J Ind Microbiol Biotechnol (2009) 36:381–389
DOI 10.1007/s10295-008-0507-6

ORIGINAL PAPER

Statistical optimization of medium composition for aspergiolide A production by marine-derived fungus *Aspergillus glaucus*

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Received: 21 October 2008 / Accepted: 1 December 2008 / Published online: 23 December 2008

Abstract Statistical methodologies were employed to optimize submerged culture medium for the production of a novel antineoplastic compound aspergiolide A by a marine-derived fungus *Aspergillus glaucus* HB1-19 for the first time. Orthogonal design was preformed to determine the initial composition. Then Plackett–Burman design was applied to evaluate the influence of related nutrients, and yeast extract paste, soybean powder and sodium glutamate were confirmed as critical factors in the medium. Response surface methodology (RSM) was finally taken as an effective approach to optimize the combination of the obtained three significant factors. The predicted maximal aspergiolide A production of 62.4 mg/L appeared at the region where the concentrations of sodium glutamate, soybean powder, and yeast extract paste were 2, 1, and 1.07 g/L, respectively. Under the proposed optimized conditions, the experimental aspergiolide A production reached 71.2 mg/L. The correlation between predicted value and measured value of these experiments proved the validity of the response model. After optimization, aspergiolide A production increased 4.22 times compared to that of the original medium. Elemental analysis was finally taken into consideration, and carbon–nitrogen ratio in the medium increased from 20.1:1 to 86.6:1. This great difference was inferred as the most important reason for production enhancement by metabolic pathway analysis.

Keywords *Aspergillus glaucus* · Aspergiolide A · Statistical methodologies · Medium optimization · Carbon–nitrogen ratio

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Introduction

Marine microorganisms such as actinomycetes and fungi were proved to be a fertile source of bioactive compounds [4, 17]. In recent years, a large number of novel bioactive substances have been isolated from marine microorganisms [5, 6, 30]. Many marine natural products hold great potential for the development of new and much needed drugs. Some have been in Phase I–III clinical trials [23].

However, the production of active metabolites by marine microorganisms is usually very low, and large-scale culture has to be involved to meet the needs of clinical trials [25]. Besides, marine environment (high pressure, high salt, hypoxia and low light intensity) is so different from the land that marine microorganisms often have special growth and production conditions. Bernan et al. [3] reported that the biomass and bioxalomycins production increased 33 and 400%, respectively, in marine *Streptomyces* sp. LL-31F508 when the content of sodium chloride increased from 0 to 2%. Osterhage et al. [24] found that more than 80% of secondary metabolites were different between marine and terrestrial *Phoma* species as determined by HPLC-DAD and HPLC-MS.

Aspergillus glaucus HB1-19, a marine-derived filamentous fungus, was obtained from the marine sediment around the mangrove roots collected in Fujian province of China. From the fermentation broth, a novel anthraquinone

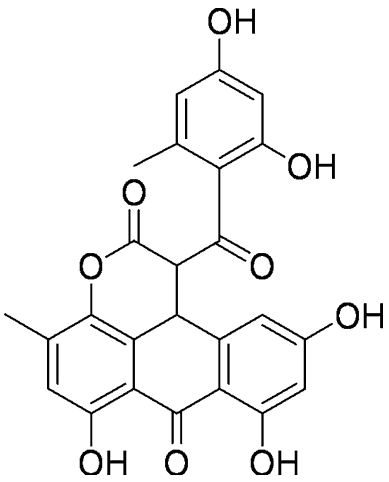

Fig. 1 Chemical configuration of aspergiolide A [10]

derivative with naphtha [1, 2, 3-de] chromene-2, 7-dione skeleton, named as aspergiolide A (Fig. 1) was isolated and identified. The compound shows new chemical configuration and exhibited cytotoxicities against tumor cell lines A-549, HL-60, BEL-7402, and P388 [10]. These characteristics are very similar to anthracyclines, which are widely used and are effective antineoplastic drugs [16]. Moreover, novel anthracycline analogues development is quite necessary because use of anthracyclines is usually limited by cumulative dose-related cardiotoxic adverse effects [2, 12]. So study on aspergiolide A is very important because it holds a great potential to be a new drug. However, the production based on the original culture medium was very low (<15 mg/L) and the loss ratio of the aspergiolide A was nearly 75% during the complicated purification procedure. Therefore, it became an important task to prepare a large amount of the compound for further animal experiment and clinical research.

To increase the production of a secondary metabolite, a good fermentation medium is very important. Several statistic methods have been successfully used in medium optimization with the good quality of high performance and factors interaction compared to conventional practice of full factorial designs and single factor optimization [7]. Orthogonal and Plackett–Burman designs were often applied to reduce the number of experiment runs and find main factors. But the main disadvantage of the two designs was that they considered only first order effects and ignored the interactions [8, 13, 28]. These limitations could be eliminated by optimizing all the affecting parameters collectively by using response surface methodology (RSM) [15, 22, 27]. RSM could be used to explain the relative significance of several affecting factors even in the presence of complex interactions [20, 21, 29].

The purpose of this paper was to develop a high aspergiolide A production medium for *Aspergillus glaucus* HB1-19. In this paper, three statistical methods were carried out to develop the new medium. Orthogonal design was first applied in the experiment for deciding the initial levels of some carbon and nitrogen sources. Then Plackett–Burman design was used to screen prominent constituents and RSM was applied further for explaining their interactions and optimizing their composition. Finally, the amounts of carbon and nitrogen elements in the medium were analyzed and carbon–nitrogen ratio was compared.

Materials and methods

Chemicals

Reagents for cultivation including soluble amylum, glucose, maltose, sucrose, and inorganic salts were purchased from Sinopharm Chemical Reagent Company, China. Yeast extract paste was bought from Lanji Technology Company, Shanghai, China. Tryptone was obtained from OXOID Ltd., England. Sodium glutamate, soybean powder and ipomoea batatas powder were obtained from local supermarket. Acetonitrile of HPLC purity was bought from J&K Chemica.

Medium

Conidiation medium contained 20 g glucose, 50 g ipomoea batatas powder, 20 g agar powder, 0.3 g $MgSO_4 \cdot 7H_2O$ and 0.5 g KH_2PO_4 per liter of artificial sea water. Original fermentation medium was composed of 10 g glucose, 20 g maltose, 20 g mannitol, 10 g sodium glutamate, 6 g yeast extract paste, 0.3 g $MgSO_4 \cdot 7H_2O$ and 0.5 g KH_2PO_4 per liter of artificial sea water. The artificial sea water contained 24.5 g sodium chloride, 3.92 g sodium sulfate, 0.91 g calcium chloride, 4.98 g magnesium chloride, 0.026 g boric acid, 0.664 g kalium chloratum, 0.096 g kalium bromatum, and 0.192 g sodium bicarbonate in the final volume of 1 L distilled water.

Strains and culture conditions

Aspergillus glaucus HB1-19 was provided by Ocean University of China [10]. Mycelium of *Aspergillus glaucus* HB1-19 was first cultured on conidiation medium to induce spore formation. After cultivation at 30°C for 5 days, about 4.4×10^7 spores were inoculated to 50 ml fermentation medium, and incubated at 28°C for 40 h with shaking at 180 rpm. Afterwards, 7 ml of the above broth were inoculated into a 250 ml Erlenmeyer flask containing 43 ml fermentation medium and then incubated at 28°C with shaking at 180 rpm for 8 days.

Analytical methods

Ten milliliter broth, 20 ml ethyl acetate and 20 glass beads were added into a 250 ml Erlenmeyer flask, and incubated at 20°C with shaking at 200 rpm for 12 h. The mixture was then centrifuged with 12,000 rpm for 5 min. The upper-liquor was collected and evaporated under reduced pressure. The obtained crude gum was dissolved with 5 ml methanol and centrifuged at 12,000 rpm for 3 min. Then the supernatant was prepared for further HPLC analysis after dilution and microfiltration.

The HPLC system of Agilent 1100 with a C18 column (Kromasil^{TM}, Sweden, 250 mm × 4.6 mm × 5 μm, 100 Å-spherical silica) was used to analyze the concentration of aspergiolide A. The sample was eluted with a mobile phase comprising 50% acetonitrile and 50% ultrapure water for 22 min followed by 100% acetonitrile for another 8 min. The elution flow rate was set at 1.0 ml/min and the absorbency was monitored at 304 nm. The concentration of aspergiolide A in fermentation broth was determined by comparing to the corresponding purified standard.

Growth of *Aspergillus glaucus* HB1-19 was determined by dry cell weight. A 10 ml broth sample was processed with an air pump filtration and washed with 1 L distilled water. Then, the biomass was dried at 75°C for 24 h. Dry weight was measured after cooling and desiccation for a constant weight.

Carbon and nitrogen elements in the tryptone, yeast extract paste and soybean powder was analyzed by Vario EL III Elemental Analyzers (Elementar, Germany).

Experimental design and data analysis

Orthogonal design

On the basis of our previous study, orthogonal design $L_{25}(5^6)$ was used to optimize the initial concentration in the medium. The five levels of soluble amylum (A), sucrose (B) and maltose (C) were 5, 10, 20, 30, 40 g/L, while those of tryptone (D), yeast extract paste (E) and soybean powder (F) were 0.5, 1, 2, 3, 4 g/L, respectively. Other components kept the level as follows, KH_2PO_4 (G) 0.5 g/L, $MgSO_4{\cdot}7H_2O$ (H) 0.3 g/L, sodium glutamate (J) 10 g/L, artificial sea water, the same as original fermentation medium. The response (Y) was aspergiolide A production.

Plackett–Burman design

The Plackett–Burman *experimental* design, a fractional factorial design, was used in this work to determine the critical medium components. Ten independent variables in 12 combinations were organized by Design Expert (version 7.0, Stat-Ease, Inc., USA). The data analysis was performed by analysis of variance (ANOVA). For each variable, a high (+1) value and a low (−1) value were tested in the experiment. The high levels of the constituents were soluble amylum (A) 50 g/L, sucrose (B) 50 g/L, maltose (C) 37.5 g/L, tryptone (D) 2.5 g/L, yeast extract paste (E) 1.25 g/L, soybean powder (F) 0.625 g/L, KH_2PO_4 (G) 0.625 g/L, $MgSO_4{\cdot}7H_2O$ (H) 0.375 g/L, sodium glutamate (J) 12.5 g/L and 100% artificial sea water (K), which were 1.25 times as the low ones. All trials were performed in triplicate.

Response surface methodology

The optimum combination of various critical factors was then investigated using the central composite design (CCD). The experimental designs were performed using the statistical software Statistical Analysis System (SAS Institute Inc.). Twenty runs were arranged, which contained six replicates at the centre point. All trials were performed in triplicate. The centre point was determined by using single factor gradient test according to the result of Plackett–Burman design.

The data analysis of the model was performed in the form of ANOVA. The fitting quality of the second-order model equation was expressed by the correlation coefficient R and determination coefficient R^2, and its statistical significance was tested by F value and p value. For each variable, the quadratic model was represented as response surface.

The general form of the polynomial model for three independent variables with three various levels was given as follows,

$$Y_i = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{2} \sum_{j=2}^{3} b_{ij} X_i X_j \qquad (1)$$

where Y_i was the predicted response; X_i, X_j were input variables which influenced the response variable; Y_i, b_0 was the offset term; b_i was the ith linear coefficient; b_{ii} was the quadratic coefficient; and b_{ij} was the ijth interaction coefficient [11].

Results and discussion

Orthogonal design

With orthogonal design, 25 experiments were carried out. Table 1 showed the detailed assignment and the results. The range value indicated that all these factors had relatively great effect on the production, so they were taken to further optimization. Numbers 9 and 25 showed good

Table 1 Experimental design for the orthogonal array

No.	A	B	C	D	E	F	Y (mg/L)
1	1	1	1	1	1	1	0.0
2	1	2	2	2	2	2	0.0
3	1	3	3	3	3	3	0.0
4	1	4	4	4	4	4	2.8
5	1	5	5	5	5	5	3.7
6	2	1	2	3	4	5	0.0
7	2	2	3	4	5	1	0.0
8	2	3	4	5	1	2	2.6
9	2	4	5	1	2	3	16.2
10	2	5	1	2	3	4	0.5
11	3	1	3	5	2	4	0.0
12	3	2	4	1	3	5	1.0
13	3	3	5	2	4	1	6.5
14	3	4	1	3	5	2	0.0
15	3	5	2	4	1	3	4.9
16	4	1	4	2	5	3	1.3
17	4	2	5	3	1	4	2.5
18	4	3	1	4	2	5	0.1
19	4	4	2	5	3	1	1.9
20	4	5	3	1	4	2	10.3
21	5	1	5	4	3	2	3.9
22	5	2	1	5	4	3	0.0
23	5	3	2	1	5	4	0.0
24	5	4	3	2	1	5	10.1
25	5	5	4	3	2	1	22.5
Range	5.99	5.51	6.43	3.86	6.76	5.01	

A soluble amylum, *B* sucrose, *C* maltose, *D* tryptone, *E* yeast extract paste, *F* soybean powder

results. The results reappeared fairly (data not shown) and No. 25 was selected.

Based on the L_{25} (5^6) orthogonal design, the concentration of the six components could be taken as soluble amylum (A) 40 g/L, sucrose (B) 40 g/L, maltose (C) 30 g/L, tryptone (D) 2 g/L, yeast extract paste (E) 1 g/L, and soybean powder (F) 0.5 g/L.

Plackett–Burman design

The Plackett–Burman experimental design details were shown in Table 2. ANOVA was applied to deal with the experimental data. The *p* values were used to check the significance of each coefficient and indicate the interactions between each independent variable [19]. *P* value < 0.05 meant that the corresponding factor was significant [9]. So as shown in Table 3, sodium glutamate (J), soybean powder (F) and yeast extract paste (E) were proved to be the critical components, and their contributions to the medium were J 47.52, F 20.81, and E 14.77%, respectively (data details not shown). The determination coefficient R^2 of the regression model ($R^2 = 0.9992$) indicated that 99.92% of the total variations could be explained by the model [26]. The adjusted determination coefficient (Adj $R^2 = 0.9916$) was also very high in order to support a high significance of the model [9, 14]. A relatively lower value of the coefficient of variation (CV = 3.16%) indicated a better precision and reliability of the experiments [1, 26].

Effect analysis was also carried out with Design Expert, and sodium glutamate (J) had a great negative effect of −7.95, while yeast extract paste (E) and soybean powder

Table 2 Plackett–Burman design matrix for 10 independent variables

No.	A	B	C	D	E	F	G	H	J	K	Y (mg/L)
1	1	1	−1	1	1	1	−1	−1	−1	1	25.4
2	−1	1	1	1	−1	−1	−1	1	−1	1	17.1
3	1	1	−1	−1	−1	1	−1	1	1	−1	18.0
4	1	−1	1	1	−1	1	1	1	−1	−1	25.4
5	1	−1	−1	−1	1	−1	1	1	−1	1	21.3
6	−1	−1	1	−1	1	1	−1	1	1	1	16.5
7	−1	1	−1	1	1	−1	1	1	1	−1	15.4
8	1	−1	1	1	1	−1	−1	−1	1	−1	14.0
9	1	1	1	−1	−1	−1	1	−1	1	1	7.0
10	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	14.0
11	−1	1	1	−1	1	1	1	−1	−1	−1	25.4
12	−1	−1	−1	1	−1	1	1	−1	1	1	9.8

Factors A–F represented constituents as same as that in Table 1

G KH_2PO_4, *H* $MgSO_4{\cdot}7H_2O$, *J* sodium glutamate, *K* artificial sea water

Table 3 ANOVA for Plackett–Burman design

Source[a]	Degree of freedom	Sum of square	*F* value	*P* > *F*
Model	10	398.43	131.438	0.0678
A	1	13.96	46.062	0.0931
B	1	4.40	14.511	0.1634
C	1	0.20	0.648	0.5685
D	1	2.12	7.002	0.2300
E	1	58.90	194.297	0.0456*
F	1	82.99	273.777	0.0384*
G	1	0.03	0.083	0.8213
H	1	27.31	90.104	0.0668
J	1	189.49	625.118	0.0254*
K	1	19.03	62.779	0.0799
Error	1	0.30		
Total	11	398.74		

Determination coefficient $R^2 = 0.9992$; adjusted determination coefficient Adj $R^2 = 0.9916$; coefficient of variation CV = 3.16%

* Significant at the 5% level ($P < 0.05$)

[a] Factors A–K represented constituents as same as that in Table 2

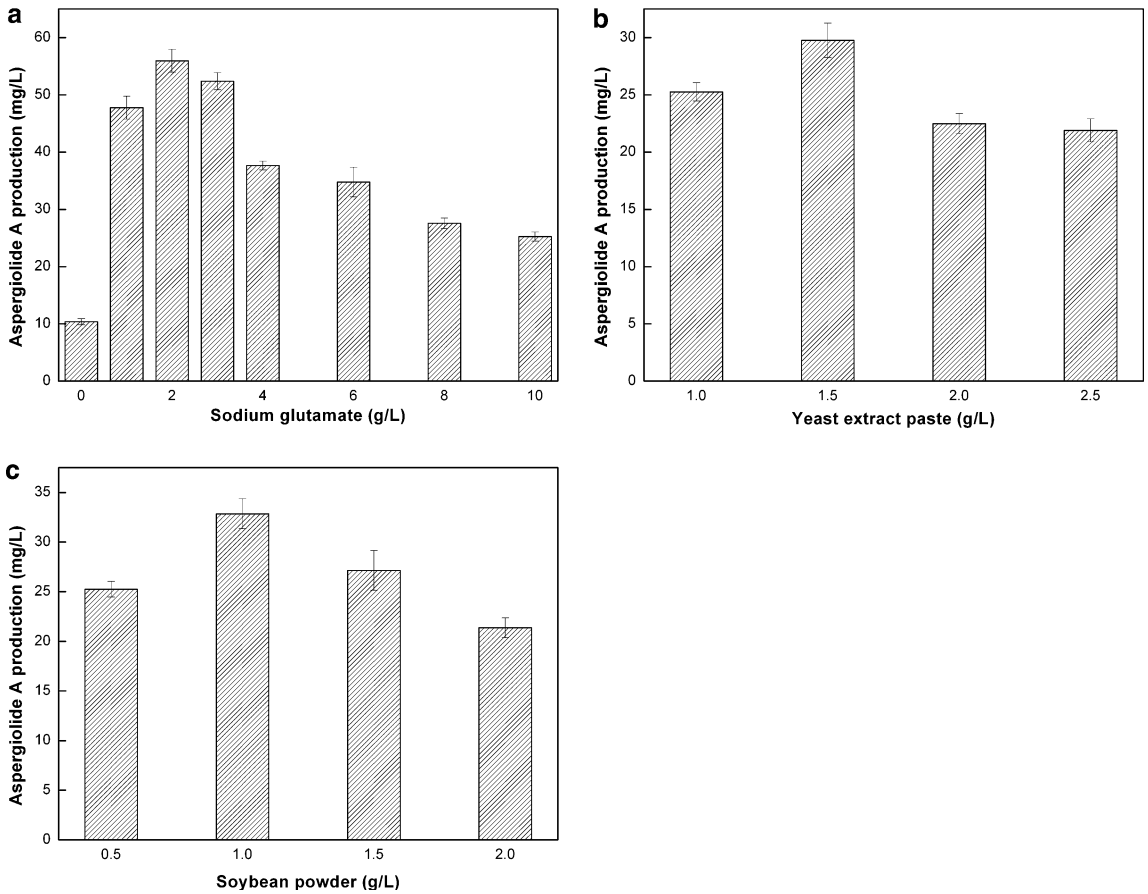

Fig. 2 Effect of single factor gradient test on aspergiolide A production. **a** Sodium glutamate, **b** yeast extract paste, **c** soybean powder

(F) showed positive effect of 4.43 and 5.26. According to the respective effect and contribution, we could conclude that change of J would have much greater effect than E and F. It was quite useful information for method selection for centre point determination in RSM.

Response surface methodology

Further experiments designed with response surface methodology were carried out to optimize the relationship of the critical components; including sodium glutamate, soybean powder, and yeast extract paste. In order to search for the optimum combination of critical components of the medium, a CCD was performed. To keep the experiments' validity, a reliable centre point must be confirmed. Because sodium glutamate was proved to have a greater contribution than others, the concentration of sodium glutamate would have the most important effect on the medium. For this reason, a single factor gradient test was used to approach the vicinity of the optimum point. The constituents kept the same concentrations as that determined by orthogonal design except the changing one. Eight concentration gradients were set for sodium glutamate (J), and four for soybean powder (F) and yeast extract paste (E). All trials were performed in triplicate. According to the experiment results (Fig. 2), the centre point was chosen as sodium glutamate 2 g/L, soybean powder 1 g/L, and yeast extract paste 1.5 g/L. The CCD design and experimental results were shown in Table 4. The five coded levels (-1.68, -1, 0, 1, 1.68) of CCD were set as 0, 0.5, 2, 3.5, 5 g/L for sodium glutamate, 0.25, 0.625, 1, 1.375, 1.875 g/L for soybean powder, and 0.5, 1, 1.5, 2, 2.5 g/L for yeast extract paste.

The experimental results were fitted with a second-order polynomial equation (Eq. 1) by applying multiple regression analysis on the experimental data. P value < 0.05 represented that the corresponding term was significant. Therefore, the second-order polynomial equation was finally modified to Eq. 2.

$$Y = 57.5 - 4.25J - 3.86F - 4.96E - 9.11J^2 - 4.61F^2 - 3.18E^2 \quad (2)$$

ANOVA of the CCD design was in Table 5. The determination coefficient R^2 with the value 0.9421 indicated that 94.21% of the total variations could be comprehended by the model, adjusted determination

Table 4 CCD plan in coded value and observed response (aspergiolide A)

No.	J (Sodium glutamate)	F (Soybean powder)	E (Yeast extract paste)	Y (mg/L) (Aspergiolide A)
1	−1	−1	−1	50.6
2	−1	−1	1	46.2
3	−1	1	−1	48.8
4	−1	1	1	39.3
5	1	−1	−1	40.9
6	1	−1	1	36.1
7	1	1	−1	34.0
8	1	1	1	28.1
9	−1.68	0	0	35.5
10	1.68	0	0	28.3
11	0	−1.68	0	53.2
12	0	1.68	0	36.0
13	0	0	−1.68	61.4
14	0	0	1.68	35.8
15	0	0	0	59.7
16	0	0	0	56.4
17	0	0	0	56.8
18	0	0	0	58.4
19	0	0	0	56.1
20	0	0	0	57.7

coefficient (Adj $R^2 = 0.9916$) close to determination coefficient R^2 further affirm the model [26]. Coefficient of variation 8.09% was a little bigger, which might be caused by the complicated extraction process. The graphical representations of the regression equation could be exhibited by the 3D response surfaces. All these response surfaces were presented in Fig. 3 from which the values of aspergiolide A for different concentrations of the variables could be predicted. The maximum predicted value was located at the highest point of the surface. This value could also be calculated by differential calculus. But all the interactions among the three factors were not significant judging from the multiple regression analysis because $P > 0.05$. So the relationship among them was almost independent of each other. With the help of the software's prediction profiler optimization, the maximum aspergiolide A production was predicted to be 59.4 ± 3.0 mg/L when sodium glutamate and soybean powder kept central point level (2 and 1 g/L, respectively) but yeast extract paste changed to 1.07 g/L (Fig. 4).

Experimental verification of theoretical optimum

Three independent repeated experiments were performed to verify the reliability of the theoretical optimum. Each experiment was carried out with five parallel samples. The average aspergiolide A production was 71.2 mg/L, which indicated a relatively good accordance with the theoretical prediction. The correlation between predicted and measured values verified the validity of the response model and the existence of an optimum point.

Time course experiments and carbon–nitrogen ratio analysis

The results of time course experiments were shown in Fig. 5. The maximum dry cell weights in original medium and optimized medium were 14.0 and 13.9 g/L, respectively, which were almost the same, and both appeared at

Table 5 ANOVA for CCD design

Source	Degree of freedom	Sum of square	Mean of square	F value	$P > F$
Model	9	2247.68	249.74	18.072	0.0001*
J	1	246.46	246.46	17.834	0.0018*
F	1	203.78	203.78	14.746	0.0033*
E	1	335.58	335.58	24.283	0.0006*
J × J	1	1195.85	1195.85	86.535	0.0001*
J × F	1	4.95	4.95	0.358	0.5630
J × E	1	1.29	1.29	0.093	0.7664
F × F	1	306.09	306.09	22.149	0.0008*
F × E	1	4.88	4.88	0.353	0.5654
E × E	1	145.59	145.59	10.536	0.0088*
Error	10	138.19	13.82		
Total	19	2385.87			

Determination of coefficient $R^2 = 0.9421$; coefficient of variation CV = 8.09%; adjusted determination coefficient Adj $R^2 = 0.8899$

* Significant at the 5% level ($P < 0.05$)

J Sodium glutamate, *E* yeast extract paste, *F* soybean powder

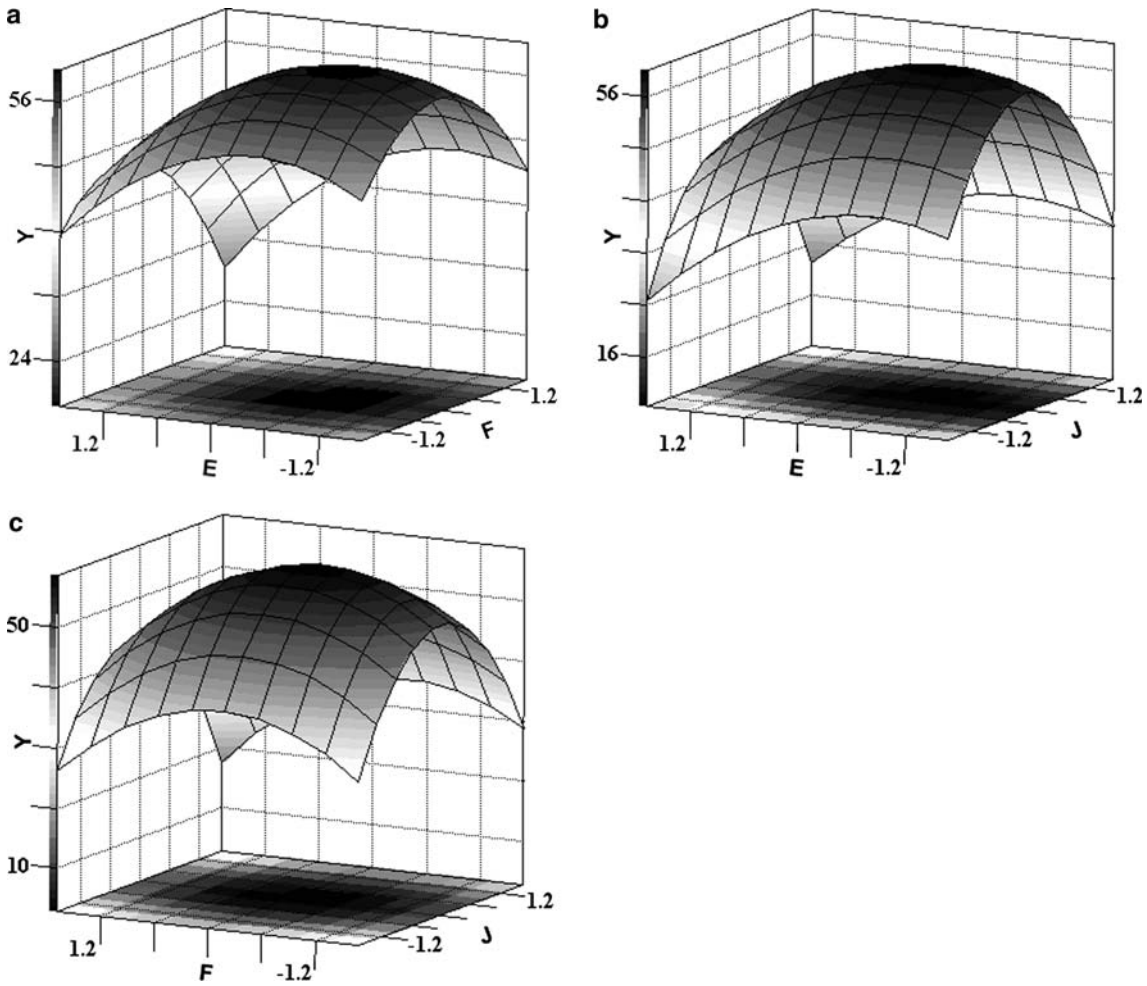

Fig. 3 Response surface plot for the effect of two various factors on aspergiolide A production. The other factors kept the central point level. **a** Soybean powder (F) versus yeast extract paste (E), **b** sodium glutamate (J) versus yeast extract paste (E), **c** sodium glutamate (J) versus soybean powder (F)

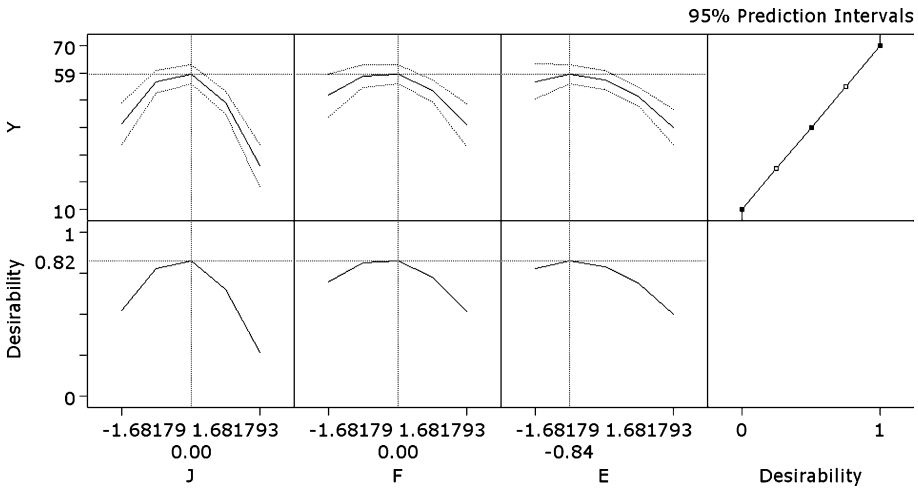

Fig. 4 Prediction for aspergiolide A production (Y) with 95% prediction intervals. Yeast extract paste (E), soybean powder (F), sodium glutamate (J)

130 h. The pH value in optimized medium was lower than that in original one during the whole period. The potential reason was that higher viscosity of optimized medium led to lower DO level and more organic acid accumulation. Aspergiolide A production reached the highest point 67.9 mg/L at 180 h in optimized medium, while it was

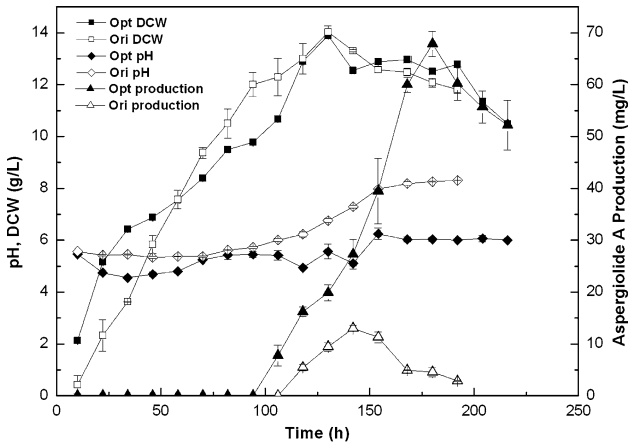

Fig. 5 Time course profiles of pH, dry cell weight (DCW) and aspergiolide A production in original medium (*open symbols*) and optimized medium (*solid symbols*)

Table 6 Carbon and nitrogen contents in original and optimized medium

Medium	Components	C (g/L)	N (g/L)	C:N
Original medium	Glucose	4	0	20.1:1
	Maltose	8.42	0	
	Mannitol	7.92	0	
	Yeast extract paste	1.78	0.51	
	Sodium glutamate	3.21	0.75	
	Sum	25.33	1.26	
Optimized medium	Soluble amylum	17.76	0	86.6:1
	Maltose	16.84	0	
	Sucrose	12.63	0	
	Soybean powder	0.44	0.07	
	Tryptone	0.89	0.26	
	Yeast extract paste	0.32	0.09	
	Sodium glutamate	0.64	0.15	
	Sum	49.52	0.57	

only 13.0 mg/L at 142 h in original medium. So it could be calculated that the highest production of aspergiolide A in optimized medium was 5.22 times as that in original medium.

To explore the difference between the original and optimal medium, carbon and nitrogen contents in the two mediums were analyzed (Table 6). After optimization, carbon–nitrogen ratio increased from 20.1:1 to 86.6:1. This might be the most important reason for production enhancement.

Generally, microorganisms will start to utilize intracellular nitrogen source when exogenous nitrogen is exhausted. At this time, adenosine monophosphate (AMP) dehydrogenase is activated and catalyzes AMP to degrade and release NH_3. Isocitrate dehydrogenase (ICDH) activity will then decrease because AMP is an activator of ICDH. Based on the above analysis, citric acid will accumulate in mitochondrion by TCA cycle. The redundant citric acid will transfer to cytoplasm and degrade to acetyl-CoA and oxaloacetic acid. Then acetyl-CoA and malonyl-CoA synthesized from acetyl-CoA enters the following fatty acid synthesis. Therefore, a relatively high carbon–nitrogen ratio will improve fatty acids synthesis [18].

Both fatty acids and polyketides are synthesized with acetyl-CoA and malonyl-CoA as starters. Coincidentally, in our case aspergiolide A is a polyketide and was also postulated to synthesize via PKS pathway from acetyl-CoA and malonyl-CoA [10]. Therefore, a relatively high carbon–nitrogen ratio would also increase aspergiolide A production. The experimental results might indirectly confirm the deduced PKS synthesis pathway of aspergiolide A.

By analyzing the carbon–nitrogen ratio, it was suggested that a new strategy of fed-batch fermentation could be used for aspergiolide A production in bioreactor. First, nitrogen source could be increased and the carbon–nitrogen ratio could be decreased to a certain value in order to avoid the inhibited effect on growth and to get more biomass in batch stage because nitrogen source is necessary for cell generation and growth. Then carbon source should be fed continuously to increase aspergiolide A production.

Conclusion

In this work, statistic methods were successfully applied for medium optimization of aspergiolide A production from marine-derived fungus *Aspergillus glaucus* HB1-19. With combined application of orthogonal design, Plackett–Burman design and central composite design, a high yield medium was successfully developed. The optimized medium resulted in 4.22-fold higher production of aspergiolide A as compared to that in original medium. However, the biomass produced in the two mediums were almost the same. The applied methods of medium optimization were efficient, relatively simple, and time and material saving. Content of carbon and nitrogen in the two mediums were also analyzed. After optimization, carbon–nitrogen ratio changed from 20.1:1 to 86.6:1. This might be the most important reason for production increase on the basis of the presumed synthesis pathway analysis.

Acknowledgments This work was supported by the National High Technology Research and Development Program of China (2006AA09Z429). We are grateful to Ocean University of China for supply of the strain and the aspergiolide A standard.

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