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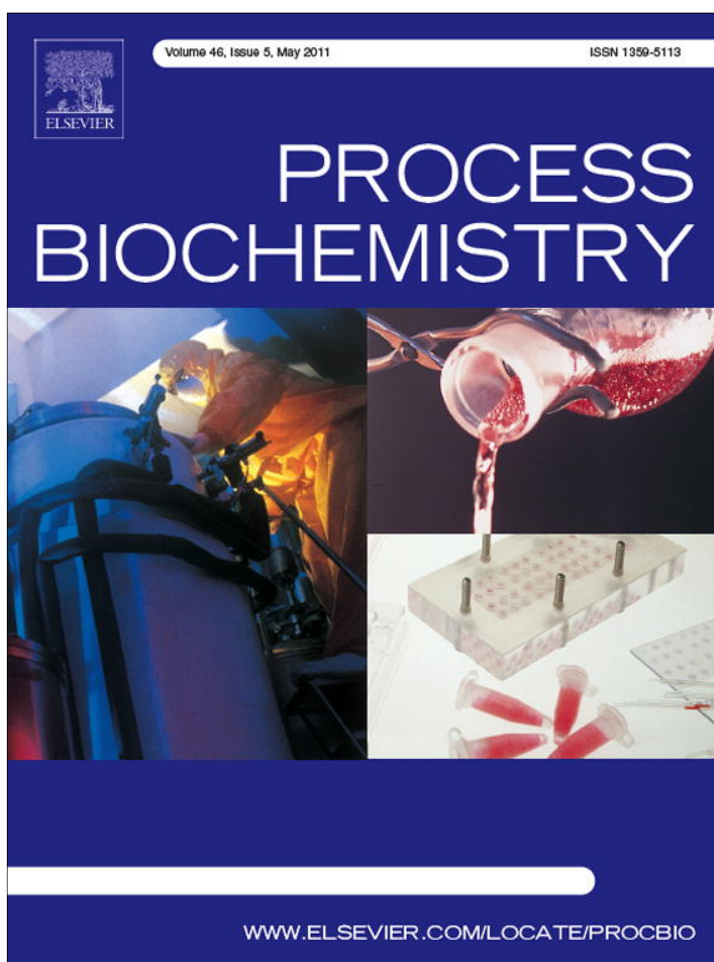

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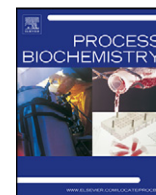

Bacillamide C production by the optimized cultivation of the *Bacillus atrophaeus* strain C89 associated with the South China Sea sponge *Dysidea avara*

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ABSTRACT

Marine natural products with biological activity represent an important resource for marine drugs and biotechnological products; however, most marine natural products, especially those derived from sponges, have not been further developed because of the limited number of sources. The scale-up cultivation of the microbial source of sponge-derived natural products could provide a possible approach to solve this bottleneck problem. In this study, statistical Plackett–Burman (PB) design and Box–Behnken response surface methodology (RSM) were used to optimize the medium components to increase the production of bacillamide C by the *Bacillus atrophaeus* strain C89, which was associated with the marine sponge *Dysidea avara*. The obtained optimal medium consisted of 3.64 g/L corn starch, 6.29 g/L $CaCO_3$, 4.00 g/L soy peptone, 6.00 g/L peptone, 0.10 g/L cysteine and 0.02 g/L tryptophan with artificial sea water (ASW). Using this optimized medium, the concentration of bacillamide C reached 70.85 mg/L in fed-batch cultivation in a 5-L fermentor at pH 7.70, which was 25.80-fold higher than the level observed in a shake flask using the basic medium. This study provides a potential approach for the production of bacillamide C and related derivatives, which was the first to optimize the cultivation of sponge-associated microbes for the production of marine natural products.

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1. Introduction

Marine sponges are known to be important sources of novel marine natural products. For example, *Dysidea* sponge-derived biologically active marine natural products include polybrominated phenyl ethers, chlorinated amino acid derivatives, furano-sesquiterpenes and polyhydroxy steroids [1–3]. However, the further research and development (R&D) of marine sponge-derived natural products have been limited by sponge collection and the low concentration of bioactive natural products in sponges. Only 0.31 g of pure halichondrin B, for example, can be isolated from one ton of wet sponge *Halichondria okadai* [4]. Thus, the strategy of direct exaction of bioactive products from sponges cannot satisfy the requirement for sponge-derived bioactive natural products.

It is known that marine sponges harbor various microorganisms [5,6]. In 1988, Stierle et al. [7] first discovered that a *Micrococcus* sp. isolated from the sponge *Tedania ignis* produced the same diketopiperazines as the sponge itself. So far, increasing evidence has demonstrated that most of the sponge-derived natural products come from sponge-associated microorganisms [8,9]. Sponge-associated microorganisms could also be involved in supplying nutrients, stabilizing sponge skeletons and protecting sponges from bio-fouling or predation [10]. Accordingly, the isolation and scale-up cultivation of sponge-associated microbes, which produce the bioactive natural products, may solve the current bottleneck problem of supply limitation [11].

To our current knowledge [12–16], bacillamides are composed of four members, bacillamides A, B, C and D (Fig. 1). Bacillamide D was first isolated from *Thermoactinomyces* sp. strain TM-64 in 1975 [12]. In 2003, bacillamide A was isolated from the marine bacterium *Bacillus* sp. SY-1 as a new algaecide against the harmful dinoflagellate *Cochlodinium polykrikoides* [13]. In 2007, the chemical study of *Bacillus endophyticus* isolated from a Bahamian hypersaline microbial mat led to the isolation of bacillamides B and C [14]. Meanwhile, bacillamide C was also isolated from a *Microbispora aerata* strain [15]. In our previous study [16], bacillamide C and a new compound of neobacillamide A were isolated from *Bacillus atrophaeus* strain C89, associated with the South China Sea sponge *Dysidea avara*.

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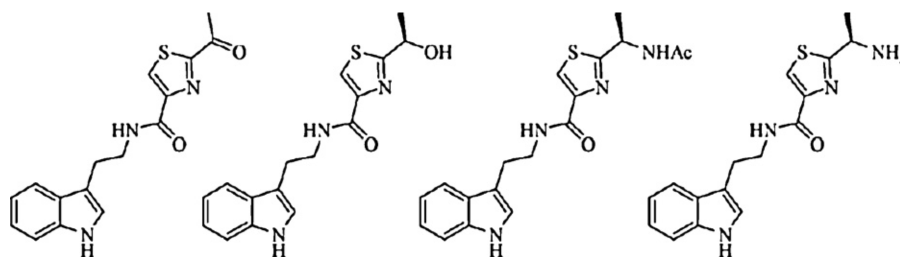

Fig. 1. Structure of bacillamides (left to right: bacillamide A, bacillamide B, bacillamide C, bacillamide D).

Bacillamides contain a tryptamide thiazole motif that serves as a building block in some potently bioactive cyclic peptides, such as the antibiotic zelkovamycin produced by a *Streptomyces* sp. [17] and the protein synthesis inhibitors A-21459 A and B from an *Actinoplanes* sp. [18]. Bacillamide A shows antibiosis against dinoflagellates and raphidophytes [13]. Different synthesized derivatives of bacillamide A (4-methoxy-bacillamide A, 4-bromo-bacillamide A, 4-chloro-bacillamide A, 4-fluoro-bacillamide A and 4-iodo-bacillamide A) can control the growth of particular species of cyanobacteria; in particular, 4-iodo-bacillamide can act as an algistatic agent against eukaryotic algae depending on its concentration [19]. Although studies on the chemical synthesis of bacillamides have been conducted recently [20], to our knowledge, bacillamides B, C and D have not been tested for algicidal activity or for other biological activity due to a lack of compound material.

In the case of metabolite production by sponge-associated microorganisms, previous research has mainly focused on marine enzymes such as lipase, chitinase and alpha-amylase [21–23]. Abundant natural small molecular products have been isolated from sponge-associated microbes [24,25], but their production by microbial cultivation has rarely been reported [11]. To date, bacillamide C production by microbial fermentation has not been reported as well as natural products from *B. atrophaeus*.

The purpose of this study was to optimize the medium components using Plackett–Burman (PB) design and Box–Behnken response surface methodology (RSM) in order to increase the production of bacillamide C by the cultivation of *B. atrophaeus* strain C89, which was associated with the marine sponge *D. avara*. Meanwhile, the effects of precursor and pH, fed-batch cultivation of *B. atrophaeus* strain C89 in a 5-L fermentor were investigated for bacillamide C production. This study lays the foundation for the mass-production of bacillamide C and related derivatives.

2. Materials and methods

2.1. Sponge, bacterial strain and media

The sponge *D. avara* (phylum Porifera, class Demospongiae, order Dictyoceratida, family Dysidea) was collected by SCUBA diving at a depth of about 20 m around Sanya Island in the South China Sea and was identified by Professor Jinghe Li, Institute of Oceanology, Chinese Academy of Sciences [26].

B. atrophaeus strain C89, which shows a significant activity against *Aspergillus niger* and *Paecilomyces variotii* [26], was isolated from *D. avara* and identified according to the 16S rRNA gene (GenBank No. DQ091007).

Medium 1 was beef extract peptone medium, containing 5.00 g/L beef extract and 10.00 g/L peptone; medium 2 was Luria-Bertani medium, containing 5.00 g/L yeast extract and 10.00 g/L peptone; medium 3 was 2216E medium, containing 1.00 g/L beef extract and 5.00 g/L peptoneand medium 4 consisted of 5.00 g/L corn starch, 5.00 g/L soy peptone, 5.00 g/L peptone and 5.00 g/L $CaCO_3$. The above media were prepared with artificial sea water (ASW) [27]. The bacterium was cultured in 250-mL shaking flasks containing 100 mL of one of the above media at 28 and 150 rpm for 96 h.

2.2. HPLC analysis of bacillamide C

The concentration of bacillamide C was determined by HPLC on a reversed phase ZORBAX Eclipse XDB-C18 column (4.60 mm × 150.00 mm; i.d. 5.00 μm) coupled to a diode array detector (DAD) set at 220 nm (Agilent, USA). The column temperature was 25 °C. The flow rate was 1 mL/min and the injection was done through a 20 μL loop. The mobile phase was 20% (v/v) acetonitrile at the beginning and gradually increased to 45% (v/v) acetonitrile in 15 min.

The reference pure compound bacillamide C was isolated according to Yu et al. [16]. A stock solution of the analytes (1 mg/mL) was prepared by dissolving pure compound in methanol. A seven-point calibration curve between the peak areas and the concentrations of bacillamide C was set up using standard solutions over the concentration range of 1.00–1000.00 μg/mL with values of LOQ (limit of quantification) and LOD (limit of detection) equal to 0.50 μg/mL and 0.15 μg/mL, respectively. The linear regression equation was as follows: $Y = 49.66397X + 132.33327$ ($r^2 = 0.9999$), where, Y was the peak area and X was the concentration of bacillamide C (μg/mL).

The fermentation broth (10 mL) of *B. atrophaeus* strain C89 was extracted twice with an equal volume of EtOAc. The residual material after evaporation under vacuum was dissolved in methanol for HPLC analysis. The HPLC profiles of a standard solution containing 100 μg/mL bacillamide C and an extract sample are shown in Fig. 2. The concentration of bacillamide C was determined according to the regression equation above.

2.3. The effect of precursors on bacillamide C production

The effects of three possible precursors (alanine, cysteine and tryptophan) on bacillamide C production were investigated at five concentration levels (20.00 mg/L, 60.00 mg/L, 100.00 mg/L, 140.00 mg/L and 180.00 mg/L) and they were added at different times (0, 10, 18, 28, 48 and 68 h) during the cultivation by a single-factor experiment.

2.4. Statistical optimization of the medium components

Optimization of the medium constituents was carried out in two stages. First, the variables that had a significant effect on bacillamide C production were identified using a two-level Plackett–Burman design method. Second, Box–Behnken response surface methodology was used to optimize the concentration of each variable [28].

2.5. Cultivation in a 5-L fermentor

A 5-L fermentor (BIOSTAT® A Plus, Sartorius Stedim, German) containing 3 L of the optimized medium was used. The inoculum volume was 5.00% (v/v) (cell density was *ca.* 0.01×10^9 cells/mL). The temperature was maintained at 28 °C and pH, agitation speed and dissolved oxygen (DO) were recorded by the Supervisory Process Control Software. DO was regulated by adjusting agitation speed under 1.00vvm. Fed-batch cultivation was carried out by adding 2-fold concentrated optimal medium at the first 24 h. Precursors were added in the late stage of the log phase of bacterial cultivation. The bacterial growth was analyzed by direct microscopic count. The total residual sugar was measured using the DNS method [29].

2.6. Statistical analytical methods

All experiments and measurements were performed in triplicates. One-way ANOVA and Duncan's multiple range test were used to determine the significant difference at $P < 0.05$. The related data analysis was done using Excel 7.0, Minitab 15 and ANOVA software.

3. Results and discussion

3.1. Selection of basic medium and the effects of precursors on bacillamide C production

Among the four media tested, medium 4 resulted in the highest bacillamide C yield (2.75 ± 0.05 mg/L) and biomass density ($16.85 \pm 0.26 \times 10^8$ cells/mL) after 96-h cultivation. The bacillamide C yields of media 1, 2 and 3 were 2.41 mg/L, 1.86 mg/L and 1.31 mg/L, respectively. Thus, medium 4 was selected as the basic medium for further optimization. In this basic medium, the *B.*

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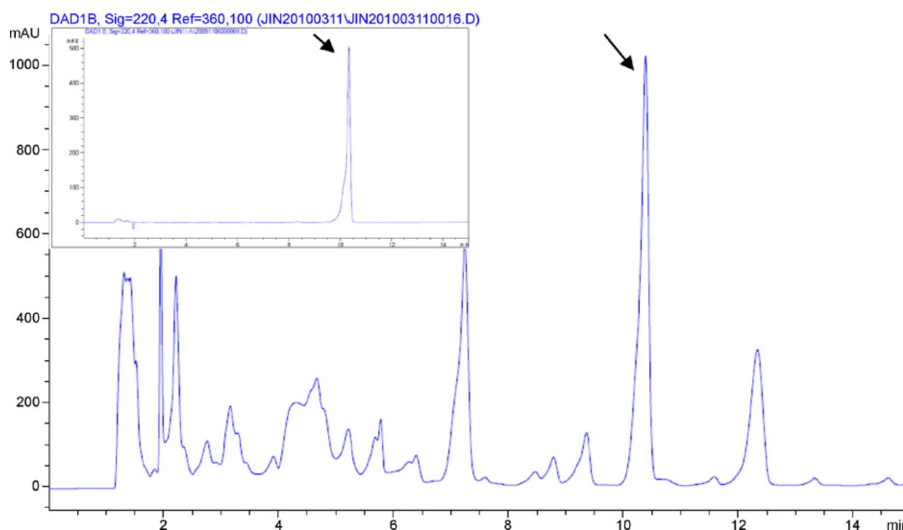

Fig. 2. HPLC of a standard solution containing 100 μg/mL bacillamide C and an extract sample containing 22.88 mg/L bacillamide C, the arrow indicates bacillamide C.

atrophaeus strain C89 reached the log phase after 10–28 h of cultivation and the stationary phase was reached at 28–96 h of cultivation (data not shown).

The comparison of bacillamide C production with 100 mg/L alanine, cysteine and tryptophan addition in the basic medium was made at different time points: 0 h, 10 h, 18 h, 28 h, 48 h and 68 h. As a result, the optimal addition time point was in the first 68 h for cysteine and tryptophan, and in the first 28 h for alanine. Meanwhile, at the relevant optimal time points, 100.00 mg/L was proved to be the optimal concentration for all the three precursors at the tested concentration range of 20.00–180.00 mg/L (data not shown). The maximum bacillamide C productivity reached 7.47 mg/L, 6.36 mg/L and 7.32 mg/L upon the addition of cysteine, tryptophan and alanine, respectively.

A possible biosynthetic pathway for neobacillamide A suggested that bacillamide C could be derived from the amino acids alanine, cysteine and tryptophan [30]. Consistent with this hypothesis, our result showed that cysteine, tryptophan and alanine had positive effects on the biosynthesis of bacillamide C.

3.2. Selection of important medium components for bacillamide C production

The statistical Plackett–Burman design method was used to optimize the medium components for improving bacillamide C production. The (−) value and (+) value of the seven components (the 4 components of medium 4 and the 3 precursors) are shown in Table 1. Cysteine, tryptophan and alanine were supplemented at 68 h, 68 h and 28 h of the fermentation period, respectively. The Plackett–Burman design for 12 trials with two concentrations of each variable and the corresponding bacillamide C yield is shown in Table 2, where the variables X_1–X_7 represented the medium constituents, D_1–D_3 represented the dummy variables/unassigned variables. The result of Plackett–Burman design is exhibited in Table 3. The variables manifesting a confidence level above 95% were selected and further optimized. Variables X_1 (corn starch), X_2 (alanine), X_4 ($CaCO_3$) and X_6 (cysteine) had confidence levels above 95% and thus were considered to be significant factors. The other three variables, X_3 (peptone), X_5 (soy peptone) and X_7 (tryptophan), were negligible at the selected concentrations because their confi-

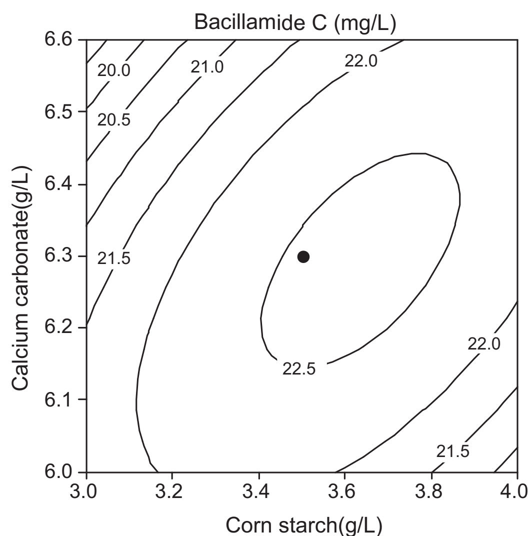

Fig. 3. Effect of corn starch and $CaCO_3$ on bacillamide C production at 0.10 g/L Cys and 0.00 g/L Ala.

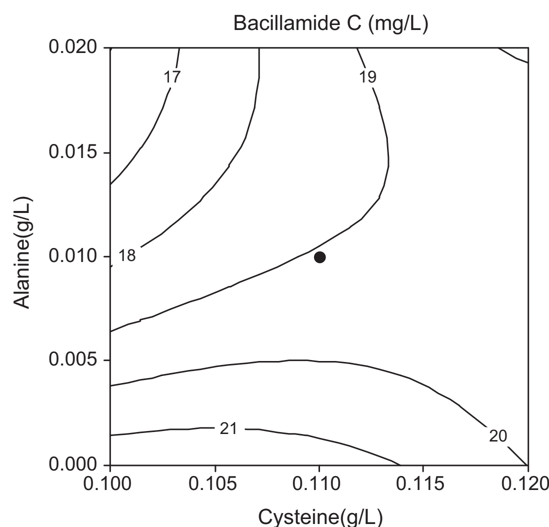

Fig. 4. Effect of Cys and Ala on bacillamide C production at 3.00 g/L corn starch and 6.00 g/L $CaCO_3$.

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Table 1
Values for the Plackett–Burman experiment.

Variable	Medium component	+Value (g/L)	−Value (g/L)
X_1	Corn starch	6.00	4.00
X_2	Alanine	0.10	0.02
X_3	Peptone	6.00	4.00
X_4	$CaCO_3$	6.00	4.00
X_5	Soy peptone	6.00	4.00
X_6	Cysteine	0.10	0.02
X_7	Tryptophan	0.10	0.02

Table 2
Two-level Plackett–Burman design matrix with bacillamide C production.

Trial no.	Variable										Bacillamide C yield (mg/L)
	X_1	X_2	D_1	X_3	X_4	D_2	X_5	X_6	D_3	X_7	
1	1	1	−1	1	1	−1	1	−1	−1	−1	5.65 ± 0.08
2	1	−1	−1	−1	1	1	1	−1	1	1	6.40 ± 0.12
3	1	1	−1	1	−1	−1	−1	1	1	1	6.18 ± 0.13
4	−1	1	−1	−1	−1	1	1	1	−1	1	7.80 ± 0.04
5	−1	1	1	−1	1	−1	−1	−1	1	1	8.31 ± 0.15
6	−1	1	1	1	−1	1	1	−1	1	−1	7.92 ± 0.14
7	−1	−1	−1	1	1	1	−1	1	1	−1	14.93 ± 0.26
8	−1	−1	1	1	1	−1	1	1	−1	1	11.04 ± 0.07
9	1	1	1	−1	1	1	−1	1	−1	−1	7.47 ± 0.23
10	1	−1	1	1	−1	1	−1	−1	−1	1	6.54 ± 0.26
11	1	−1	1	−1	−1	−1	1	1	1	−1	6.56 ± 0.21
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	8.14 ± 0.14

dence levels were below 95%. Accordingly, they were set at 6.00 g/L (+), 4 g/L (−) and 0.02 g/L (−), respectively.

3.3. Optimization of the component concentrations for bacillamide C production

According to Plackett–Burman design experiment, four significant variables X_1 (corn starch), X_2 (alanine), X_4 ($CaCO_3$) and X_6 (cysteine) were selected for further optimization using Box–Behnken RSM to determine the respective optimal values. The coded values of the variables at various levels in Box–Behnken RSM analysis are given in Table 4. The Box–Behnken experimental design and the obtained bacillamide C yield are shown in Table 5. As a result, the following equation was obtained by Minitab 15.0 analysis: $Y = +19.7274 - 1.4022X_1 + 0.0043X_2 + 1.3838X_3 - 0.9908X_4 - 0.8747X_1{}^2 - 0.5857X_2{}^2 - 0.7474X_3{}^2 + 0.9654X_4{}^2 - 1.6616X_1X_2 + 0.9425X_1X_3 - 0.2349X_1X_4 + 1.0453X_2X_3 + 1.4585X_2X_4 + 0.6261X_3X_4$, where Y is the predicted response, and X_1, X_2, X_3 and X_4 are the coded values for corn starch, cysteine, $CaCO_3$ and alanine, respectively. According to the ANOVA analysis in Table 6, the values of Model F and Model Prob. > F were found to be 13.89 and 0.001, respectively, which implied that the model was significant. Analysis of variance and regression based on Table 6 using MINITAB 15 showed that the coefficient of determination (R^2) was 0.95 (data not shown), indicating a good agreement between the experimental and predicted values of Bacillamide C production.

The contour plots shown in Figs. 3 and 4 were based on the regression equation obtained by holding two variables constant at the level of −1, while varying the other two within their experimental range. Fig. 3 shows the interaction between corn starch and $CaCO_3$, and Fig. 4 depicts the combined effect of cysteine and alanine. Consequently, the optimized medium for bacillamide C production obtained was as follows: 3.64 g/L corn starch, 6.29 g/L $CaCO_3$, 4.00 g/L soy peptone, 6.00 g/L peptone, 0.10 g/L cysteine and 0.02 g/L tryptophan with ASW.

Finally, the maximal bacillamide C production and bacterial growth were compared by cultivating the bacterium in the basic medium and the optimized medium in the shake flask (Fig. 5). In the optimized medium, a concentration of 22.80 mg/L bacillamide C was achieved after 96 h cultivation, which was 8.3-fold higher than that in the basic medium (2.75 mg/L). It was only 0.77% higher than the concentration of 22.62 mg/L predicted according to the equation from the Minitab 15.0 analysis, which indicated proficiency of the model used for optimizing bacillamide C production. Moreover, the maximum biomass of *B. atrophaeus* strain C89 reached 1.78×10^9 cells/mL in the optimized medium. The ratio of bacillamide C production per cell increased from 1.63×10^{-12} mg in the basic medium to 1.28×10^{-11} mg in the optimized medium, suggesting the improved ability of single cell to produce bacillamide C.

The conventional method for medium optimization is time-consuming and cannot depict the combined effect of multiple factors involved [31]. The Plackett–Burman method is widely used for the identification of significant variables as well as their

Table 3
Effect estimates for bacillamide C production based on the Plackett–Burman design.

Factor	Medium component	Effect	S.E.	T	P	Confidence level (%)
X_1	Corn starch	−3.221	0.291	−5.53	0.005	99.50
X_2	Alanine	−1.714	0.291	−2.94	0.042	95.80
X_3	Peptone	1.265	0.291	2.17	0.096	90.40
X_4	$CaCO_3$	1.778	0.291	3.05	0.038	96.20
X_5	Soy peptone	−1.032	0.291	−1.77	0.151	84.90
X_6	Cysteine	1.837	0.291	3.15	0.034	96.60
X_7	Tryptophan	−0.734	0.291	−1.26	0.277	72.30

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Table 4
Coded and real concentration values of the important variables in Box–Behnken design.

Coded value (level)	Real value of variable			
	Corn starch (g/L)	$CaCO_3$ (g/L)	Cysteine (g/L)	Alanine (g/L)
−1	3.00	6.00	0.10	0.00
0	3.50	6.30	0.11	0.01
1	4.00	6.60	0.12	0.02

Table 5
Box–Behnken design matrix with the experimental and predicted values of bacillamide C.

Trial no.	Variables/levels					Bacillamide C yield (mg/L)	
		Corn starch (coded value)	Cysteine (coded value)	$CaCO_3$ (coded value)	Alanine (coded value)	Experiment	Predicted
1	1	0	0	−1	1	16.94 ± 0.15	17.15
2	1	1	1	0	0	15.64 ± 0.13	15.42
3	1	0	0	1	1	21.49 ± 0.15	21.17
4	1	−1	−1	0	0	18.18 ± 0.30	18.21
5	1	−1	1	0	0	21.33 ± 0.11	21.54
6	1	0	0	0	0	19.13 ± 0.45	19.94
7	1	0	0	−1	−1	20.27 ± 0.17	20.39
8	1	1	−1	0	0	19.14 ± 0.18	18.73
9	1	0	0	1	−1	22.32 ± 0.10	21.90
10	2	1	0	0	−1	19.99 ± 0.34	19.34
11	2	1	0	0	1	16.30 ± 0.20	16.89
12	2	0	−1	1	0	18.21 ± 0.29	18.43
13	2	0	0	0	0	19.65 ± 0.28	19.43
14	2	−1	0	0	−1	22.26 ± 0.24	21.68
15	2	0	−1	−1	0	17.81 ± 0.01	17.75
16	2	0	1	−1	0	15.84 ± 0.28	15.67
17	2	−1	0	0	1	19.47 ± 0.26	20.17
18	2	0	1	1	0	20.42 ± 0.03	20.53
19	3	−1	0	1	0	20.11 ± 0.50	20.04
20	3	0	1	0	−1	19.03 ± 0.10	19.73
21	3	0	−1	0	−1	21.86 ± 0.27	22.46
22	3	1	0	−1	0	14.25 ± 0.25	14.47
23	3	0	1	0	1	21.30 ± 0.10	20.67
24	3	−1	0	−1	0	19.49 ± 0.40	19.16
25	3	0	0	0	0	20.40 ± 0.25	19.82
26	3	1	0	1	0	18.64 ± 0.16	19.12
27	3	0	−1	0	1	18.30 ± 0.18	17.74

Table 6
Analysis of variance and regression for bacillamide C production.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	Prob. > *F*
Block	1.258	2	0.6290	1.150	0.355
Model	106.349	14	7.5964	13.890	0.001
Linearity	58.353	4	14.588	26.670	0.000
Square	18.731	4	4.683	8.560	0.003
Interaction	29.265	6	4.878	8.920	0.002
Error	5.469	10	0.547		
Total	113.077	26			

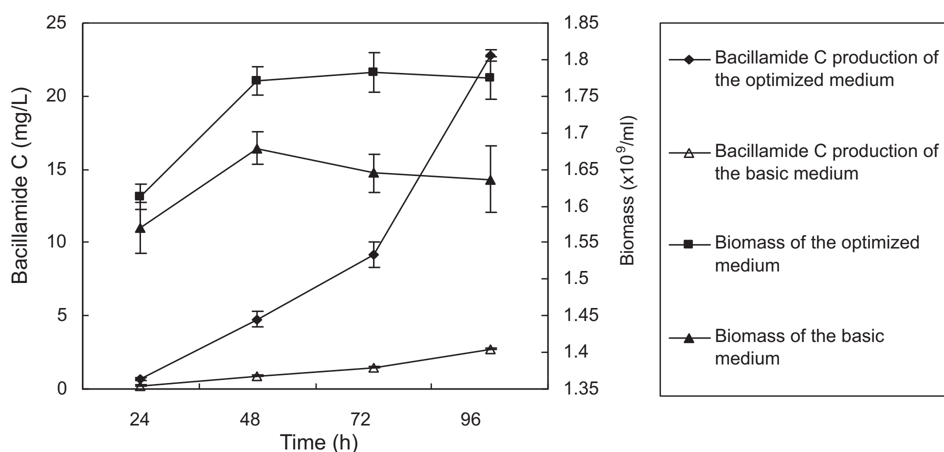

Fig. 5. A comparison of bacillamide C production and cell biomass between the optimized medium and the basic medium in the shake flask ($n = 3$).

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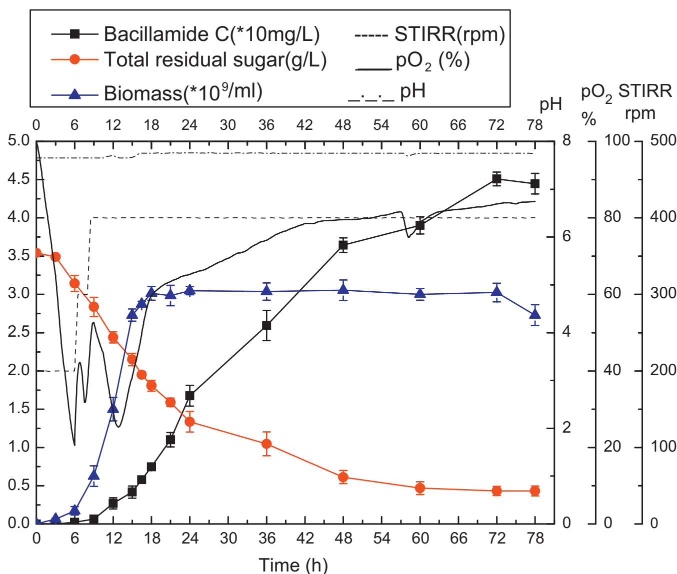

Fig. 6. Batch cultivation profiles in a 5-L fermentor at pH 7.70, biomass (▲), total residual sugar (●), bacillamide C (■), pH (_._._), pO_2 (−−) and STIRR (−).

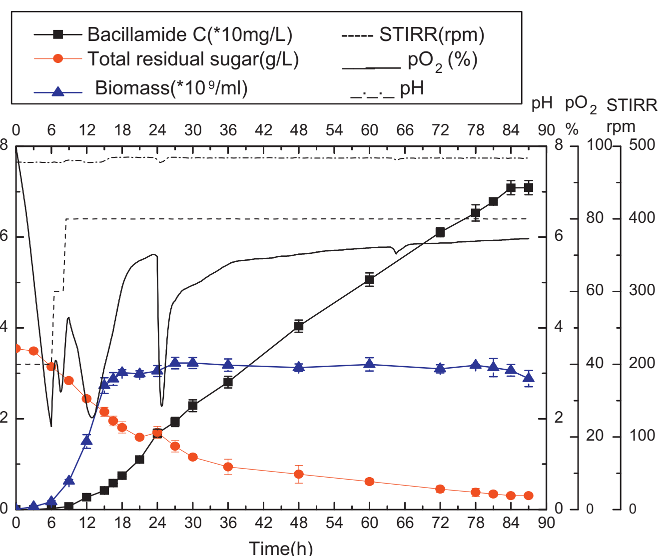

Fig. 7. Fed-batch cultivation profiles in a 5-L fermentor at pH 7.70, biomass (▲), total residual sugar (●), bacillamide C (■), pH (_._._), pO_2 (−−) and STIRR (−).

significance levels [26]. Response surface methodology (RSM) is an important statistical technique for revealing interactions among the variables and screening the optimum factors for desirable responses [32]. As shown in the results above, the Plackett–Burman design and RSM are powerful tools for identifying the significant factors and their values for bacillamide C production.

3.4. Fed-batch cultivation of B. atrophaeus strain C89 in a 5-L fermentor for bacillamide C production

Compared with cultivation in the shake flask, the growth speed, maximum biomass and production of bacillamide C were improved notably in a 5-L fermentor using the optimized medium with 0.10 g/L cysteine and 0.02 g/L tryptophan supplement in the first 58 h. For example, the cell growth entered the stationary phase after 18 h cultivation, the cultivation period was reduced from 96 h in the shake flask to 72 h in a 5-L fermentor. In particular, the maximum biomass and yield of bacillamide C reached 2.39×10^9 cells/mL and 34.80 mg/L, respectively (cultivation profiles not shown), which were higher than those observed in the shake flask (1.78×10^9/mL, 22.80 mg/L).

Naturally, the cultivation pH changed from 7.20 to 8.40 during the batch cultivation. In order to evaluate the effect of pH on the cell growth and bacillamide C production, the batch cultivation in the 5-L fermentor was carried out at pH 7.20, pH 7.70, pH 8.20 and uncontrolled pH respectively. As a result, the production of bacillamide C at 72 h were 39.12 mg/L, 45.09 mg/L, 23.58 mg/L and 34.80 mg/L, respectively. Meanwhile, the maximum biomass (3.05×10^9 cells/mL) was obtained at pH 7.70, while the total residual sugar (0.43 g/L) was the least at this pH. Thus pH 7.70 was maintained in the following fed-batch cultivation.

During the batch cultivation at pH 7.70 (Fig. 6), the total sugar declined significantly from 3 h to 24 h, while the accumulation of bacillamide C increased sharply from 9 h to 24 h and reduced after 24 h, thus, in fed-batch cultivation (Fig. 7), 200 mL of 2-fold optimized medium without cysteine and tryptophan was supplemented in the first 24 h cultivation, while 0.10 g/L cysteine and 0.02 g/L tryptophan were supplemented after 64 h. Compared with batch cultivation (Fig. 6), in fed-batch cultivation (Fig. 7), an approximately 1.6-fold increase in bacillamide C yield (70.85 mg/L) was achieved though the maximum biomass remained nearly unchanged (3.225×10^9 cells/mL). Meanwhile, the stationary phase of bacterial growth extended to 84 h, the total residual sugar after fermentation decreased to 0.31 g/L.

4. Conclusions

In this study, an optimized medium for the cultivation of *B. atrophaeus* strain C89 to produce bacillamide C was obtained using Plackett–Burman design and Box–Behnken response surface methodology. The optimal medium consisted of 3.64 g/L corn starch, 6.29 g/L $CaCO_3$, 4.00 g/L soy peptone, 6.00 g/L peptone, 0.10 g/L cysteine and 0.02 g/L tryptophan with ASW. Using the optimized medium and fed-batch cultivation strategy in a 5-L fermentor at pH 7.70, bacillamide C reached a concentration of 70.85 mg/L, which was 25.80-fold higher than in the 250-mL shake flask containing basic medium.

Sponge-associated microorganisms represent a treasure trove of biodiversity for the discovery of marine natural products with biotechnological potential [33,34]. Sponge-associated microbial fermentation will provide a strategy to overcome the bottleneck problem of supply limitation in the research and development of sponge-derived marine natural products. This study was the first to optimize the cultivation of sponge-associated microbes for the production of marine natural products. According to this study, the *B. atrophaeus* strain C89 from the South China Sea sponge *D. avara* is a potential strain that may be used for the mass-production of bacillamide C and bacillamide derivatives.

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