Enhanced production of questin by marine-derived *Aspergillus flavipes* HN4-13

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Abstract

Questin has favorable applications. Fractional factorial design, Box–Behnken design, and response surface methodology were adopted to optimize the fermentation conditions of the marine-derived fungus, *Aspergillus flavipes* HN4-13, thereby enhancing questin production. Optimal fermentation conditions in a 500-mL conical flask with 200 mL of medium were 4% soluble starch, 0.9% beef extract, 4% NaCl, 0.05% Na₂HPO₄, pH 6, 2% inoculum size, and shaking at 28 °C and 160 rpm/min for 7 days. The production of questin can achieve 64.93 ± 4.55 mg/L, with no significant difference from the predicted value (66.27 mg/L). Thus, this optimized process of questin production is feasible. Such production is 17-fold higher than that of the basal Sabouraud's dextrose medium. Results indicate the potential of *A. flavipes* HN4-13 in the large-scale production of questin through fermentation.

Keywords Questin · *Aspergillus flavipes* HN4-13 · Fermentation optimization · Response surface methodology

Introduction

The probability of searching for novel antimicrobial agents from terrestrial microorganisms is becoming small. The ocean covers approximately 71% of the earth's surface and accounts for at least 95% of the total earth's biosphere, making it a huge treasure trove of bioresources and pharmaceutical substances (Jin et al. 2016). Most of the early marine drugs were derived from the sea animals or plants. However, realizing the industrial production of these drugs is difficult due to the limited biomass of the sea animals or plants, the

difficulty of collection, and the complicated separation and purification involved. However, marine microorganisms are renewable resources, which are easy to collect and reproduce rapidly at the industrial scale through fermentation technology. At the same time, many studies have shown that marine microorganisms are the real metabolic sources of bioactive substances from marine animals or plants (Gerwick and Fenner 2013). Therefore, marine microorganisms are becoming the research hotspot of bioactive natural products (Wang et al. 2016; Guo et al. 2019a; Song et al. 2014). Marine-derived fungus exhibits high pharmacological potential attributed to its special physical and chemical conditions (Gomes et al. 2015; Guo et al. 2016; Jin et al. 2016; Zhao et al. 2016a).

Questin (Fig. 1) is an anthraquinone compound derived from marine *Aspergillus flavipes* HN4-13, which shows favorable antimicrobial effects against *Vibrio harveyi* (Guo and Wang 2017; Guo et al. 2019a, b). Further study on the antibacterial efficacy of this compound in vivo will contribute to the development of novel antibiotics. However, the yield of questin from *A. flavipes* HN4-13 is low, which limits its pharmaceutical applications. Thus, an effective technique should be adopted to enhance questin production from *A. flavipes* HN4-13.

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Fig. 1 Chemical structure of questin

This work aimed to obtain a sufficient quantity of questin to investigate its antagonistic efficacy and mechanism of action. Single-factor experiments, two-level fractional factorial design (FFD), Box–Behnken design (BBD), and response surface methodology (RSM) were adopted to optimize the culture medium and parameters for the production of questin through a marine-derived fungus, *A. flavipes* HN4-13.

Materials and methods

Materials and chemicals

Aspergillus flavipes HN4-13 strain (CCTCC No. AF 2,015,022) was maintained on a Sabouraud dextrose (SD) agar plate (3% glucose, 1% peptone, 2% agar, dissolved in natural aged seawater). Standard questin (purity > 94%) was purified and identified in accordance with the literature (Guo and Wang 2017). *A. flavipes* HN4-13 and questin were maintained at the Laboratory of Marine Natural Products Chemistry in Huaihai Institute of Technology. Other biochemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Shake-flask fermentation

The seed culture was prepared by incubating spores from a fresh culture of HN4-13 on a SD agar plate into 500 mL Erlenmeyer flask containing 200 mL of liquid SD medium (3% glucose, 1% peptone, dissolved in naturally aged seawater) and cultured at 28 °C for 24–48 h with shaking at 160 rpm/min. The procedures were optimized by adding the seed culture into a 500 mL conical flask containing 200 mL of liquid fermentation medium.

Preparation of active extracts

The harvested fermentation cultures were filtered through cheesecloth filtration to isolate the broths from mycelia. The filtrates were extracted three times with an equal volume of ethyl acetate (EtOAc) to obtain an EtOAc solution. Next, the active extracts were obtained through concentrating the

EtOAc solution under reduced pressure and dissolving it in CH₃OH to the concentration of 10 mg/mL to determine the questin content.

Single-factor experiments

SD medium was used as the initial medium. First, the constituents of the medium were screened. For screening the carbon source, glucose was replaced with soluble starch, sucrose, maltose, or fructose and the nitrogen source in the medium was kept constant. For screening the nitrogen source, peptone was replaced with yeast extract, beef extract, KNO₃, or sodium glutamate, while the carbon source was kept constant. For screening inorganic salts, MgSO₄, CaCl₂, KCl, Na₂HPO₄, and K₂HPO₄ were added into the medium, while carbon and nitrogen sources were kept constant. Salinity was screened, and 0, 1%, 2%, 3%, 4% and 5% NaCl and distilled water were used to replace naturally aged seawater. The other fermentation parameters were as follows: 1% inoculum size, natural pH value, and 28 °C at 160 rpm/min for 7 days.

Second, the fermentation parameters, such as inoculum size, initial pH and culture days were screened. Thereafter, 0, 1%, 2%, 3%, 4%, and 5% of the inoculum size were used and the inoculum was cultured at 28 °C and 160 rpm/min for 7 days. The pH (5, 6, 7, 8, 9) was screened with an inoculum size of 2% (v/v) at 28 °C and 160 rpm/min for 7 days. The culture days were screened with an inoculum size of 2% (v/v) at 28 °C and 160 rpm/min for 4, 5, 6, 7, and 8 days. The best single factors or their levels were determined in accordance with the production of questin.

Two-level fractional factorial design

The fermentation variables which have significant effects on the production of questin were obtained through a two-level FFD. Accordingly, the three main factors could be selected from seven variables using only eight experiments (Guo and Wang 2017). Each variable had two levels coded as −1 and +1, and the center points (0) of all the eight, variables were determined on the basis of the results of the single-factor experiments. The FFD experiments (Table 1) were designed using JMP 7 (SAS, USA) software. The *t* test was used to determine the significance of the regression coefficients. The variable with a *P* value less than 0.05 was considered significant for the production of questin (Zhao et al. 2016b).

Box–Behnken design

BBD is a type of RSM used to optimize the values of significant variables. Design Expert 7.0.0 (Stat-Ease, Minneapolis, USA) was used to analyze the experimental data

Table 1 The (+) and (−) value for each single variable and production of questin in the FFD experiment

No.	Soluble starch (%)	Beef extract (%)	Na ₂ HPO ₄ (%)	NaCl (%)	Inoculum size (%)	pH	Culture days (d)	Production (mg/L)
1	− 1 (2)	− 1 (0.5)	− 1 (0.025)	− 1 (3)	+ 1 (3)	+ 1 (7)	+ 1 (8)	7.97
2	− 1 (2)	− 1 (0.5)	+ 1 (0.075)	+ 1 (5)	− 1 (1)	− 1 (5)	+ 1 (8)	19.59
3	− 1 (2)	+ 1 (1.5)	+ 1 (0.075)	− 1 (3)	+ 1 (3)	− 1 (5)	− 1 (6)	3.71
4	− 1 (2)	+ 1 (1.5)	− 1 (0.025)	+ 1 (5)	− 1 (1)	+ 1 (7)	− 1 (6)	6.99
5	+ 1 (4)	− 1 (0.5)	+ 1 (0.075)	− 1 (3)	− 1 (1)	+ 1 (7)	− 1 (6)	65.89
6	+ 1 (4)	− 1 (0.5)	+ 1 (0.075)	+ 1 (5)	+ 1 (3)	− 1 (5)	− 1 (6)	61.47
7	+ 1 (4)	+ 1 (1.5)	− 1 (0.025)	− 1 (3)	− 1 (1)	− 1 (5)	+ 1 (8)	19.61
8	+ 1 (4)	+ 1 (1.5)	+ 1 (0.075)	+ 1 (5)	+ 1 (3)	+ 1 (7)	+ 1 (8)	11.07

Table 2 Estimates of each variable for the production of questin based on FFD

Variable	Soluble starch	Beef extract	Na ₂ HPO ₄	NaCl	Inoculum size	pH	Culture days
<i>t</i> Ratio	6.41	− 6.07	0.23	0.1	− 1.49	− 0.67	− 4.27
Prob> <i>ltl</i>	0.0158	0.0178	0.8396	0.9256	0.2572	0.5649	0.0383
Ranks	1	2	6	7	4	5	3

and establish the model (Liu et al. 2010). BBD consisted of 17 experimental points, including 12 factorial points and 5 central points. The dependent variable (*Y*, mg/L) was the production of questin, while soluble starch (*X*₁), beef extract (*X*₂), and culture days (*X*₃) were chosen as independent variables. The range and values of the three independent variables are shown in Table 3. The optimized objective was to achieve the maximum questin production using this software. The experimental data of BBD were fit with the following second-order polynomial equation:

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

where *Y* represents the predicted response, β_0 , β_i , β_{ii} and β_{ij} represent the constant coefficients, while *X*_{*i*} and *X*_{*j*} are the independent variables.

Analysis of the production of questin

The content of questin in active extracts was determined using HPLC and standard curves (Zhao et al. 2016b; Guo et al. 2017). The standard curve between the concentrations of questin and the peak areas was established using an ultimate 3000 HPLC instrument (Thermo Fisher Scientific, USA) on an ODS column (Shim-Pack CLC-ODS, 6.0 mm × 150 mm, 5 μm, 1.0 mL/min). The concentrations of the standard solutions ranged from 0.03125 mg/mL to 0.5 mg/mL, and the ultraviolet detection was at 280 nm. The mobile phase used a gradient elution procedure consisting of water and methanol. The gradient

Table 3 The coded (actual) value for each independent variable and production of questin in the BBD experiment

No.	Soluble starch (%)	Beef extract (%)	Culture days (d)	Production (mg/L)
1	− 1 (3)	− 1 (0.2)	0 (6)	11.88
2	+ 1 (5)	− 1 (0.2)	0 (6)	14.37
3	− 1 (3)	+ 1 (1.0)	0 (6)	17.93
4	+ 1 (5)	+ 1 (1.0)	0 (6)	16.34
5	− 1 (3)	0 (0.6)	− 1 (5)	22.03
6	+ 1 (5)	0 (0.6)	− 1 (5)	13.99
7	− 1 (3)	0 (0.6)	+ 1 (7)	27.87
8	+ 1 (5)	0 (0.6)	+ 1 (7)	25.84
9	0 (4)	− 1 (0.2)	− 1 (5)	30.88
10	0 (4)	+ 1 (1.0)	− 1 (5)	24.7
11	0 (4)	− 1 (0.2)	+ 1 (7)	21.91
12	0 (4)	+ 1 (1.0)	+ 1 (7)	72.43
13	0 (4)	0 (0.6)	0 (6)	57.09
14	0 (4)	0 (0.6)	0 (6)	60.17
15	0 (4)	0 (0.6)	0 (6)	65.93
16	0 (4)	0 (0.6)	0 (6)	48.05
17	0 (4)	0 (0.6)	0 (6)	61.06

procedure was as follows: 0–5 min 10% (v/v) CH₃OH, 5.1–10 min 10–100% CH₃OH, 10.1–20 min 100% CH₃OH, 20.1–21 min 100–10% CH₃OH, and 21.1–25 min 10% CH₃OH. The following linear regression equation was obtained: $Y = 0.0047X - 0.0119$ ($R^2 = 0.988$). *X* is the peak area of questin and *Y* is the concentration. The yield

of questin under each cultural condition was calculated using the above linear equation.

Results

Single-factor investigation

The effects of different carbon sources, nitrogen sources, inorganic salts, NaCl concentration, inoculum size, pH and culture days on the production of questin by *A. flavipes* HN4-13 were examined. The experimental results indicated that 3% soluble starch, 1% beef extract, 0.05% Na₂HPO₄, 4% NaCl, 2% inoculum size, pH 6, and 7-day culture are favorable for the production of questin.

Screening of significant factors through FFD

All the single variables, including soluble starch, beef extract, Na₂HPO₄, NaCl, inoculum size, pH, and culture days, were screened through two-level FFD on the basis of single-factor experimental results. Table 1 shows the FFD design and responses of different experiments. The relevant *t* values and significance are shown in Table 2. The level of significance follows the order starch > beef extract > culture days > inoculum size > pH > Na₂HPO₄ > NaCl. *P* values of starch, beef extract, and culture days were lower than 0.05 and were significant variables (Guo et al. 2017). The increased amount of starch had a positive effect on the production of questin, whereas beef extract and culture days had negative effects (Zhao et al. 2016b). Hence, soluble starch, beef extract, and culture days were the significant variables for questin production. The other variables were set at their mediate level.

Optimization of significant factors through BBD

On the basis of the FFD results, BBD was applied to further optimize the levels of the three significant variables. Three levels (−1, 0, and +1) of each factor were set as 3%, 4% and 5% for starch (A); 0.2%, 0.6% and 1.0% for beef extract (B); and 5, 6, and 7 days (C) for culture days, respectively. The matrices and results of BBD are shown in Table 3. Based on the parameter estimates, the application of RSM can offer the empirical relationships between response variable and independent variables. Thus, the following second-order polynomial equation was established:

$$Y = 58.46 - 1.15A + 6.55B + 7.06C - 1.02AB + 1.50AC + 14.18BC - 29.19A^2 - 14.14B^2 - 6.84C^2 \quad (2)$$

Table 4 shows the results of ANOVA. The values of “*P* > *F*” (0.0016), determination *R*² (0.9410), and lack of fit (0.2889) revealed that the model was significant (Zhao et al. 2016b). Moreover, B, C, BC, A², and B² were the significant terms of the model (*P* < 0.05).

Figure 2 shows the interactive effects of two variables on questin production. When soluble starch, beef extract, and culture days were 4%, 0.9%, and 7, respectively, the predicted maximal production of questin (66.27 mg/L) was achieved by the model. Therefore, the optimized fermentation parameters for questin production in a 500-mL conical flask with 200 mL liquid medium were 4% starch, 0.9% beef extract, 4% NaCl, 0.05% Na₂HPO₄, pH 6, 2% inoculum size, 28 °C culture temperature, and 160 rpm/min for 7 days.

Model validation

Verified experiments under the optimal fermentation conditions were conducted to confirm the predicted result and optimized effects. The practical yield of questin was

Table 4 Analysis of variance for the relationships between response variable and independent variables

Source	Sum of squares	<i>df</i>	Mean square	<i>F</i> value	Prob > <i>F</i>	Sig.
Model	6555.57	9	728.40	12.40	0.0016	**
A	10.51	1	10.51	0.18	0.6850	
B	342.70	1	342.70	5.83	0.0464	*
C	398.33	1	398.33	6.78	0.0352	*
AB	4.16	1	4.16	0.071	0.7978	
AC	9.03	1	9.03	0.15	0.7067	
BC	803.72	1	803.72	13.68	0.0077	**
A ²	3587.30	1	3587.30	61.07	0.0001	**
B ²	842.00	1	842.00	14.34	0.0068	**
C ²	196.92	1	196.92	3.35	0.1098	
Lack of fit	235.43	3	78.48	1.79	0.2889	

***P* < 0.01, *0.01 < *P* < 0.05

Fig. 2 Response surface plots showing effects of pairwise factors on the production of questin and their interactions

64.93 ± 4.55 mg/L ($n = 3$), which showed no significant difference with the predicted value (66.27 mg/L), and thus indicated the validity of this RSM model. Meanwhile, the production of questin in the optimized conditions was 17-fold higher than that of the initial SD medium.

Discussion

Many useful metabolites can be produced by microbial fermentation (Cheng et al. 2013; Dey et al. 2018; Wang et al. 2018; Zhao et al. 2016b). Fermentation conditions depend on many variables, and the interactions among them are complicated. Therefore, optimizing the microbial culture medium and fermentation parameters is important. Various optimization methods in mathematical statistics have been widely used to optimize of microbial fermentation, among which RSM is the most effective. RSM is a statistical method that uses reasonable experimental design

and specific data obtained through experiments to fit the functional relationships between factors and response values through a multiple quadratic regression equation (Guo et al. 2014). Subsequently, the optimal fermentation conditions are determined by analyzing the regression equation. RSM is an effective method to optimize processing parameters, improve product quality, and solve practical problems in production, and it is widely used in agriculture, biology, food, chemistry, manufacturing, and other fields.

In the present study, the fermentation conditions for questin production from *A. flavipes* HN4-13 by RSM were divided into four steps: First, single-factor experiments were carried out to investigate the single factors that affect the production of questin by *A. flavipes* HN4-13; Second, the significant factors were investigated and identified as starch, beef extract, and culture days through two-level FFD; Third, tri-factors and tri-level BBD were adopted to optimize the levels of significant factors. RSM was used to analyze the experimental data, build the model and

predict the optimal values of significant variables. Finally, the experiment was verified to ensure the validity of the RSM model.

In summary, the optimized fermentation conditions for the production of questin through *A. flavipes* HN4-13 in a 500-mL conical flask with 200 mL of medium were 4% soluble starch, 0.9% beef extract, 4% NaCl, 0.05% Na₂HPO₄, pH 6, 2% inoculum size, and shaking at 28 °C and 160 rpm/min for 7 days. The production of questin achieved 64.93 ± 4.55 mg/L and was 17-fold higher than that of the initial medium. The results suggest the potential of *A. flavipes* HN4-13 in the large-scale production of questin through fermentation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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