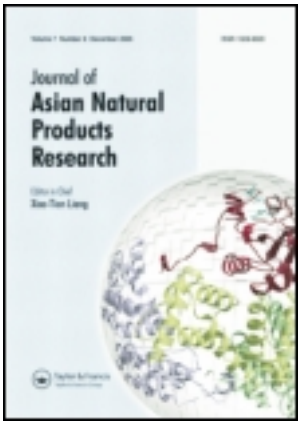

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Medium optimization for enhanced co-production of two bioactive metabolites in the same fermentation by a statistical approach

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This paper describes improved optimization method that combines the one-factor-at-a-time method (OFAT), Plackett–Burman design, and the response surface method (RSM), which were used to optimize the medium for the production of fumigaclavine C (FC) and helvolic acid (HA) from endophytic *Aspergillus fumigatus* CY018 simultaneously. The ideal carbon and nitrogen sources for the two compounds were assessed initially via the one-factor-at-a-time method. Three key cultivation factors (pH, phosphate, and inoculum size) were chosen based on the results of Plackett–Burman design, and subsequently optimized by the central composite design. The two metabolites were amply afforded when the cultivation was carried out with the inoculum size of 2.45% at pH 4.2 and 28°C for 19 days in the medium containing (g/l): mannitol 50, sodium succinate 5.4, $NaNO_3$ 2, $MgSO_4{\cdot}7H_2O$ 0.3, $FeSO_4{\cdot}7H_2O$ 0.01, and KH_2PO_4 0.67. The highest yields of FC and HA achieved herein were 17.26 and 16.88 mg/l. This work might be the first endeavor leading to the improved simultaneous production of two complex active metabolites with a single strain.

Keywords: fumigaclavine C; helvolic acid; co-production; Plackett–Burman design; response surface methodology

1. Introduction

Microbes are a rich source of bioactive small-molecule metabolites, of which some have been developed as life-rescuing antibiotics such as penicillin and streptomycin. In continuation of our characterization of bioactive natural products from symbiont cultures [1], *Aspergillus fumigatus* CY018 living originally inside the leaves of the salinity-tolerant plant *Cynodon dactylon* was ascertained to be an efficient producer of fumigaclavine C (FC) and helvolic acid (HA) [2], both being structurally unique (Figure 1) and functionally promising. FC is a prenylated indole alkaloid possessing four chiral carbons, which could only be isolated from the cultures of some *A. fumigatus* strains. In addition to its vasorelaxant action in isolated rat aortic rings [3], this alkaloid improves experimental colitis in mice via downregulating Th1 cytokine production and matrix metalloproteinase activity [4], and it attenuates additionally the concanavalin A-induced liver injury in mice mainly via inhibiting TNF-α (tumor necrosis factor-α) production and lymphocyte adhesion to extracellular matrices [5]. Moreover, as a clavine, FC shares the core structure with most ergopeptine clinic drugs such as bromocriptine, cabergoline, and ergometrine, and therefore can be easily modified to these drugs, even stronger neurotransmitter regulator compounds curing Parkinsonism and other nervous disease as precursor

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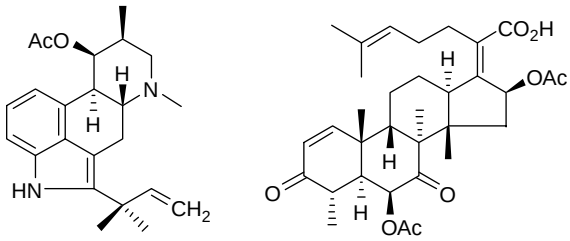

Fumigaclavine C **Helvolic acid**

Figure 1. Structures of FC and HA.

through chemical, bioconversion, or directed biosynthesis methods.

HA, a structurally complex triterpene antibiotic of a fusidane carbon skeleton was first characterized in 1943 from the culture filtrate of *A. fumigatus* [6]. It was later reisolated from other fungal species including *Cephalosporium caerulens*, *Fusidium coccineum*, and *Alternaria* sp. [7,8]. HA is an antibacterial agent active not only against ordinary clinic pathogens *Staphylococcus aureus* and *Helicobacter pylori*, but also against rare archaea such as *Sulfolobus solfataricus* [9–11]. Interestingly, HA has recently been disclosed to be substantially synergistic with penicillin and erythromycin against a set of multidrug-resistant *S. aureus* strains [12]. Furthermore, this triterpene acid was demonstrated to be phytotoxic directly or indirectly via its antibiotic-like action on the rhizosphere organisms [13]. Recently, much endeavor has been put on the biosynthesis of this compound. The key oxidosqualene cyclase and relative tailoring process had been found and clarified [14]. Structurally, another two commercial antibiotics fusidic acid and cephalosporin P1 have minute difference with HA of less than five groups, and so HA can be available as a direct source of these antibiotics production only if obtained with high yield and simple industrial process. The aforementioned observations highlighted the significance of the two fungal metabolites in terms of function and applicability. Accordingly, the reliable availability of FC and HA is highly desired for usage-oriented developments, and the approach has to be set up for a limitless mass supply. Since the structural complexity of the two metabolites foretells that both could not be chemically synthesized in a cost-effective manner, the ample generation of FC and HA seems to be largely or exclusively expected from the scaled-up microbial fermentation. Moreover, it has been rarely reported to enhance the co-production of two or more drug(-like) molecules with a single strain although optimizations aiming at the improved accumulation of a single compound are frequently encountered [15]. Wei *et al.* [16] reported the co-production of fumaric acid and chitin from a nitrogen-rich lignocellulosic material (dairy manure) using a pelletized filamentous fungus *Rhizopus oryzae* ATCC 20344. Kang *et al.* [17] proposed a novel strategy for succinate and polyhydroxybutyrate co-production in *Escherichia coli*. Above works focused on the primary metabolites. Our experiment aims at secondary metabolites.

Previously, we recognized that CY018 is a generous co-producer of FC and HA [2], which share the earlier steps of the biosynthetic pathways [18,19]. Additionally, in our previous work of FC and HA extraction from solid state fermentation, we found that their characteristics, belonging to alkaloid and acid, respectively, will allow a feasible downstream process at lower cost. Conventional FC extraction, an acid

aqueous solution (pH 2) partition followed by alkalization with sodium carbonate (pH 10), may be an effective way to separate the two compounds simultaneously. These gave us collectively a strong impetus to establish a protocol that may allow the improved co-production of FC and HA by *A. fumigatus* CY018. Due to the similarity in multiple factors including the carbon source, nutrition component, and cultivation time course obtained by OFAT experiments, we supposed whether the optimized condition for one compound can also increase the yield of the other. As detailed subsequently, the production of the two metabolites by the fungus was actually co-enhanced remarkably with the maximum yield of FC and HA up to 17.26 and 16.88 mg/l, respectively, being approximately 2- and 10-fold increases relative to those afforded in the basal culture condition.

2. Results and discussion

2.1 Optimization of medium carbon and nitrogen source

2.1.1 Effect of carbon source

In the present investigation, the first experimentation was designed for evaluating comparatively the effect of the different carbon sources on the fungal growth as well as on FC and HA productions. As a result, cultivation with mannitol, sucrose, and glucose permitted better fungal growth (dry weight of cells: ~4 g/l), indicating that monosaccharide, disaccharide, and sugar alcohol were more propitious to the biomass of the strain. This observation rationalized our finding that mannitol favored the highest FC yield of 10.16 mg/l (Figure 2). And the results were also in harmony with Moussa's [20] observation about the production of alkaloids with the core ergoline ring existing in FC similarly, which demonstrated that the slowly metabolized carbon sources including sucrose and mannitol remain sufficient for the secondary metabolism at the later stage of the fermentation. Different from *Claviceps purpurea* [21] and *Penicillium roquefortii* [22] of ergot alkaloid producing capacity, this study showed that *A. fumigatus* CY018 was able to effectively utilize slowly releasing carbon sources in its vegetative period. The highest yield of HA was observed when glucose was used as the carbon source. As the optimum carbon resource for FC production, meanwhile, mannitol is also the third optimum carbon nutrition for HA. Therefore, we take mannitol as the carbon resource for FC optimization, which may also have positive effect on HA production based on the observation.

2.1.2 Effect of nitrogen source

As shown in Figure 3, the strain was able to utilize a wide range of nitrogen sources. However, the inorganic nitrogen sources including $NaNO_3$ and $(NH_4)_2SO_4$ seemed more supportive for biomass accumulation and FC formation than organic nitrogen sources urea and yeast extract did. This observation was distinct from Moussa's [20]

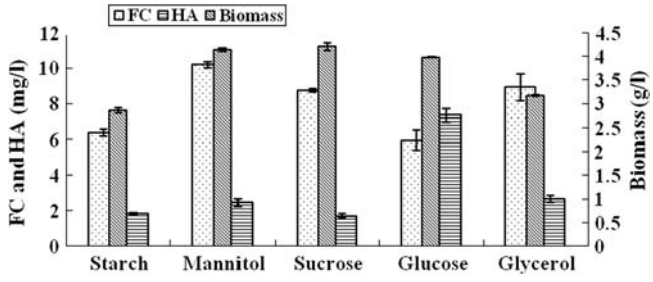

Figure 2. Effect of carbon sources on the fungal growth and production of FC and HA.

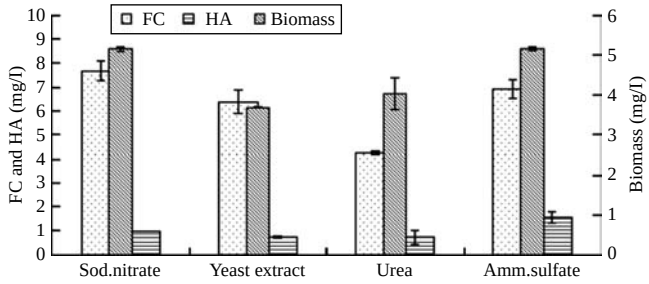

Figure 3. Effect of nitrogen sources on the fungal growth and production of FC and HA.

report that ammonium salt was capable of inhibiting the ergot alkaloid biosynthesis. As a matter of fact, the highest biomass accumulation (5.14 g/l) and FC production (7.67 mg/l) were recorded when the strain was fed on sodium nitrate. This is in agreement with the recent observation that structural gene of transcription and concomitant metabolite synthesis can be repressed by high levels of nitrogen and alkaline pH in some species of fungi [23]. In addition, the ions are readily adsorbed and assimilated by fungal cells. Furthermore, the nitrogen sources given at $N = 0.4$ g/l met the requirements both for the vegetative growth and for the subsequent secondary metabolism. Interestingly, the highest content of HA was also detected when ammonium sulfate and sodium nitrate were applied, demonstrating that inorganic nitrogen sources favored the HA production. Accordingly, as we described HA enhancement at the end of last paragraph, we chose sodium nitrate for subsequent experiment (Table 1).

2.1.3 *Selection of critical physicochemical components*

The design aimed at the yield of FC as dependent variable instead of HA because, from the view point of their production sites, the mainly spores derived FC seemed harder to enhance in liquid cultivation compared to mycelium derived HA. A wide variation in FC production was observed in Plackett–Burman experiments. This variation reflected the importance of optimization to obtain the high yield of FC. Analyzed by Statistica 6.0, the data of regression are shown in Table 2. A first-order model was fitted to the result obtained from the 12 experimental runs:

$$\begin{aligned} Y(\mathrm{mg/l}) = {} & 15.86 - 0.02\mathrm{A} - 0.69\mathrm{B} \\ & + 3.00\mathrm{C} - 2.64\mathrm{D} + 8.00\mathrm{E} \\ & - 2.01\mathrm{F} + 95.83\mathrm{G} - 0.10\mathrm{H} \\ & - 0.02\mathrm{I} \\ & (\mathrm{A}: \mathrm{M}, \quad \mathrm{B}: \mathrm{SN}, \quad \mathrm{C}: \mathrm{P}, \\ & \mathrm{D}: \mathrm{Mg}, \quad \mathrm{E}: \mathrm{Fe}, \quad \mathrm{F}: \mathrm{pH}, \\ & \mathrm{G}: \mathrm{IS}, \quad \mathrm{H}: \mathrm{T}, \quad \mathrm{I}: \mathrm{RS}). \end{aligned} \tag{1}$$

The coefficient of determination (R^2) of the first-order model was 0.9820, explaining that nearly 99% of the variability in the response could be indicated by the model. The *t*-test was employed to identify the effect of every factor on FC production. Table 2 showed that the concentration of phosphate, initial pH, and inoculum size were the most significant factors ($P < 0.05$). Hence, all these factors were selected for further optimization to obtain a maximum response.

2.1.4 *Time course of the fungal growth and production profile of FC and HA*

The growth curve of the title strain and its production profile of FC and HA were carried out with time. Notably, both FC and HA increases were observed since the 13th day of fermentation, and reached the maxima on days 19 and 16, respectively.

Table 1. Plackett–Burman experimental design matrix for screening of important variables for FC production.

	Variables											
No.	M ($g\,l^{-1}$)	SN ($g\,l^{-1}$)	P ($g\,l^{-1}$)	Mg ($g\,l^{-1}$)	Fe ($g\,l^{-1}$)	pH	IS (%)	T (°C)	RS ($r\,m^{-1}$)	Dummy 1	Dummy 2	Yield ($mg\,l^{-1}$)
1	1 (70.00)	−1 (1.00)	1 (1.00)	−1 (0.20)	−1 (0.005)	1 (4.00)	−1 (2)	1 (32)	1 (140)	−1	1	5.12 ± 0.31
2	1 (70.00)	1 (3.00)	−1 (0.50)	1 (0.40)	−1 (0.005)	−1 (3.00)	−1 (2)	1 (32)	1 (140)	1	−1	3.95 ± 0.22
3	−1 (30.00)	1 (3.00)	1 (1.00)	−1 (0.20)	1 (0.010)	−1 (3.00)	−1 (2)	−1 (28)	1 (140)	1	1	7.44 ± 0.45
4	1 (70.00)	−1 (1.00)	1 (1.00)	1 (0.40)	−1 (0.005)	−1 (3.00)	1 (5)	−1 (28)	−1 (120)	1	1	9.82 ± 0.38
5	1 (70.00)	1 (3.00)	−1 (0.50)	1 (0.40)	1 (0.010)	1 (4.00)	−1 (2)	−1 (28)	−1 (120)	−1	1	2.81 ± 0.09
6	1 (70.00)	1 (3.00)	1 (1.00)	−1 (0.20)	1 (0.010)	−1 (3.00)	1 (5)	1 (32)	−1 (120)	−1	−1	8.16 ± 0.62
7	−1 (30.00)	1 (3.00)	1 (1.00)	1 (0.40)	−1 (0.005)	1 (4.00)	1 (5)	−1 (28)	1 (140)	−1	−1	5.93 ± 0.07
8	−1 (30.00)	−1 (1.00)	1 (1.00)	1 (0.40)	1 (0.010)	1 (4.00)	−1 (2)	1 (32)	−1 (120)	1	−1	6.14 ± 0.14
9	−1 (30.00)	−1 (1.00)	−1 (0.50)	1 (0.40)	1 (0.010)	−1 (3.00)	1 (5)	1 (32)	1 (140)	−1	1	7.87 ± 0.33
10	1 (70.00)	−1 (1.00)	−1 (0.50)	−1 (0.20)	1 (0.010)	1 (4.00)	1 (5)	−1 (28)	1 (140)	1	−1	6.40 ± 0.59
11	−1 (30.00)	1 (3.00)	−1 (0.50)	−1 (0.20)	−1 (0.005)	1 (4.00)	1 (5)	1 (32)	−1 (120)	1	1	5.67 ± 0.11
12	−1 (30.00)	−1 (1.00)	−1 (0.50)	−1 (0.20)	−1 (0.005)	−1 (3.00)	−1 (2)	−1 (28)	−1 (120)	−1	−1	6.89 ± 0.28

Note: M, mannitol; SN, sodium nitrate; P, KH_2PO_4; Mg, $MgSO_4{\cdot}7H_2O$; Fe, $FeSO_4{\cdot}7H_2O$; IS, inoculum size; T, temperature; RS, rotation speed.

Table 2. Estimated effect, regression coefficient, and corresponding T and P values for FC production Plackett–Burman design experiment.

Variables	Effect	Coefficient	T Value	P Value
M	−0.61	−0.01	−1.77	0.217
SN	−1.38	−0.69	−4.00	0.057
P	1.50	3.00	4.36	0.048
Mg	−0.52	−2.63	−1.52	0.266
Fe	0.24	48.00	0.69	0.558
PH	−2.01	−2.01	−5.83	0.028
IS	1.91	95.83	5.55	0.030
T	−0.39	−0.09	−1.15	0.368
RS	−0.46	−0.02	−1.34	0.311

Note: M, mannitol; SN, sodium nitrate; P, KH_2PO_4; Mg, $MgSO_4{\cdot}7H_2O$; Fe, $FeSO_4{\cdot}7H_2O$; IS, inoculum size; T, temperature; RS, rotation speed.

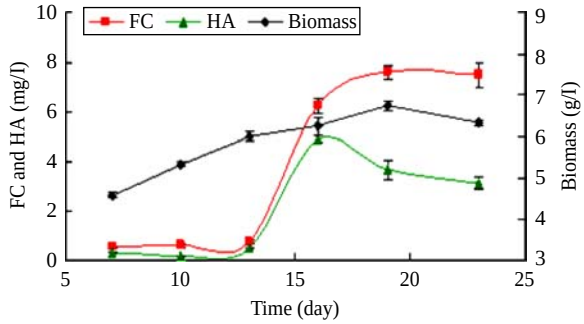

Figure 4. Time course of growth, FC, and HA production by *A. fumigatus* CY018.

After 'peak days,' the production of both metabolites declined at different rates (Figure 4). In accordance with this phenomenon, the growth rate of the fungus started reducing since the 13th day of fermentation, on which the conversion from trophophase to idiophase occurred. The co-decrease of biomass and the two metabolites after day 19 aroused most probably from nutrient stress. So we choose day 19 as the harvest day, when FC presented its highest yield and the yield of HA still retained in higher level.

2.2 *Optimization by RSM (Response Surface Method)*

Based on Plackett–Burman experiments (Table 1), inorganic phosphate concentration (X_1), pH (X_2), and inoculum size (X_3) were selected for further optimization by RSM because of their significant effects on FC production (P value: X_1, 0.048; X_2, 0.028; X_3, 0.030). Mannitol and sodium nitrate, proven to be the optimal carbon and nitrogen sources for FC synthesis, were also acceptable for HA production. The Central Composite Design (CCD) for FC was directly adopted to examine the yield of HA. To assess the combined effect on FC production of these medium components (independent variables), a central composite factorial design of $2^3 = 8$ plus 2 center points and 6 ($2 \times 3 = 6$) star points leading to a total of 16 experiments were carried out. Equation (5) represents the mathematical model relating to the independent process variables, X_i and the second-order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Statistica 6.0. A CCD matrix of the independent variables in the form of coded

Table 3. The CCD matrix of independent variables in code and actual values with their corresponding responses in terms of production of FC.

No.	P (g/l)	pH	IS (%)	FC (mg/l)
1	−1.00 (0.50)	−1.00 (2.00)	−1.00 (1.00)	8.90 ± 0.13
2	−1.00 (0.50)	−1.00 (2.00)	1.00 (3.00)	5.54 ± 0.04
3	−1.00 (0.50)	1.00 (4.00)	−1.00 (1.00)	14.35 ± 0.39
4	−1.00 (0.50)	1.00 (4.00)	1.00 (3.00)	18.68 ± 0.54
5	1.00 (1.50)	−1.00 (2.00)	−1.00 (1.00)	10.41 ± 0.02
6	1.00 (1.50)	−1.00 (2.00)	1.00 (3.00)	3.46 ± 0.25
7	1.00 (1.50)	1.00 (4.00)	−1.00 (1.00)	14.74 ± 0.09
8	1.00 (1.50)	1.00 (4.00)	1.00 (3.00)	11.75 ± 0.01
9	−1.68 (0.16)	0.00 (3.00)	0.00 (2.00)	10.11 ± 0.09
10	1.68 (4.68)	0.00 (3.00)	0.00 (2.00)	10.13 ± 0.42
11	0.00 (1.00)	−1.68 (1.32)	0.00 (2.00)	0.93 ± 0.02
12	0.00 (1.00)	1.68 (4.68)	0.00 (2.00)	16.03 ± 0.20
13	0.00 (1.00)	0.00 (3.00)	−1.68 (0.32)	14.95 ± 0.06
14	0.00 (1.00)	0.00 (3.00)	1.68 (3.68)	7.37 ± 0.40
15	0.00 (1.00)	0.00 (3.00)	0.00 (2.00)	15.03 ± 0.02
16	0.00 (1.00)	0.00 (3.00)	0.00 (2.00)	15.96 ± 0.67

Table 4. Regression results of CCD.

	Regression	SE	*T*	*P*	−95%	+95%
Mean/Interc.	−24.54	8.64	−2.83	0.029	−45.70	−3.38
X_1	21.47	6.14	3.49	0.012	6.44	36.50
X_1^2	−6.29	2.18	−2.88	0.027	−11.63	−0.95
X_2	15.64	3.69	4.23	0.005	6.59	24.68
X_2^2	−2.15	0.54	−3.94	0.007	−3.48	−0.81
X_3	1.58	3.07	0.51	0.623	−5.92	9.10
X_3^2	−1.20	0.54	−2.20	0.069	−2.53	0.13
X_1 by X_2	−1.49	1.17	−1.27	0.250	−4.36	1.37
X_1 by X_3	−2.72	1.17	−2.32	0.058	−5.59	0.14
X_2 by X_3	1.45	0.58	2.48	0.047	0.020	2.89

and actual values along with responses of each experimental trial is given in Table 3. The results were analyzed by Statistica 6.0 and cited in Tables 4 and 5.

A second-order polynomial model fitted to the experimental results for the yield of FC was developed according to the regression results of CCD (Table 4):

$$Y = -24.54 + 21.47X_1 - 6.29X_1^2 + 15.64X_2 - 2.15X_2^2 + 1.58X_3 - 1.20X_3^2 - 1.49X_1X_2 - 2.72X_1X_3 + 1.45X_2X_3, \quad (2)$$

where the FC production as yield (Y) is a function of inorganic phosphate concentration (X_1), pH (X_2), and inoculum size (X_3).

The quality of fit of the polynomial model equation was expressed by the coefficient of regression (R^2), which was found to be 0.9558 (namely, 95.58% of the confidence level of the model to predict the response in our study). The 'Adj R^2' was 0.8895, which was in agreement with R^2, indicating that the model was fit well. And the final experiment was conducted to validate the CCD model developed.

The special features of the RSM tool, 'contour plot generation' and 'point prediction' were also studied to find the optimum value of the combination of three significant factors for the maximum yield of FC (Figure 5). Three-dimensional graphs of pH value and inoculum size, which

Table 5. Analysis of variance for the experimental results of the central composite design.

	SS	df	MS	*F*
X_1	3.68	1	3.68	1.33
X_1^2	22.85	1	22.85	8.31
X_2	234.57	1	234.57	85.28
X_2^2	42.81	1	42.81	15.56
X_3	34.53	1	34.53	12.55
X_3^2	13.39	1	13.39	4.87
X_1 by X_2	4.45	1	4.45	1.62
X_1 by X_3	14.88	1	14.88	5.41
X_2 by X_3	16.94	1	16.94	6.16
Error	16.50	6	2.75	
Total SS	373.55	15		

display significant mutual interaction, were generated, while keeping the phosphate at its center point level. From the center point of the contour plot or from the bump of the 3D plot, the optimal composition of the medium component was identified. The predicted values were experimentally verified, indicating that the maximum FC production (17.26 mg/l) was afforded when the cultivation was accomplished in 1 liter shaking flask containing 200 ml of the medium with the inoculum size of 2.45%, KH_2PO_4 at 0.67 g/l, and pH 4.2. Interestingly, the production of HA in this condition

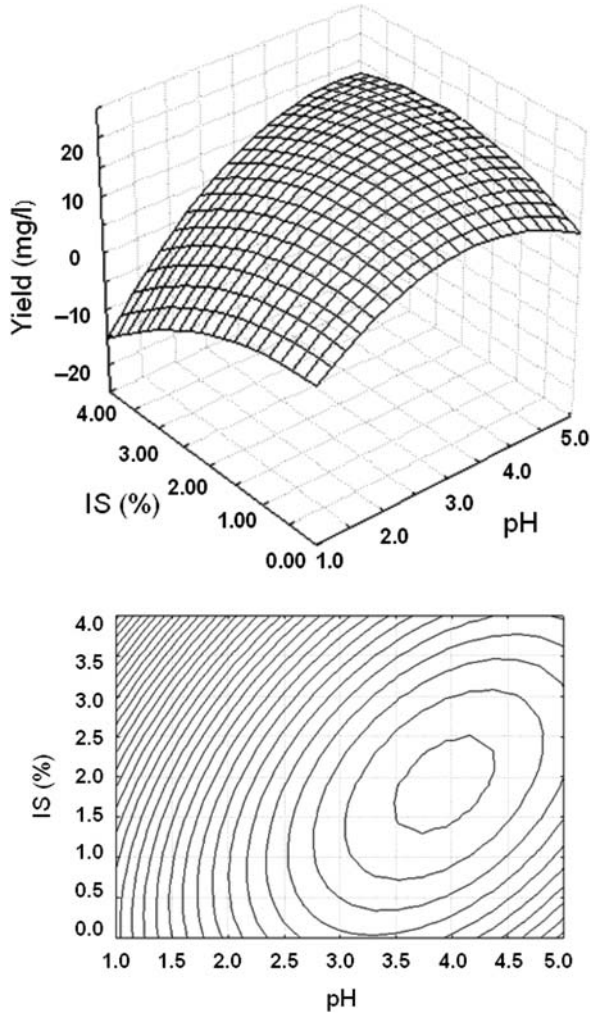

Figure 5. 3D response surface curve and 2D contour plot of the combined effects on the FC production of medium volume and pH at given inorganic phosphate concentration.

could reach to 16.88 mg/l, 9.89-fold increase from that obtained in the basal culture condition. In addition, the ratio of HA/FC was able to increase from 1:4 to 1:1, indicating that both metabolites could be expected as the main products from the optimized fermentation.

3. Materials and methods

3.1 Materials

Mannitol, sucrose, glucose, starch, glycerol, sodium succinate, sodium nitrate, ammonium sulfate, urea, yeast extract, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, KH_2PO_4, methanol, and ethyl acetate were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); HPLC grade methanol was supplied by TEDIA Company, Inc. (Fairfield, CA, USA); Supelco C18 solid phase extraction cartridge (3 ml tube) was purchased from Sigma–Aldrich Company (China) (Shanghai, China). All other solvents used in this study were of AR grade unless indicated otherwise. Authentic samples FC and HA were purified (purity $>99\%$) and identified (MS and NMR data) in our laboratory as before [2].

3.2 Microorganism and inoculum preparation

The fungal strain *A. fumigatus* CY018, previously isolated from the *C. dactylon* leaves [2], was maintained on 2.0% w/v of potato carrot agar slants, subcultured once in a month, and stored at 4°C. The seed was prepared by inoculating five activated fungal tablets ($d = 5$ mm) from agar Petri dish into 200 ml liquid potato dextrose medium preloaded in each of 1 liter Erlenmeyer flask, followed by cultivation for 5 days at 28°C and 150 rpm.

3.3 Medium and fermentation

Fermentation was carried out in 1-liter-sized Erlenmeyer flasks, each containing 200 ml of the sterile medium modified from that reported earlier [20]. The components of basal medium are as follows (g/l): sucrose, 50; yeast extract, 2; sodium succinate, 5.4; $MgSO_4{\cdot}7H_2O$, 0.3; $FeSO_4{\cdot}7H_2O$, 0.01; KH_2PO_4, 1; and H_2O (added up to 1 liter). The pH was adjusted to 5.4 with concentrated ammonia. The medium was inoculated with 5 ml seed and incubated for 15 days on a rotary shaker at 28°C and 150 rpm. All experiments were carried out at least in triplicate. The effect of different carbon sources on the production of FC and HA was checked by substituting sucrose in basal medium sequentially with glycerol, glucose, soluble starch, and mannitol (all at $C = 20$ g/l). Similarly, the effects of different nitrogen sources on FC and HA generations were assessed by substituting yeast extract with organic (urea) and inorganic nitrogen sources including sodium nitrate and ammonium sulfate (all at $N = 0.4$ g/l). The effects of pH, temperature, inorganic phosphate concentration, Mg^{2+}, Fe^{2+}, inoculum size, and rotation speed were all evaluated by Plackett–Burman factorial designs.

3.4 Optimization of carbon source and nitrogen source

In order to obtain the better carbon source both for FC and HA production, mannitol, glucose, starch, and glycerol (all at $C = 20$ g/l) were applied to replace separately sucrose in the basal medium.

For selecting the suitable nitrogen nutrition both for FC and HA production, sodium nitrate, ammonium sulfate, and urea (all at $N = 0.4$ g/l) were added to substitute separately the yeast extract used in the basal medium.

3.5 Production profile of FC and HA

To obtain the time course of FC and HA, cultures were harvested at each 3-day interval (starting on the 7th day) during the

22-day inoculation. All other conditions were maintained as described above. The peak time on which the strain CY018 gave the higher FC and HA yields was fixed as the harvest time for the subsequent experimentation.

3.6 Plackett–Burman design

In order to optimize the factors that have strong or substantial impacts on the target metabolite production, a total of nine parameters including carbon resource concentration, nitrogen resource concentration, pH, temperature, inorganic phosphate concentration, Mg^{2+}, Fe^{2+}, inoculum size, and rotation speed were examined by Plackett–Burman design [24]. The nine factors plus two dummy variables were tested in 12 experimental runs with each independent variable having low (−1) and high (1) levels (Table 1). The fitted first-order equation is as follows:

$$Y = \beta_0 + \sum \beta_i x_i, \tag{3}$$

where Y is the predicted response, β_0 and β_i stand for constant coefficients, and x_i represents the coded independent factors. The statistical software Statistica 6.0 package was used for designing and analyzing the experimental data.

3.7 Biomass estimation and metabolite extraction

The broth was centrifuged at 6000 *g* for 20 min to separate fungal cell and filtrate. The cell was dehydrated at 100°C to a constant weight after washing with distilled water three times. The filtrate was extracted twice with an equal volume of ethyl acetate. The *in vacuo* evaporation of solvent at 40–50°C from the combined extracts gave a dryness, which was resolved with methanol, then preserved in a refrigerator to remove salt and fat prior to FC and HA quantifications.

3.8 FC and HA quantification

A given quantity of the crude extract (10 mg) was dissolved in 100 μl methanol, followed by sonicating (10 min) and warming (10 min) the solution on a water bath at approximately 45°C to dissolve the sample thoroughly. Thus obtained solution was centrifuged for 10 min at 10,000 *g*. To get rid of saccharides, proteins, and others that might interfere with the FC and HA quantifications, 80 μl supernatant was filtered successively over the Supelco C18 solid phase extraction cartridge (eluted with 4 ml of HPLC grade methanol) through 0.45 μm membrane. The contents of FC and HA were subsequently determined by HPLC (Agilent Technologies 1200 series, Singapore, Singapore) over a reversed phase column (Phenomenex, 250 × 4.6 mm, Gemini 5 μm C_{18} 110A, California, USA). For co-analyzing FC and HA, the elution condition was modified from that reported by Lee-Parsons *et al.* [25]. Thus, FC and HA were simultaneously quantified by HPLC carried out within 25 min at a flow rate of 1 ml/min, and using as the mobile phase aqueous acetonitrile (linearly programed from 2% through 47%) in water containing 1% trifluoroacetic acid as the mobile phase. The injection volume was 10 μl, and the detection wavelength was set at 254 nm. All experiments were carried out at 25°C. Yields were calculated as follows:

$$\text{FC or HA (mg)} = \frac{c \times 4 \times 1.25 \times m}{10},$$

where 'm' represents weight of crude extract (mg), 'c' stands for calculated concentration (mg/ml) via external standard method, and equations of calibration curve are as follows:

$$\text{FC}: \text{Area} = 4780.9557c\,(\text{mg/ml}) - 0.1780 \quad (R^2 = 0.99999),$$

$$\text{HA}: \text{Area} = 5913.9099c(\text{mg/ml}) - 4.2217 (R^2 = 0.99997).$$

3.9 Optimization of fermentation process by RSM

RSM, an empirical statistical modeling technique employed frequently for multiple regression analysis, was applied to quantify FC. Briefly, a central composite design for three independent variables was used to give the combination of values that optimized the response within the region of 3D observation spaces, which was required for suggesting a minimal number of experiments designed using the software package, Statistica 6.0.

The medium components (independent variables) selected for the optimization were the factors that are key to FC and HA productions. Regression analysis was carried out on the data obtained from the experiments.

Coding of the variables was done according to the following equation:

$$x_i = \frac{X_i - X_{\mathrm{cp}}}{\Delta X_i}, \quad i = 1, 2, 3, \ldots, k, \quad (4)$$

where 'x_i' represents dimensionless value of an independent variable, 'X_i' symbolizes real value of an independent variable, 'X_{cp}' stands for real value of an independent variable at the center point, and 'ΔX_i' means step change of real value of the variable 'i' corresponding to a variation of a unit for the dimensionless value.

The relationship of the independent variables and the response was calculated by the second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i X_j + \sum i_{i<j} \sum j \beta_{ij} X_i X_j, \quad (5)$$

where 'Y' is the predicted response, 'β_0' a constant, 'β_i' the linear coefficient; 'β_{ii}' the squared coefficient, 'β_{ij}' the cross-product coefficient, and 'k' the number of factors.

The second-order polynomial coefficients were calculated using the software package Statistica 6.0 to estimate the responses of the dependent variables. Response surface plots were also obtained using Statistica 6.0.

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1. Equally contributed to the work.

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