53.4	1658.2
20	10 (0)	13.6 (2)	100 (0)	6 (0)	1116.1 ± 22.3	999.8
21	10 (0)	10 (0)	90 (-2)	6 (0)	1443.5 ± 29.7	1415.2
22	10 (0)	10 (0)	110 (2)	6 (0)	1291.8 ± 58.8	1326.9
23	10 (0)	10 (0)	100 (0)	4 (-2)	1409.1 ± 29.0	1434.0
24	10 (0)	10 (0)	100 (0)	8 (2)	1060.9 ± 30.0	1042.8
Central points						
25	10 (0)	10 (0)	100 (0)	6 (0)	2100.2 ± 11.1	2018.9
26	10 (0)	10 (0)	100 (0)	6 (0)	1971.8 ± 25.2	2018.9
27	10 (0)	10 (0)	100 (0)	6 (0)	2055.9 ± 16.2	2018.9
28	10 (0)	10 (0)	100 (0)	6 (0)	2040.3 ± 18.8	2018.9
29	10 (0)	10 (0)	100 (0)	6 (0)	1931.2 ± 41.5	2018.9
30	10 (0)	10 (0)	100 (0)	6 (0)	2103.8 ± 14.1	2018.9
31	10 (0)	10 (0)	100 (0)	6 (0)	1929.3 ± 28.9	2018.9

components, a four-factor, five-level rotatable central composite design was carried out. The design matrix and corresponding experimental data are given in Table 3. The regression coefficient, *F* value, *p* value and standard error are summarized in Table 4. *F* value and *p* value indicate the significance of the regression coefficient. From Table 4, the linear coefficients  $x_1$ ,  $x_5$  and  $x_8$ , the quadratic coefficient  $x_1^2$ ,  $x_5^2$  and  $x_7^2$  as well as the interaction coefficient  $x_1x_5$ ,  $x_1x_7$ ,  $x_1x_8$  and  $x_7x_8$  are all significant at a 1 % significance level. Therefore, the linear coefficients  $x_1$ ,  $x_5$  and  $x_8$ , the quadratic coefficient  $x_1^2$ ,  $x_5^2$  and  $x_7^2$  as well as the interaction coefficient  $x_1x_5$ ,  $x_1x_7$ ,  $x_1x_8$  and  $x_7x_8$  are the most influential factors. On the other hand, the linear effect  $x_7$  and the interactive effects of  $x_5x_7$  and  $x_5x_8$  on ECB yield

were all slight, as indicated by the large *p* value. By applying multiple regression analysis on the experimental data, the following second-order polynomial Eq. (4) was established to explain the production of ECB:

$$Y = 2018.92 - 121.77x_1 - 164.60x_5 - 97.79x_8 + 105.29x_1x_5 + 105.47x_1x_7 + 96.47x_1x_8 + 83.54x_7x_8 \tag{4}$$

where *Y* is the predicted response, and  $x_1$ ,  $x_5$ ,  $x_7$ , and  $x_8$  are the coded values for peptone, K<sub>2</sub>HPO<sub>4</sub>, mannitol, and L-ornithine, respectively.

The optimal conditions for maximum ECB production, calculated by setting the partial derivatives of Eq. (4) to zero with respect to the corresponding variables, were as

**Table 4** Result of regression analyses of central composite design

Variables	Coefficient	Standard error	F value	p value Prob. > F
Model	2016.74	45.75	44.078	0.0001
$x_1$	-121.76	24.71	-4.928	0.0001**
$x_5$	-164.6	24.71	-6.661	0.0001**
$x_7$	-22.07	24.71	-0.893	0.385
$x_8$	-97.79	24.71	-3.957	0.0012*
$x_1^2$	-85.32	22.64	-3.769	0.0022*
$x_5^2$	-171.93	22.64	-7.595	0.0002**
$x_7^2$	-161.42	22.64	-7.131	0.0002**
$x_8^2$	-195.59	22.64	-8.596	0.0002**
$x_1x_5$	105.29	30.26	3.479	0.0032*
$x_1x_7$	105.47	30.26	3.485	0.0032*
$x_1x_8$	96.47	30.26	3.188	0.0062*
$x_5x_7$	-17.81	30.26	-0.588	0.565
$x_5x_8$	53.73	30.26	1.775	0.095
$x_7x_8$	83.54	30.26	2.761	0.0143

Values of probability of failure (“Prob. > F”) less than 0.0010 and 0.0100 indicate that model terms are very significant (\*\*) and significant (\*), respectively

$x_1$  peptone,  $x_5$   $K_2HPO_4$ ,  $x_7$  mannitol,  $x_8$  L-ornithine

follows: peptone 8.6 g/L,  $K_2HPO_4$  8.4 g/L, mannitol 97.3 g/L, L-ornithine 5.3 g/L. The maximum response value for ECB production was estimated as 2277.2 mg/L.

With ECB production value as the response, the three-dimensional response surfaces and two-dimensional contour plots are displayed in Fig. 2a–d. These figures demonstrated the relative effects of two variables on the production of ECB while the third value is kept constant. Figure 2a–d depicts the three-dimensional response surfaces and two-dimensional contour plots for the optimization of the medium compositions for the production of ECB. Each response surface plot presented a clear peak and the corresponding contour plot depicted the effect of two variables (among them peptone,  $K_2HPO_4$ , mannitol and L-ornithine) on the production of ECB within their experimental range, while the other variable was constant at its central point level. As shown in Fig. 2a–d, the maximum ECB production was predicted within the range of this study, indicating that further increase of peptone,  $K_2HPO_4$ , mannitol and L-ornithine concentrations would not cause a further increase in ECB production. Figure 2a represents the interaction between peptone and  $K_2HPO_4$  and its effect on the level of ECB produced. The ECB production generally increased to a peak value with an increase in peptone concentration and a decrease in  $K_2HPO_4$  concentration, and then decreased with a further increase in peptone concentration or a decrease in  $K_2HPO_4$  concentration. The variable of peptone concentration is relatively more significant than the  $K_2HPO_4$  concentration on the production of ECB. The two-dimensional contour plot shows a symmetrical mound shape with a coordinate

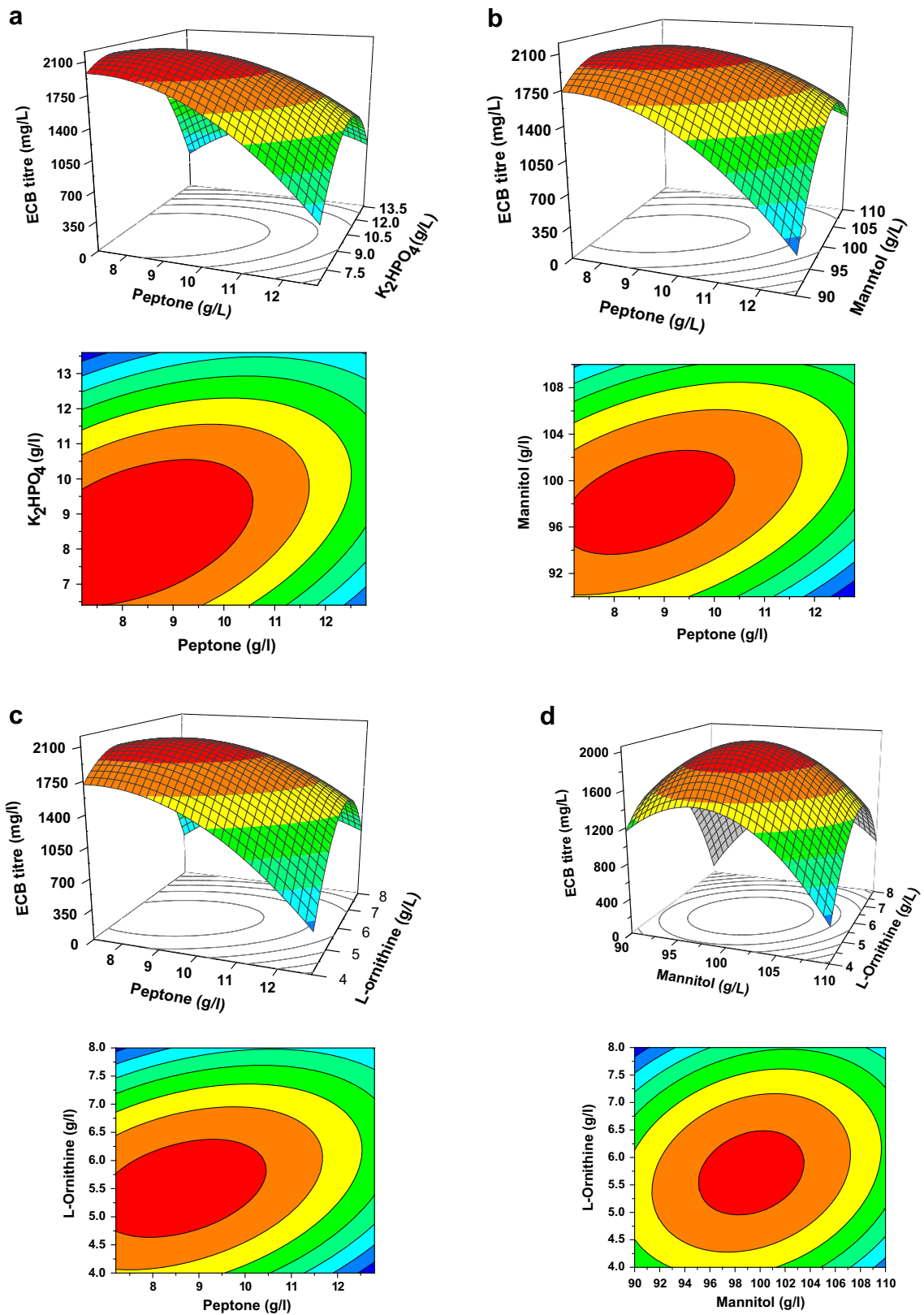
axis and the p value (0.0032, ANOVA) is far lower than 0.05, indicating a significant effect on the interaction between  $K_2HPO_4$  and peptone concentrations. Similar result was also found in the significant interaction effect on ECB production against peptone and mannitol (Fig. 2b), peptone and L-ornithine (Fig. 2c), mannitol and L-ornithine (Fig. 2d).

### Experimental verification of theoretical optimum

To validate the model of the optimization approach, confirmation experiments were carried out in triplicate under conditions as predicted by the model: peptone 8.6 g/L,  $K_2HPO_4$  8.4 g/L, mannitol 97.3 g/L, L-ornithine 5.3 g/L and a mean experimental value of ECB production  $2285.6 \pm 35.6$  mg/L was obtained. There was a good agreement between the predicted value (2277.2 mg/L) and the empirical experimental results ( $2285.6 \pm 35.6$  mg/L) verifies the validity of the model. ECB production using the predicted optimal medium was significantly enhanced, being 1.4-fold higher than that with original medium ( $1583.1 \pm 40.9$  mg/L). It demonstrates that the CCD design is a successful approach for estimating optimal medium parameters for ECB production.

### Discussion

There have been no reports made concerning the development of an *Aspergillus* strain via mutagenesis for the production of ECB. In our study, the strain improvement



**Fig. 2** Three-dimensional response surface curves and contour plot of the echinocandin B production as a function of peptone and  $K_2HPO_4$  (a), mannitol and peptone (b), peptone and L-ornithine (c), mannitol and L-ornithine (d)

using sequential mutagenesis proved to be an effective method in enhancing ECB production from *A. nidulans*. Four hundred and sixty colonies were screened after sequential mutation that involved the use of physical UV radiations and chemical mutagens (lithium chloride and sodium nitrite) treatment, and a total of 15 mutants was detected with notably improved level of ECB production. The mutant *A. nidulans* ULN-59 exhibited the highest ECB production ( $1583.1 \pm 40.9$  mg/L) and improved the yield 2.1 times as compared to production by parent strain (Suppl. Fig. S3). These results suggested that physical and chemical mutations are considered to have the potential to change the echinocandin-type compound content of fungal strain. Kanda also reported increased echinocandin-type compound FR901379 production by a non-foaming mutant of *Coleophoma empetri* F-11899 which was generated through UV mutagenesis. Under the optimum culture conditions, the mutant produced 5.0 U/mL FR901379, while 1.0 U/mL of production was achieved in wild type strain [9, 14]. Masarekar et al. isolated *Zalerion arboricola* MF5415 by two chemical mutagens (*N*-nitroso-*N*-methylurethane and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine) treatment which was found to be increased 2.5-fold yield of pneumocandins  $A_0$  compared to their parent strain [20]. The stability of strains is considered an important and useful criterion for industrial fermentation. As shown in Suppl. Fig. S4, the mutant *A. nidulans* ULN-59 maintained the same production yield after being subcultured ten times and hence should have great potential use in the industrial production of ECB.

Productivity of any fungal fermentation is affected by media composition and process parameters and therefore the further investigation was performed to statistically optimize the culture medium for the production of ECB from *A. nidulans* ULN-59 using FFD and CCD methodologies. All significant variables involved in ECB production were evaluated by FFD because it can test a large number of variables while avoiding the loss of any essential information in subsequent optimization studies. Based on analysis of FFD, four key variables, viz., peptone, mannitol,  $K_2HPO_4$  and L-ornithine were all positively significant factors for ECB production (Table 2). Among the four factors, peptone, as a nitrogen source, had the most influence on ECB yield ( $p < 0.002$ ). It was also a supplementary nitrogen source and benefited other lipopeptide antibiotics (such as pneumocandin  $A_0$ , FR901379, and iturin A) production by fungi [14, 20]. Mannitol, the most significant medium component effecting ECB production by *A. nidulans* ULN-59, was also used as an important carbon source for producing echinocandins-type pneumocandin  $A_0$  by *Zalerion arboricola* [20]. It is rich cost-effective, making it a valuable nutrient source in the fermentation industry. The biosynthesis of many different

types of antibiotics and other secondary metabolites is regulated by phosphate.  $K_2HPO_4$ , as an available phosphate and inorganic salts, is often an important factor for the production of secondary metabolites by certain fungi.  $K_2HPO_4$  had significant effects on the production of streptolydigin in *Streptomyceslydicus* AS 4.2501 [19]. In the present study,  $K_2HPO_4$  appeared to have an important role in ECB production. In addition, according to the chemical structure of ECB (Fig. 1), L-ornithine can be a biosynthetic precursor of ECB production; thus, it is reasonable why L-ornithine has obvious effect on ECB production. This was followed by use of a multifactorial response surface approach employing CCD, an effective design strategy, for studying the effects of key variables and their mutual interactions. The optimum medium contained: 8.6 g peptone, 97.3 g mannitol, 8.4 g  $K_2HPO_4$  and 5.3 g L-ornithine per liter. The optimized medium provided another 1.4-fold increase in final ECB concentration to  $2285.6 \pm 35.6$  mg/L compared to the original medium. The results of the validation experiment and the predicted value matched perfectly, proving that the statistically based experimental designs had been effective in enhancing the production of ECB.

To explore the difference between the original and optimized medium, carbon and nitrogen contents in the two mediums were analyzed and carbon–nitrogen ratio was calculated in Table 5. Comparing the original medium, carbon–nitrogen ratio of the optimized medium was increased from 9.23:1 to 18.09:1 (Table 5), which might be the most important reason for ECB production enhancement. ECB is a cyclic hexapeptide with a fatty acid (linoleoyl) side chain (Fig. 1). Fatty acids are synthesized

**Table 5** Carbon and nitrogen components and carbon–nitrogen ratio of the basic and optimized medium

Medium	Components	C (g/L)	N (g/L)	C:N
Original medium	Soybean meal	18.98	2.8	9.23:1
	Peptone	1.04	1.3	
	Peanut oil	14.52		
	Glycerin	3.91		
	L-Proline	0.521	0.12	
	Sum	38.97	4.22	
Optimized medium	Soybean meal	18.98	2.8	18.09:1
	Mannitol	38.455		
	Peptone	0.894		
	Peanut oil	14.52		
	Glycerin	3.91		
	L-Ornithine	2.39	1.11	
	L-Proline	2.605	0.61	
	Sum	81.75	4.52	



with acetyl-CoA and malonyl-CoA starters [5, 6, 30]. Leman reported that a relatively high carbon–nitrogen ratio would improve fatty acids synthesis from acetyl-CoA and malonyl-CoA [17]. Coincidentally, in our case, ECB has a fatty acid side chain and it was postulated to be synthesized from acetyl-CoA and malonyl-CoA. Therefore, a relatively high carbon–nitrogen ratio would also improve the ECB production.

Low yield and high production costs in the currently available commercial ECB have limited its widespread use [9]. Thus there is a need for high ECB producing strain and improvement of ECB productivity. In this work, a combined approach of microbial strain improvement and statistical optimization was successfully developed for enhanced production of echinocandin B by *A. nidulans* ZJB-0817. The overall results obtained show a remarkable 3.0-fold (from 750.8 to 2285.6 mg/L) improvement in ECB production when compared to that using the original medium. The applied methods of sequential mutagenesis and statistical optimization were efficient, relatively simple, and time and material saving. These results of this work promise to improve the present production of ECB and may be adopted to enhance other objective products' production by fungal strains.

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