

Optimized production and isolation of antibacterial agent from marine *Aspergillus flavipes* against *Vibrio harveyi*

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Abstract Statistical methodologies, including Plackett–Burman design and Box–Behnken design, were employed to optimize the fermentation conditions for the production of active substances against aquatic pathogen *Vibrio harveyi* by marine-derived *Aspergillus flavipes* strain HN4-13. The optimal crucial fermentation values for maximum production of active substances against *V. harveyi* were obtained as follows: X_1 (peptone) = 0.3%, X_2 (KCl) = 0.25%, and X_3 (inoculum size) = 4.5%. The predicted diameter of inhibitory zone against *V. harveyi* was 23.39 mm, and the practical value reached 23.71 ± 0.98 mm with a 62.3% increase. Bioassay-guided fractionation resulted in the acquisition of two compounds whose structures were identified as questin (**1**) and emodin (**2**). Questin exhibited the same antibacterial activity against *V. harveyi* as streptomycin (MIC 31.25 µg/mL). This is the first time to report questin as a potential antibacterial agent against aquatic pathogen *V. harveyi*.

Keywords *Aspergillus flavipes* · *Vibrio harveyi* · Fermentation optimization · Response surface methodology · Bioassay-guided fractionation · Questin

Introduction

For several decades, the aquaculture industry of China acquired the prompt development and the yield of aquatic products ranks the first in the world. Meanwhile some animal diseases continuously appeared. Infectious vibriosis caused by *Vibrios* has been identified as a main economic challenge to the aquaculture (Morya et al. 2014; Guo et al. 2016b). Among them, *Vibrio harveyi* is considered to be a major pathogen, which induced animal mortality during early larval stages, resulting in serious losses in the production and marketing of aquatic animals, especially on farmed and wild shrimp (Thompson et al. 2010; Morya et al. 2014). At present, the application of antibiotics in the administration of aquaculture was widely used. However, the long-term application or abuse of antibiotics may be adverse to the environment protection and human health, including the transfer of antibiotic resistance to human pathogens, other aquatic pathogens, and the accumulation of antibiotic residues (Harikrishnan et al. 2010; Cao et al. 2011). Hence, other than the optional control strategies, for example, improving husbandry and water quality, lowering stocking densities, nourishing better nutrition, the application of favorable microbes or their metabolites is also broadly expected to become an alternative way for the prevention and control of vibriosis (Cao et al. 2011; Xu et al. 2014).

Living in a unique environment characterized as high salt, oligotrophic, and weak alkali, marine fungi could metabolize different natural products (NPs) from their

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terrestrial counterparts (Zhao et al. 2013). To date, about 800 new NPs with antimicrobial, antitumor, and antiviral activities have been identified from the marine fungal metabolites (Blunt et al. 2015; Gomes et al. 2015). The antimicrobial reports on marine fungal NPs were mainly focused on human pathogens (Zheng et al. 2013; Schueffler and Anke 2014; Song et al. 2014; Sun et al. 2014); however, there are rarely reports on the research of aquatic pathogens (Barakat and Gohar 2012).

To discover the potential use of marine fungal NPs in the prevention and treatment of aquatic pathogens, a fungal strain HN4-13 identified as *Aspergillus flavipes* was purified from Lianyungang coastal sediment. The metabolites of *A. flavipes* strain HN4-13 showed multiple antimicrobial activities against *V. harveyi*, *V. anguillarum*, *V. cholerae*, and *V. Parahemolyticus*, especially for *V. harveyi*. Thus, we used the inhibition zone against *V. harveyi* as an indicator to optimize the fermentation conditions by Plackett–Burman design, Box–Behnken design and response surface methodology and to identify the antibacterial products. The results will be report in this paper.

Materials and methods

Microorganism

Strain HN4-13 (CCTCC AF 2015022) was isolated from marine sediment collected in Lianyungang coast and identified as a member of *Aspergillus flavipes* based on the morphological characters and internal transcribed spacer sequence (GenBank No. JX287370) (Guo et al. 2013). *V. harveyi*, *V. anguillarum*, *V. cholerae*, and *V. Parahemolyticus* were provided by Dr. Zhenxia Su. All strains were kept at the Marine Microbial Natural Products Chemistry Laboratory of Huaihai Institute of Technology (Lianyungang, Jiangsu, China).

Shake-flask culture

The seed culture was prepared by adding mycelia on a sabouraud's dextrose agar medium (peptone 1%, glucose 4%, agar 1.5%, dissolved in natural seawater) into a 500-mL conical bottle with 200 mL of liquid sabouraud's dextrose medium (peptone 1%, glucose 4%, dissolved in natural seawater). Cultures were incubated in a rotary shaker with the rotating speed (160 rpm/min) at 28 °C for 1 day. The optimization process were conducted by adding the harvested seed culture into 500-mL conical bottle including 200 mL of liquid fermentation medium with shaking at 160 rpm/min as follows.

Statistical fermentation optimization

To obtain the maximal antibacterial activity of *A. flavipes* strain HN4-13, the optimization procedures of fermentation conditions were divided into the following three parts.

Two-level Plackett–Burman design

The objective of this optimization procedure was to determine which fermentation factors have significant effects on the antibacterial activity of active extracts. The key variables of fermentation conditions were investigated using a two-level Plackett–Burman design, which permits for screening of nine variables in just twelve experiments. Factors were applied at two levels coded as -1 and $+1$. The center points (0) of all the nine variables were determined according to the results of single-factor experiments. The software JMP 7 (SAS, USA) was used to design the experiments (Table 1).

Path of steepest ascent (descent)

The path of steepest ascent (descent) was applied to further optimize the variables screened by Plackett–Burman design to guide the triers nearly approach the optimum of the surface. The path initiated from the center of Plackett–Burman design, to run away from the center of Plackett–Burman design along the way of steepest ascent (descent) (Zhao et al. 2016). This routine should be ceased in favor of a more precise experiment once the way of steepest ascent (descent) no more led to increase (Zhang and Sang 2012). We moved -0.2 , 0.05 , and 1% in peptone, KCl, and inoculum size, respectively.

Box–Behnken design

The optimization of the values of significant factors was conducted by response surface methodology. Data analysis and model building were carried out by Design Expert 7.0.0 (Stat-Ease, Minneapolis, USA) (Guo et al. 2016a). Box–Behnken design consists of five central points and twelve factorial points; one of the response surface methodologies was employed to ascertain the response mode and establish the model. The dependent variable (Y , %) was the diameters of inhibition zone against *V. harveyi*, while peptone (X_1), KCl (X_2), and inoculum size (X_3) were selected for independent factors. The range and values of three independent factors are shown in Table 4. By this software, the optimization objective of fermentation condition was to achieve the maximum diameter of inhibition zone. The pattern of the system was evaluated by the following second-order polynomial equation:

Table 1 Experimental results of Plackett–Burman design of *Aspergillus flavipes* strain HN4-13

No.	Glucose (%)	Peptone (%)	KCl (%)	NaH ₂ PO ₄ (%)	NaCl (%)	PH	Inoculum size (%)	Temperature (°C)	Fermentation time (d)	Inhibition zone (mm)
1	4	1.5	0.15	0.15	4	8	3	36	10	16.38
2	2	1.5	0.05	0.15	4	8	1	28	6	10.63
3	2	0.5	0.15	0.05	4	8	3	28	6	23.78
4	4	0.5	0.05	0.15	2	8	3	36	6	21.31
5	2	1.5	0.05	0.05	4	6	3	36	10	9.07
6	2	0.5	0.15	0.05	2	8	1	36	10	22.12
7	2	0.5	0.05	0.15	2	6	3	28	10	19.71
8	4	0.5	0.05	0.05	4	6	1	36	6	14.77
9	4	1.5	0.05	0.05	2	8	1	28	10	12.52
10	4	1.5	0.15	0.05	2	6	3	28	6	19.45
11	2	1.5	0.15	0.15	2	6	1	36	6	11.39
12	4	0.5	0.15	0.15	4	6	1	28	10	17.93

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

where Y represents the predicted response, β_0 , β_i , β_{ii} and β_{ij} are constant coefficients, while X_i and X_j are the independent variables.

Preparation of the active extracts

Harvested fermentation broths were filtered through cheesecloth to isolate broth from mycelia. The filtrate was extracted with an equivalent volume of ethyl acetate for three times to give an ethyl acetate solution. Ethyl acetate solution was further enriched under reduced pressure to produce active extracts, which was dissolved in methanol (10 mg/mL).

Bioactivity-guided fractionation of active compounds

The fungus *A. flavipes* strain HN4-13 was cultivated in 500-mL conical bottles including 200 mL optimal fermentation medium that was composed of peptone 0.3%, glucose 3%, KCl 0.25%, NaH₂PO₄ 0.1%, NaCl 3%, inoculum size 4.5% and distilled water (pH 7.0) and was grown at 32 °C for 8 days with shaking at 160 rpm/min. The fermented whole broths (20 L) were filtered through cheesecloth to separate mycelia from broth. Then the filtrates were extracted by an equivalent volume of ethyl acetate three times, and concentrated under reduced pressure to give ethyl acetate extract (7.1 g).

The ethyl acetate extract was subjected to a vacuum column chromatography over silica gel, eluting with a

gradually gradient of petroleum ether–CH₂Cl₂ (1:1 and 0:1) and then of CH₂Cl₂–CH₃OH (100–0%) to achieve nine primary fractions (Fr.1–Fr.9). Fr.1–Fr.9 were screened for antibacterial activity against *V. harveyi* by the Oxford cup method. The Fr.5 (0.45 g) eluted with CH₂Cl₂–CH₃OH (25:1) showed the antibacterial activity against *V. harveyi*, which was further purified into three sub-fractions (Fr.5-1 to Fr.5-3) by Sephadex LH-20 after elution with CH₃OH. Then Fr.5-2 (0.1 g) was purified by preparative high-performance liquid chromatography (PHPLC) eluting with 80% MeOH–H₂O to give compounds **1** (4.0 mg, t_R 7.3 min) and **2** (8.5 mg, t_R 13.5 min).

Evaluation of antibacterial activity

The antibacterial activity against *V. harveyi* of the active extracts was evaluated by the Oxford cup method. The medium was beef extract peptone medium (BP) composed of beef extract 0.3%, peptone 1%, NaCl 0.5%, agar 1.5%, and the pH was adjusted to 7.0 with NaOH. These cups were placed on plates previously inoculated with the pathogens. 100 µL of each extract (10 mg/mL) was added in Oxford cups and incubation at 37 °C for 12 h. The experiments were conducted in triplicate, and the average inhibition zone (mm in diameter) was recorded as the antibacterial activity of active extracts.

Compounds were dissolved in methanol, diluted into different concentrations with sterile distilled water by continuous twofold dilution. These solutions were directly used to determine their antibacterial activities against aquatic *V. harveyi*, *V. anguillarum*, *V. cholerae*, and *V. parahaemolyticus* using above method. The minimum inhibitory concentration (MIC) was defined as the lowest

concentration of compounds that produced the inhibition zone against the tested pathogens (Sun et al. 2014). The experiments were performed in duplicate with full agreement between both results.

Results

Screening the significant factors using Plackett–Burman design

On the basis of single-factor experimental results, two-level Plackett–Burman design was employed to choose the most key fermentation factors. The experimental design and responses of different experiments are shown in Table 1. Table 2 shows regression coefficients of the variables on the responses, the associated *t* values, and significant level. The fit quality of first-order model was evaluated by the determination coefficient R^2 , which was determined as 0.983. The results indicated that the model could explain 98.3% of the variability in the response (Li et al. 2008). The analysis of variance showed the model was significant. In Table 2, *P* value of the three variables such as peptone, KCl and inoculum size was less than 0.10, which was identified as significant, while others were not significant. It also can be seen from Table 2, the increase of KCl and inoculum size had the positive effects on inhibition zone, whereas peptone had a negative effect. Therefore, peptone, KCl and inoculum size were chosen to make further optimization.

Approximating the optimum combination using path of steepest ascent (descent)

The path of ascent (descent) was decided to find the appropriate direction of changing variables, and then the concentration was increased or decreased according to the

sign of the main effect on increasing the diameter of inhibition zone. The path of steepest ascent (descent) began from the zero level of Plackett–Burman design and shifted along the path in which KCl and inoculum size increased, while peptone decreased. Other variables were set at their zero level. The design and data obtained from the experiment are shown in Table 3. The biggest inhibition zone was 23.28 mm when the concentrations of peptone, KCl and inoculum size were 0.4, 0.25, and 5%, respectively. It offered that the point was near the region of maximum inhibition zone response. Further optimization with Box–Behnken design and response surface methodology was around this point.

Identifying the optimal fermentation conditions using Box–Behnken design

Based on the results of Plackett–Burman design and path of ascent (descent) trial, peptone (X_1), KCl (X_2), and inoculum size (X_3) were chosen for further optimization by the trial of tri-factor, tri-level Box–Behnken design. Based on the parameter estimates, the application of response surface methodology can offer an empirical relationship between the response factors and the test variables. The matrices of Box–Behnken design are shown in Table 4. By applying multiple regression analysis on the experimental data, the equation for the diameter of inhibition zone was established as:

$$Y = 22.76 - 1.24X_1 - 0.082X_2 - 1.15X_3 + 0.64X_1X_2 - 0.052X_1X_3 - 0.65X_2X_3 - 1.16X_1^2 - 0.88X_1^2 - 1.06X_3^2 \quad (2)$$

The value of determination R^2 (0.9113) for Eq. (2) is reasonably close to 1, which indicates the regression models defined well the true behavior of the system (Guo et al. 2014). The corresponding analysis of variance is shown in Table 5. The values of “ $P > F = 0.0056$ ” indicated that the fitness of the model was significant. In addition, the lack of fit value of the model showed 0.7156, without significant difference (Guo et al. 2016a). The results also indicated that the linear effects of peptone and inoculum size are very significant ($P < 0.01$). The quadratic effect of peptone, KCl, and inoculum size ($P < 0.05$) is also significant.

The interactions between three variables (peptone, KCl and inoculum size) and the inhibition zone were exhibited by the response surface and contour plots (Fig. 1). The effects of peptone interaction with each of the two other factors on the diameter of inhibition zone are shown in Fig. 1a, b. The diameter of inhibition zone increased to a high value with increasing peptone from 0.2 to 0.3, but then, with increasing the concentration of peptone, the

Table 2 Analysis of the two-level Plackett–Burman design

Variable	Regression coefficient	<i>t</i> value	Sig.	Ranks
Glucose	0.472	1.09	0.3887	7
Peptone	− 3.348	− 7.76	0.0162	1
KCl	1.92	4.45	0.0470	2
NaH ₂ PO ₄	− 0.363	− 0.84	0.4886	8
NaCl	− 1.162	− 2.69	0.1148	5
pH	1.202	2.78	0.1085	4
Inoculum size	1.695	3.93	0.0592	3
Temperature	− 0.748	− 1.73	0.2252	6
Fermentation time	− 0.3	− 0.69	0.5590	9

Table 3 Experimental levels and results of the path of steepest ascent (descent)

No.	Peptone (%)	KCl (%)	Inoculum size (%)	Inhibition zone (mm)
1	1.0	0.10	2	22.19
2	0.8	0.15	3	20.08
3	0.6	0.20	4	22.88
4	0.4	0.25	5	23.28
5	0.2	0.30	6	22.30

inhibition zone could decrease, which indicated that peptone has significant effect on the diameter of inhibition zone. A similar phenomenon is observed in Fig. 1b, c with inoculum size and the other two factors. The increase in inoculum size enhanced the diameter of inhibition zone, but after that, with further increasing the inoculum size over 4.5%, the inhibition zone would be decreased. The effects of KCl interaction with each of the two other factors on the diameter of inhibition zone are shown in Fig. 1a, c, which imply that KCl is less significant than the other two variables.

The predicted optimal levels of peptone (X_1), KCl (X_2), and inoculum size (X_3) were obtained by applying the regression analysis to Eq. (2): $X_1 = 0.3\%$, $X_2 = 0.25\%$, and $X_3 = 4.49\%$. To confirm the result, the rechecking experiment was performed under the optimum fermentation conditions as follows: 0.3% peptone, 3% glucose, 0.25% KCl, 0.1% NaH_2PO_4 , 3% NaCl, pH7.0, 4.5% inoculum size, 32 °C, 160 rpm/min for 8 days. The

practical value of inhibition zone reached (23.71 ± 0.98) mm, which was not significant difference to the predicted value (23.39 mm), indicating that the model was accurate and adequate. Compared with that under the original liquid SD fermentation conditions (1% peptone, 4% glucose, natural seawater, 1% inoculum size, 28 °C, 160 rpm/min for 7 days), the diameter of inhibition zone increased 62.3%.

Identification of questin and emodin

The EtOAc extract of strain HN4-13 was subjected to bioassay-guided fractionation over silica gel column, Sephadex LH-20 column and C18 column to give compounds **1** and **2** (Fig. 2). The structures **1** and **2** were identified as 1,6-dihydroxy-8-methoxy-3-methylanthracene-9,10-dione (questin) and 1,3,8-trihydroxy-6-methylanthracene-9,10-dione (emodin), respectively, by UV, ^1H - and ^{13}C -NMR, DEPT and ESI-MS data (Fig. S1–S6).

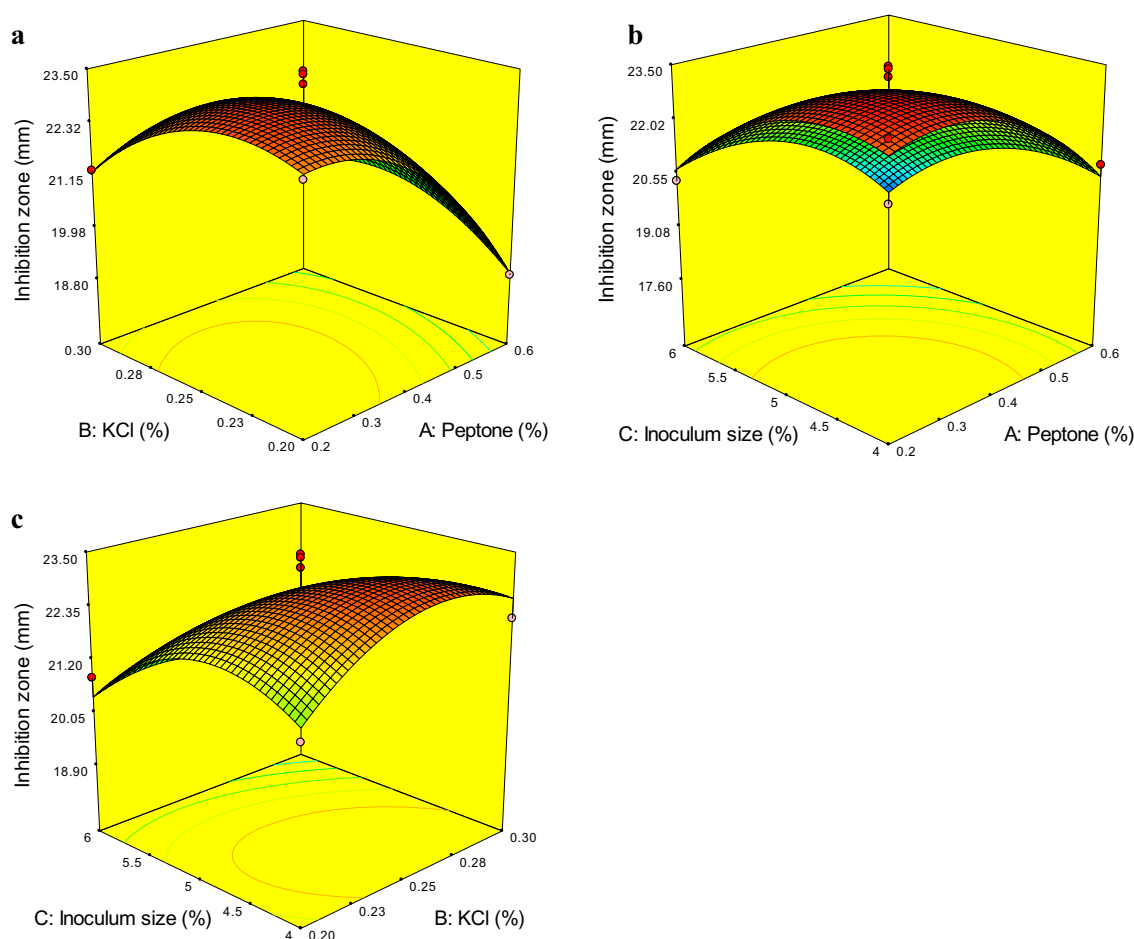
1,6-dihydroxy-8-methoxy-3-methylanthracene-9,10-dione (questin, **1**): orange powder. ^1H -NMR (600 MHz, $\text{DMSO}-d_6$) δ 13.23 (br s, 1H, HO-1), 7.18 (br s, 1H, H-2), 7.41 (br s, 1H, H-4), 7.10 (br s, 1H, H-5), 6.82 (br s, 1H, H-7), 3.88 (s, 3H, H_3CO -8), 2.37 (s, 3H, H_3 -11). ^{13}C -NMR (150 MHz, $\text{DMSO}-d_6$) δ 163.4 (C, C-1), 119.1 (CH, C-2), 146.6 (C, C-3), 124.2 (CH, C-4), 132.0 (C, C-4a), 107.0 (CH, C-5), 161.7 (C, C-6), 105.0 (CH, C-7), 164.6 (C, C-8), 112.5 (C, C-8a), 186.3 (C, C-9), 114.4 (C, C-9a), 182.3 (C, C-10), 136.8 (C, C-10a), 56.3 (CH_3 , CH_3O -8), 21.4 (CH_3 ,

Table 4 Box–Behnken design and experimental results of strain HN4-13

No.	Coded levels			Y (%)
	X_1 : Peptone (%)	X_2 : KCl (%)	X_3 : Inoculum size (%)	Inhibition zone (mm)
1	− 1 (0.2)	− 1 (0.2)	0 (5)	22.58
2	+ 1 (0.6)	− 1 (0.2)	0 (5)	18.88
3	− 1 (0.2)	+ 1 (0.3)	0 (5)	21.28
4	+ 1 (0.6)	+ 1 (0.3)	0 (5)	20.14
5	− 1 (0.2)	0 (0.25)	− 1 (4)	23.26
6	+ 1 (0.6)	0 (0.25)	− 1 (4)	20.81
7	− 1 (0.2)	0 (0.25)	+ 1 (6)	20.37
8	+ 1 (0.6)	0 (0.25)	+ 1 (6)	17.71
9	0 (0.4)	− 1 (0.2)	− 1 (4)	21.12
10	0 (0.4)	+ 1 (0.3)	− 1 (4)	22.11
11	0 (0.4)	− 1 (0.2)	+ 1 (6)	20.83
12	0 (0.4)	+ 1 (0.3)	+ 1 (6)	19.22
13	0 (0.4)	0 (0.25)	0 (5)	22.36
14	0 (0.4)	0 (0.25)	0 (5)	21.45
15	0 (0.4)	0 (0.25)	0 (5)	23.17
16	0 (0.4)	0 (0.25)	0 (5)	23.45
17	0 (0.4)	0 (0.25)	0 (5)	23.38

Table 5 Analysis of variance for the quadratic model of the Box–Behnken design experiments

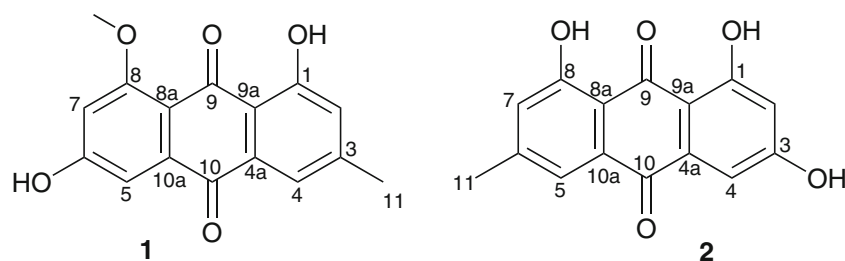
Source	Sum of squares	DF	Mean square	F value	P value	Sig.
Model	41.56	9	4.62	8.20	0.0056	**
X_1	12.38	1	12.38	21.97	0.0022	**
X_2	0.05	1	0.05	0.10	0.7649	
X_3	10.51	1	10.51	18.66	0.0035	**
X_1X_2	1.64	1	1.64	2.91	0.1319	
X_1X_3	0.01	1	0.01	0.02	0.8927	
X_2X_3	1.69	1	1.69	3.00	0.1269	
X_1^2	5.69	1	5.69	10.10	0.0155	*
X_2^2	3.26	1	3.26	5.79	0.0471	*
X_3^2	4.75	1	4.75	8.43	0.0228	*
Residual	3.94	7	0.56			
Lack of fit	1.04	3	0.35	0.48	0.7156	

* $P < 0.05$ and ** $P < 0.01$ **Fig. 1** Response surface plots showing effects of pairwise factors on the inhibition zone and their interaction

C-11). ESI-MS m/z 285.1 $[M + H]^+$. These data are consistent with literature report (Park and Kim 2011).

1,3,8-trihydroxy-6-methylantracene-9,10-dione (emodin, **2**): orange powder. $^1\text{H-NMR}$ (600 MHz, $\text{DMSO-}d_6$) δ 12.02 (s, 1H, HO-1), 7.16 (br s, 1H, H-2), 7.49 (br s, 1H,

H-4), 7.11 (br s, 1H, H-5), 6.59 (d, 1H, $J = 2.1$ Hz, H-7), 12.08 (1H, s, HO-8), 2.41 (s, 3H, H-11). $^{13}\text{C-NMR}$ (150 MHz, $\text{DMSO-}d_6$) δ 164.5 (C, C-1), 108.0 (CH, C-2), 165.7 (C, C-3), 108.9 (CH, C-4), 135.1 (C, C-4a), 120.5 (CH, C-5), 148.3 (C, C-6), 124.2 (CH, C-7), 161.4 (C, C-8),

Fig. 2 Chemical structure of questin (1) and emodin (2)

113.4 (C, C-8a), 189.6 (C, C-9), 108.8 (C, C-9a), 181.4 (C, C-10), 132.8 (C, C-10a), 21.5 (CH₃, C-11). ESI-MS m/z 271.3 [M + H]⁺. These data are consistent with literature report (Lv et al. 2011).

Antibacterial activities of emodin and questin against aquatic pathogens

The antibacterial activities of emodin and questin were determined by the Oxford cup assay using the aquatic pathogens *V. harveyi*, *V. anguillarum*, *V. cholerae* and *V. parahaemolyticus*. Questin showed the same activity to the positive control streptomycin against *V. harveyi* (MIC 31.25 µg/mL). Questin also exhibited antibacterial activity against *V. anguillarum*, *V. cholerae* and *V. parahaemolyticus* with the MIC values of 62.5, 62.5 and 125 µg/mL (Table 6), while emodin was not active against the tested aquatic pathogens.

Discussion

The fungus *A. flavipes* has been reported to produce pectinase (Martínez-Trujillo et al. 2011), L-methioninase (El-Sayed 2011), alpha-galactosidase (Ozsoy and Berkkan 2003), butyrolactones (Nagia et al. 2012), cerebrosides (Jiang et al. 2004), isobenzofuran (Kwon et al. 2012), alkaloids such as spiroquinazoline (Barrow and Sun 1994) and aspochalasin (Rochfort et al. 2005). This is the first report of questin which can be secreted by the fungus *A. flavipes*.

Questin (emodin 8-methyl ether) can be isolated from plant such as *Uvaria kurzii* (Lv et al. 2011) and *Cassia tora*

(Park and Kim 2011), and be secreted by marine-derived fungus *Aspergillus* sp. B-F-2 (Liu et al. 2006), marine mangrove plant-derived endophytic *Eurotium rubrum* (Li et al. 2009), *Trewia nudiflora* commensal fungus *Aspergillus* sp. F1 (Lin et al. 2009) and the antarctic sponge-derived *Pseudogymnoascus* sp. fungus (Figueroa et al. 2015). It has previously been shown to exhibit moderate or weak cytotoxicity, DPPH radical scavenging activity and antimicrobial activity against phytopathogenic *Fusarium oxysporum*. Besides, it is very interesting that the analogue of questin, physcion (parietin, emodin 3-methyl ether, emodin 6-methyl ether) has been used widely in the agriculture of China as a highly active botanical pesticide against phytopathogens. However, to the best of our knowledge, this is the first report of questin possessing antimicrobial activity against aquatic pathogenic *Vibrios*.

We report here the optimized production of antibacterial substances against aquatic pathogenic *V. harveyi* from *A. flavipes* strain HN4-13 with potential for aquatic applications. Statistical methods, Plackett–Burman design, Box–Behnken design and response surface methodology were combined and employed to identify the optimum fermentation conditions for the production of antibacterial active compounds as follows: 0.3% peptone, 3% glucose, 0.25% KCl, 0.1% NaH₂PO₄, 3% NaCl, pH7.0, 4.5% inoculum size, 32 °C, 160 rpm/min for 8 days. Such optimization showed a 62.3% increase in antibacterial activity. Then, two compounds, emodin and questin, were separated from the ethyl acetate extract of fermented broth of *A. flavipes* HN4-13 by activity-guided fractionation. Compared to streptomycin, questin exhibited significant antimicrobial activity against aquatic pathogen *V. harveyi* with a MIC value of 31.25 µg/mL. Work on

Table 6 Antibacterial activities against aquatic pathogens of questin

Compound	MIC (µg/mL)			
	<i>V. harveyi</i>	<i>V. anguillarum</i>	<i>V. cholerae</i>	<i>V. parahaemolyticus</i>
Streptomycin	31.25	31.25	31.25	31.25
Questin	31.25	62.50	62.50	125.0
Emodin	—	—	—	—

“—” Shows no activity

pharmacological effect and action mechanism of questin against aquatic pathogen *V. harveyi* in vitro and in vivo would provide the favorable prospect in aquatic application.

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Compliance with ethical standards

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

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