JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2015. Vol. **9**(3), p. 1783-1792

Optimization of Medium to Improve the Antifungal Activity of *Bacillus atrophaeus* XW2 using Response Surface Methodology

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(Received: 28 April 2015; accepted: 04 June 2015)

***Bacillus atrophaeus* XW2, isolated from poplar leaves, shows a strong inhibitory effect against the fungus *Colletotrichum gloeosporioides*, which causes poplar anthracnose. The effects of different medium components on antifungal activity against *C. gloeosporioides* were examined with a view to improving the antifungal activity of XW2. Plackett-Burman design revealed that among eight components within the basal medium, glucose, peptone, and yeast extract had positive effects on antifungal activity. The steepest ascent method determined the local maximums for these important factors. Central composite design then determined the optimum levels of these factors to be 22.64 g/L (glucose), 11.93 g/L (peptone), and 1.85 g/L (yeast extract). Overall, a 52.15% increase in antifungal activity was obtained after growth in optimized medium compared with basal medium. The antifungal activities of the *B. atrophaeus* XW2 lipopeptides and proteins were effectively increased by 44.84% and 44.83%, respectively.? Moreover, after optimization, strain XW2 displayed strong antagonism towards fungal phytopathogens belonging to various taxonomic groups, with inhibition rates ranging from 69.55% to 100%.**

Key words: *Bacillus atrophaeus*, antifungal activity, medium optimization, Plackett-Burman design, Central composite design, response surface methodology.

In recent years, many species and strains of the *Bacillus* genus have been used to effectively control plant diseases[1-3]. It is widely accepted that *Bacillus* species accomplish their biocontrol performance by producing a variety of antifungal metabolites (lipopeptides, proteins, and volatiles)[4, 5]. In addition, many of the antifungal metabolites produced by genus *Bacillus* have favorable toxicity and allergenic profiles in humans and animals, they are highly biodegradable and compatible with the environment, and they show a broad spectrum of antibiotic and surface activity[6, 7]. Therefore, they are classed as environmentally safe biological pesticides[8].

We previously showed that *Bacillus atrophaeus* XW2, isolated from poplar leaves, exhibited a strong inhibitory effect towards *Colletotrichum gloeosporioides*, a fungus that causes poplar anthracnose. The proteins and lipopeptides produced by strain XW2 were secreted into the culture medium, and all showed strong antifungal activity against *C. gloeosporioides*[9]. These antifungal compounds show promise as biological fungicides against *C. gloeosporioides*. The production of these antifungal compounds depends largely on the nutritional composition of the media used in laboratory or industrial scale experiments[10, 11]. Therefore, selecting appropriate nutrients and optimizing their levels in the culture medium is important if we are to increase production and reduce the costs of antifungal metabolites secreted by *B. atrophaeus* XW2.

Several methods have been used to improve the yields of biologically active substances. The one-factor-at-a-time method is

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one of the most frequently used methods. This method is simple, but has many shortcomings. It is both time consuming and labor intensive, and ignores possible interactions between factors. For these reasons, this method frequently fails to identify the region of optimum response[12, 13]. However, statistical experimental designs offer a powerful approach that can eliminate the limitations of single optimization methods, can evaluate the important factors involved, and can identify a desirable response[14]. Among these methods, response surface methodology (RSM) is widely used to optimize fermentation processes and to improve the yield of biologically active substances[10, 15].

In this study, we used RSM to determine the optimal combination of the medium composition to improve the antifungal metabolites content, enhance the control effect, and lay basis in real production application. After optimization, the effects of medium optimization on the antifungal activity of lipopeptides and proteins were determined, and the antifungal activities of *B. atrophaeus* XW2 against fungal phytopathogens belonging to various taxonomic groups were evaluated.

MATERIALS AND METHODS

Microorganisms

Bacillus atrophaeus XW2 was isolated from poplar leaves and deposited in the China General Microbiological Culture Collection Center (no. 7698). Eleven fungal phytopathogens strains (*Colletotrichum gloeosporioides* CFCC80308, *Cytospora chrysosperma* CFCC89600, *Cytosporamali* CFCC50029, *Cytospora ambiens* CFCC89623, *Botryosphaeria dothidea* CFCC82975, *Fusarium oxysporum* CFCC82468, *Verticillium dahliae* CFCC82516, *Mucor* sp. CFCC80870, *Absidia* sp. CFCC80375, *Rhizopus* sp. CFCC81898, and *Alternaria tenuissima* CFCC84533) were provided and maintained in the China Forestry Culture Collection Center (CFCC). Among them, *Colletotrichum gloeosporioides* was isolated from lesions on infected leaves of poplar and used as the indicator fungus during the medium optimization experiments. *B. atrophaeus* XW2 stocks were stored at "80°C in Luria-Bertani broth (LB) supplemented with 30% glycerol (v/v). The fungi were maintained at 4°C on potato dextrose agar (PDA) slants and cultured on PDA plates at 28°C.

Basal medium, fermentation conditions, and preparation of sterile culture filtrate

The basal medium comprised glucose (10.0g/L), NH_4Cl (3.0g/L), KH_2PO_4(1.0g/L), Na_2HPO_4(1.0g/L), $MgSO_4 \cdot 7H_2O$ (0.50g/L), yeast extract (1.0g/L), peptone (5.0g/L),and soybean meal (5.0g/L).The pH was adjusted to 7.0.

An Erlenmeyer flask (250 mL) containing 100mL of LB medium was inoculated with strain XW2 and incubated at 28°C in a rotatory shakerat 200 rev min^{-1} for 24 h to prepare the inoculum. Next, a sample of the inoculum was added aseptically to a flask (500 mL) containing100 mL of medium (final concentration, 2% (v/v)) and incubated at 28°C in rotatory shaker at 200 rev min^{-1} for 4 days. The cultures were then centrifuged (10,000 x*g*, 30 min, 4°C) to pellet the cells. The supernatants were then passed through a 0.22µm bacterial filter (Pall, Ann Arbor, USA) to yield SCF, which was stored at 4°C until required.

Antifungal activity assay

The antifungal activity of *B. atrophaeus* XW2 was measured by assaying the mycelium growth inhibition rate of *C. gloeosporioide* as previously described[16]. Briefly, 2 mL of sterile test liquid was mixed with 20 mL PDA at 45°C~50°C in a culture plate or without sterile test liquid as controls. After the medium solidified, amycelial plug (6 mm) of *C. gloeosporioide* (collected from a freshly growing mycelium culture) was inoculated into the center of the plateusing a sterile steel borer.The plates were then incubated in the dark at 28°C for 5days. The diameter of the *C. gloeosporioide* colonies was measured using the criss-cross method. Each experiment was performed in triplicate. The inhibition rate was calculated using Equation 1[17]:

$$P\ (\%) = 100 \times [(C - d) - (T - d)]/(C - d) \quad ...(1)$$

where P is the inhibition rate, C is the diameter of a control colony, T is the diameter of a treatment colony, and d is the diameter of the mycelium disc.

Plackett-Burman design

To identify the medium components that significantly affect the antifungal activity of *B. atrophaeus* XW2, individual components were

evaluated using the PB design. Each variable wasre presented at two levels, high and low, which were denoted by (+1) and ("1), respectively. The level of the components in the "high" category was 1.5 times that in the "low" category (Table 1). Eight variables were screened during 12 experimental runs. The matrix design is shown in Table 2. Experimental data were analyzed using the Minitab 16 statistical software package (Minitab Inc., USA).

Path of steepest ascent

The important variables identified by PB design were further optimized using the steepest ascent method. The path of the steepest ascent started from the zero level of each variable screened in the PB design. A series of exploratory runs were performed on the basis of the results obtained from the initial experiments. The point at which the path of steepest ascent reached a plateau was close to the optimal value and was used as the center point for further optimization using CCD[18].

Central composite design

Once the ranges of the screened variables were identified, CCD (a class of response surface methodology (RSM)) with five coded levels was used to determine the optimum concentration of each of the screened medium components. The following processing variables were chosen for the CCD experiments: glucose (A), peptone (B),and yeast extract (D). The coded levels for each independent variable are shown in Table 4.

As shown in Table 5, a set of 20 experiments was carried out and the experimental data were analyzed using the statistical software package, Design-Expert (version 8.0, Stat-Ease., Minneapolis, USA). When the experiments were completed, the average inhibition rates were denoted as the response (Y). A second-order polynomial equation was then fitted to the data using a multiple regression procedure, the relationships between the independent variables and the response were then calculated (Equation 2)[19]:

$$Y = \beta_0 + \sum\beta_i x_i + \sum\beta_{ii} x_i^2 + \sum\beta_{ij}x_ix_j \quad ...(2)$$

where Y is the predicted response, $\beta_0$is the intercept term, x_i and x_j are the coded independent variables, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and $\beta_{ij}$is the interaction coefficient. The optimum values for glucose, peptone, and yeast extract were obtained by solving the regression equation and by analyzing the response surface/contour plots.

Validation of the experimental model

To determine the accuracy of the model and to verify the optimization results, strain XW2 was fermented in either basal medium or in the predicted optimal medium under the following conditions: inoculum volume, 2%; capacity, 100 mL (in an 500 mLErlenmeyer flask); temperature, 28°C; shaking speed, 200 rev min^{-1}; fermentation time, 4 days. The cultures were then centrifuged and the supernatants filtered. Antifungal activity was assayed as described above.

Effects of medium optimization on the antifungal activity of lipopeptides and crude Proteins

SCFs were collected according to the procedure described above andcrude protein extractswere obtained as previously described[9]. Lipopeptideswere also obtained aspreviously described, but with some modifications[9]. Briefly, the pH of the SCFs was adjusted to 2.0 using 6 M HCl and the supernatants were refrigerated at 4°C overnight. The SCFs were then centrifuged, and the precipitates were collected and extracted three times with methanol. The methanol layers were combined and dried using a rotary evaporator (Heidolph, its-science, Shanghai, China). The dried solids were dissolved in sterile water. The volume of the liquids containing the crude protein and lipopeptides was one half of the SCF. Finally, the lipopeptides and proteins were centrifuged and filtered. Antifungal activity was determined as described above.

Antifungal spectrum of *B. atrophaeus* XW2

SCF was collected after optimization and used to determine the antifungal spectrum of *B. atrophaeus* XW2 against fungal phytopathogens belonging to varioustaxonomic groups. The SCF was tested against *C. gloeosporioide s*,*C. chrysosperma*, *C. mali*, *C. ambiens*, *B. dothidea*, *F. oxysporum*, *V. dahliae*, *Mucor* sp., *Absidia* sp., *Rhizopus* sp., and *A. tenuissima*. The antifungal activity was determined as described above. The colony diameters of the fungi were measured when the colony diameter of the controls covered more than three quarters of the culture dish.

RESULTS AND DISCUSSION

Optimization by Plackett-Burman design

The PB experiment (Table 2) was conducted over 12 runs to examine the importance of each variable. The experiment revealed wide variations in antifungal activity. These variations reflected the importance of optimization to attain higher antifungal activity. The Pareto chart (Figure 1) shows the effect of each variable on the response and its significance level. Confidence levels were accepted only when >95% (p<0.05). Statistical analysis revealed that the levels of glucose (A), peptone (B), and yeast extract (D) had a significant effect on the antifungal activity of *B. atrophaeus* XW2. Therefore, we selected the concentrations of glucose (A), peptone (B), and yeast extract (D) for further optimization.

Many studies showed that some medium components, such as carbon sources, nitrogen sources, and metal ions, have a positive effect on the yield of biologically active substances[11, 20]. In the present study, PB design revealed that glucose, peptone, and yeast extract had significant effects on the antifungal activity of *B. atrophaeus* XW2. The positive effects of glucose and peptone may be associated with an increased availability of the carbon source and nitrogen sources, respectively, and which would be beneficial to the production of antifungal substances through prolonging the duration of production[21]. Also, yeast extract may be an important source of nitrogen and microelements as it provides high amounts of free amino acids, short peptides, and growth factors, which may induce the production of antifungal substances[22].

Optimization by the steepest ascent path method

Based on the regression results obtained byPB design, we next used the path of the steepest ascent to determine the direction of change for each variable. As the concentrations of glucose, peptone, and yeast extract increased, the antifungal activity of *B. atrophaeus* XW2 improved. The concentrations of the other medium components remained fixed. The experimental design and corresponding results are shown in Table 3. According to the path of steepest ascent, the inhibition rate increased up untilrun 5, after which it began to decrease. This indicates that maximum antifungal activity occurred around run 5. Therefore, run 5 was used as the central point for CCD and reallocated as follows: glucose, 21.50 g/L; peptone, 11.25 g/L; yeast extract, 1.86g/L.

Optimization by CCD

According to the results presented above, the key variables (glucose, peptone, and yeast extract) and their central points were chosen for the experimental design. CCD was used to determine the effects of the key variables on antifungal activity. Each key variable was studied at five coded levels (-1.68, -1, 0, 1, and 1.68) (Table 4). The matrix for this design, along with the experimental and predicted responses, is presented in Table 5. The responses were analyzed by analysis of variance (ANOVA) and the results were used to derive a second-order polynomial equation

Table 1. Levels of the variables of Plackett-Burman design

Variable code	Component	Levels (g/L) Low value (-1)	High value (+1)
A	Glucose	10.00	15.00
B	Peptone	5.00	7.50
C	Soybean meal	5.00	7.50
D	Yeast extract	1.00	1.50
E	KH_2PO_4	1.00	1.50
F	$MgSO_4 \cdot 7H_2O$	0.50	0.75
G	NH_4Cl	3.00	4.50
H	Na_2HPO_4	1.00	1.50

Table 2. The Plackett-Burman design for the screening of significant variables

Run	Variables A	B	C	D	E	F	G	H	Inhibition rate (%)
1	1	-1	1	-1	-1	-1	1	1	48.69
2	1	1	1	-1	1	1	-1	1	58.35
3	1	-1	1	1	-1	1	-1	-1	55.74
4	1	-1	-1	-1	1	1	-1	-1	52.82
5	-1	-1	-1	-1	-1	-1	1	-1	52.72
6	-1	1	-1	-1	-1	1	-1	1	50.10
7	-1	-1	1	1	1	-1	-1	1	49.90
8	-1	1	1	-1	1	-1	-1	-1	56.14
9	-1	1	1	1	-1	1	1	-1	52.72
10	-1	-1	-1	1	1	1	-1	1	52.52
11	1	1	-1	1	-1	-1	-1	1	61.57
12	1	1	-1	1	1	-1	1	-1	66.60

(Equation 3), which describes the function of each key variable and their second-order interactions with respect to antifungal activity:

$$Y=76.16+1.66A+1.68B-1.31D+2.90AB+0.87AD+0.43BD-3.11A^2-2.88B^2-3.92D^2 \quad ...(3)$$

where Yis the inhibition rate (%) and A, B,and D are the code values corresponding to the amounts of glucose, peptone, and yeast extract, respectively.

The quadratic model was checked using ANOVA, and the results are shown in Table 6. Amodel *F*-value of 20.18 implied that the model was significant. There was only a 0.01% chance that a "Model *F*-value" this large would be due to noise. Values of "Prob>*F*" <0.05 indicated that the model terms were significant. Values > 0.1 indicatedthat the model terms were not significant. In this case, A, B, D, AB, A^2, B^2, and D^2 all showed significant effects. The coefficient of determination (R^2) for antifungal activity was calculated as 0.9478, which implies that the sample variation of more than 94.78% was attributable to the key variables and less than 5.22% of the total variance could not be explained by the model. The adjusted determination coefficient (AdjR^2=0.9080) was also high, suggesting that the model showed a high level of significance. The high correlation coefficient, R= 0.96241, indicated good agreement between the experimental and predicted values for the inhibition rate. The low coefficient of variation (C.V. =2.57%) showed that the experiments were both precise and reliable. These measures indicate that the polynomial model has good accuracy and general applicability, and can be used to analyze the response trends.

The 3D response surface and the 2D contour plots described by the regression model were then plotted to better understand the interaction effects between variables and to determine the optimum level of each component required to achieve a maximal response. Each three dimensional plot and its corresponding contour plot represents the effects of two variables on antifungal activity when the third variable is held at zero (Figure2). The optimal concentrations of glucose, peptone, and yeast extract required for maximum antifungal activity were determined by response surface analysis and also estimated using a regression equation. The predicted results are shown in Table 5. The predicted values obtained from the regression equation were in good agreement with the actual values obtained from the experiments. The model predicted a maximum inhibition rate of 77.08% at the following

Table 3. Experimental design and response of the steepest ascent path

Run	Normal variables			Actual variables (g/L)			Inhibition rate (%)
	A	B	D	A	B	D	
	0.90	1.00	0.61	2.25	1.25	0.15	
1	0	0	0	12.50	6.25	1.25	55.31
2	0.90	1.00	0.61	14.75	7.50	1.40	66.55
3	1.80	2.00	1.22	17.00	8.75	1.56	66.78
4	2.70	3.00	1.83	19.25	10.00	1.71	71.31
5	3.60	4.00	2.44	21.50	11.25	1.86	73.98
6	4.50	5.00	3.05	23.75	12.50	2.01	71.66
7	5.40	6.00	3.66	26.00	13.75	2.17	69.80

Table 4. Ranges of the independent variables used in the central composite design

Variable	Levels				
	-1.68	-1	0	1	1.68
A:glucose (g/L)	17.72	19.25	21.50	23.75	25.03
B: peptone (g/L)	9.15	10.00	11.25	12.50	13.35
D: yeast extract (g/L)	1.60	1.71	1.86	2.01	2.12

component concentrations: glucose, 22.64 g/L; peptone, 11.93 g/L; and yeast extract,1.85 g/L. These results show that the steepest ascent method is a necessary part of the analysis because it pointed us towards the vicinity of the optimum surface.

Validation of the experimental model

The statistical model was then validated in shake-flasks. The antifungal effect of *B. atrophaeus* XW2 against *C. gloeosporioide* was inhibited by 50.43% innon-optimized medium and by 76.73% in optimized medium (Fig. 3). These results show that RSM optimization effectively increased the antifungal activity of *B. atrophaeus* XW2 by 52.15%. The inhibition rate obtained from the experiments was very close to the predicted inhibition rate (77.08%), which confirms the accuracy and validity of the model.

The model described herein enabled us to optimize the growth medium for antifungal production by *B. atrophaeus* XW2. Such optimization may make the large scale production of antifungals both possible and economical. However, microorganism fermentation is a complex process. It not only depends on the nutritional content of the medium, but also on the environmental conditions (i.e., fermentation time, temperature, agitation rate, inoculation volume, medium capacity, and initial pH)[14, 16].Many studies report that environmental conditions play an important role in the production of secondary metabolites by *Bacillus* genus. Kim *et al.*[11] reported that temperature is one of the most significant factors as it affects the production of biosurfactants by *B. subtilis* JK-1, and El-Sersy *et al.*[14] showed that both incubation time and culture volume had positive effects on antibacterial peptide production. Therefore, to generate maximum antifungal activity by *B. atrophaeus* XW2 and to reduce the cost of fermentation, further studies will attempt to optimize the environmental conditions during culture.

Table 5. Experimental design and results of central composite design

Run	Variables			Inhibition rate (%)	
	A	B	D	Experimental	Predicted
1	0	0	0	71.50	70.84
2	0	0	0	75.03	76.16
3	-1	-1	-1	61.66	62.46
4	-1	-1	1	62.69	61.62
5	1	-1	-1	70.34	72.48
6	-1	1	1	65.35	65.12
7	1.68	0	0	71.81	72.50
8	0	0	0	69.98	68.42
9	0	-1.68	0	63.64	62.87
10	0	1.68	0	72.99	70.15
11	-1.68	0	0	76.19	76.16
12	1	1	-1	62.57	64.20
13	1	-1	1	77.72	76.16
14	0	0	-1.68	74.64	76.16
15	-1	1	-1	62.56	64.57
16	0	0	1.68	76.88	76.16
17	0	0	0	65.37	65.19
18	0	0	0	63.30	63.20
19	1	1	1	67.34	67.27
20	0	0	0	76.34	76.16

Effects of medium optimization on the antifungal activity of lipopeptides and proteins

In our study, the inhibition rates for lipopeptides produced by *B. atrophaeus* XW2 against *C. gloeosporioide* were 51.06% (non-optimized medium) and 92.55% (optimized medium), whereas for proteins were 49.87% and 90.40%, respectively. After optimization, the antifungal activities of lipopeptides and proteins were effectively enhanced by 44.84% and 44.83%, respectively. These results revealed that glucose, peptone, and yeast extract had significant effects on the yield of antifungal lipopeptides and proteins. This finding is in agreement with those of previous studies. For example, Mizumoto *et al.*[12] reported that glucose had a significant effect on the production of iturin A by *B. subtilis*. El-Sersy

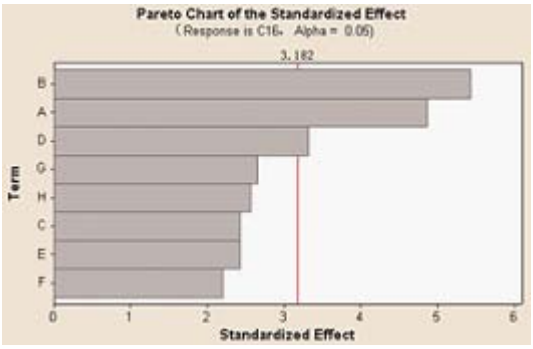

Fig. 1. Pareto chart showing the effects of different medium components on the antifungal activity of *B. atrophaeus* XW2(B, peptone; A, glucose; D, yeast extract)

et al.[14] reported that yeast extract had a positive effect on antibacterial peptide production by *B. licheniformis* SN2.

Antifungal spectrum of *B. atrophaeus* XW2

Numerous studies have reported that the culture filtrates of many species and strains of the *Bacillus* genus have a broad spectrum of activities[23]. Foldes *et al*[24] have reported abouta *B. subtilis* strain showing antagonism against 17 fungi and eight bacteria. McKeen *et al*[25] have reported that *B. subtilis* B-3 has antifungal activity against 15 fungal species. In our study, SCF derived from *B. atrophaeus* XW2 showed strong activity against the eleven fungi. After optimization, the inhibition rates were range from 69.55% to 100% (Table 7). SCF showed the strongest inhibitory effect against *C. chrysosperma* (100%) and *C. ambiens* (100%), and the weakest against *C. mali* (69.55%). Taken together, these studies suggest that antifungal substances produced by *B.*

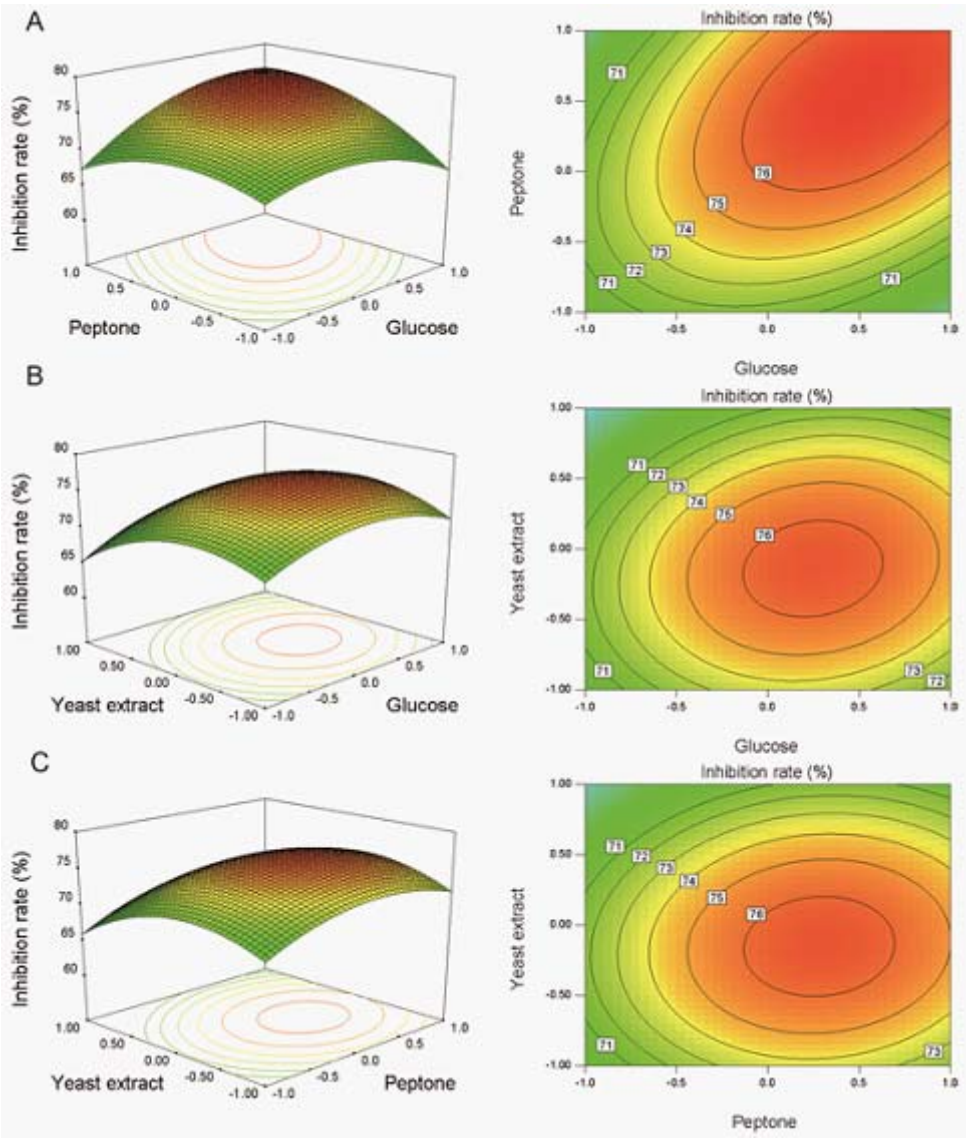

Fig. 2. Response surface plot and contour plot. A: The interaction effects of glucose and peptone on the antifungal activity of *B. atrophaeus* XW2, B: The interaction effects of glucose and yeast extract on the antifungal activity of *B. atrophaeus* XW2, C: The interaction effects of peptone and yeast extract on the antibiotic activity of *B. atrophaeus* XW2

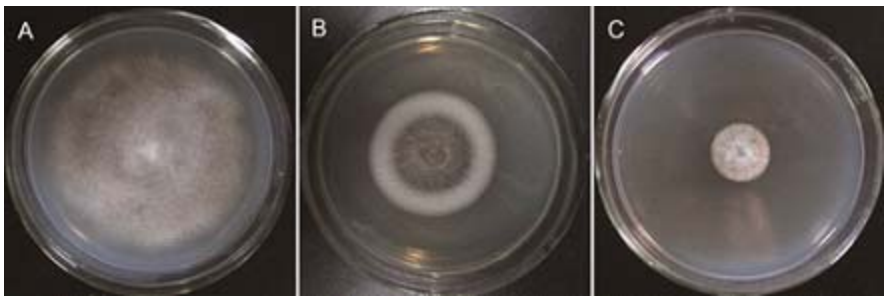

Fig. 3. Size of*C. gloeosporioide*colonies on potato dextrose agar (PDA) (after 5 days). A: control, B: PDA mixed with non-optimized sterile culture filtrate (SCF), C: PDA mixed with optimized SCF

Table 6. Analysis of variance (ANOVA) for the quadratic model

Source	SS	Df	MS	*F*-Value	Prob>*F*	Significance level
Model	576.67	9	64.07	20.18	< 0.0001	**
A	37.41	1	37.41	11.78	0.0064	**
B	38.65	1	38.65	12.17	0.0058	**
D	23.53	1	23.53	7.41	0.0215	*
AB	67.08	1	67.08	21.12	0.0010	**
AD	6.04	1	6.04	1.90	0.1978	N
BD	1.49	1	1.49	0.47	0.5084	N
A^2	139.30	1	139.30	43.87	< 0.0001	**
B^2	119.19	1	119.19	37.53	0.0001	**
D^2	221.06	1	221.06	69.62	< 0.0001	**
Residual	31.75	10	3.18			
Lack of fit	25.19	5	5.04	3.84	0.0830	N
Pure error	6.56	5	1.31			
Core total	608.43	19				

Note.R^2= 0.9478, C.V. (%) = 2.57, AdjR^2= 0.9008; Significant at the p< 0.01, **; Significant at the p< 0.05, *; Non-significant at the pe"0.05, N; Sum of squares, SS; Degree of freedom, DF; Mean square, MS.

Table 7. Antifungal Spectrum of *B. atrophaeus* XW2

Fungal phytopathogen	Inhibition rate (%)
Colletotrichumgloeosporioides	76.73±0.86d
Verticilliumdahliae	75.85±5.09d
Fusariumoxysporum	83.24±1.87c
Alternariatenuissima	86.32±0.00bc
Cytosporachrysosperma	100±0.00a
Cytosporamali	69.55±0.81e
Cytosporaambiens	100±0.00a
Botryosphaeriadothidea	98.40±0.00a
Rhizopus sp.	74.56±1.67d
Mucor sp.	88.17±0.93b
Absidia sp.	74.39±1.32d

Note. Values with the different letter are significantly different at the $p < 0.05$ according to Duncan's test, Numbers follow by the "±" are standard errors (SE).

atrophaeus XW2 may be useful for preventing/ treating plant diseases.

CONCLUSIONS

In the present work, the statistical experimental design was used to study the effect of the medium components and their interactions on the antifungal activity of *B. atrophaeus* XW2. Glucose, peptone and yeast extract were selected as positive influence factors on antifungal activity. Optimum levels of these significant factors were predicted at glucose, peptone, and yeast extract of 22.64 g/L, 11.93 g/L, and 1.85 g/L, respectively. The results of verification experiments indicated that antifungal activity of *B. atrophaeus* XW2 was effectively enhanced 52.15% after optimization, and

showed a close concordance between the experimental and predicted activity level. After optimization, the antifungal activity of lipopeptides and proteins were enhanced 44.84% and 44.83%, respectively. Moreover, *B. atrophaeus* XW2 displayed strong antagonism towards fungal phytopathogens belonging to various taxonomic groups, with inhibition rates ranging from 69.55% to 100%. Taken together, the results of this shake-flask fermentation study lay the foundations for the large scale fermentation of *B. atrophaeus* XW2. Our future studies will focus on scaling up the process to enable fermentation on an industrial scale.

ACKNOWLEDGEMENTS

The research was supported by Special Fund for Forest Scientific Research in the Public Welfare (201204501).

REFERENCES

1. Asaka, O., Shoda, M. Biocontrol of *Rhizoctoniasolani* Damping-Off of Tomato with *Bacillus subtilis* RB14. *Appl. Environ. Microbiol.*, 1996; **62**(11): 4081-4085.
2. Ongena, M., Jacques, P., Touré, Y., Destain, J., Jabrane, A., Thonart, P. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.*, 2005; **69**(1): 29-38.
3. Pérez-García, A., Romero, D., De Vicente, A. Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. *Curr.Opin.Biotechnol.*, 2011; **22**(2): 187-193.
4. Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B., Urdaci, M.C. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. *Res. Microbiol.*, 2002; **153**(5): 269-276.
5. Zhang, X.Y., Li, B.Q., Wang, Y.G., Guo, Q.G., Lu, X.Y., Li, S.Z., Ma, P. Lipopeptides, a novel protein, and volatile compounds contribute to the antifungal activity of the biocontrol agent *Bacillus atrophaeus* CAB-1. *Appl. Microbiol. Biotechnol.*, 2013; **97**(21): 9525-9534.
6. Delcambe, L., Peypoux, F., Besson, F., Guinand, M., Michel, G. Structure of iturin and iturin-like substances [proceedings]. *Biochem. Soc. T.*, 1977; **5**(4): 1122-1124.
7. Gu, X.B., Zheng, Z.M., Yu, H.Q., Wang, J., Liang, F.L., Liu, R.L. Optimization of medium constituents for a novel lipopeptide production by *Bacillus subtilis* MO-01 by a response surface method. *Process Biochem.*, 2005; **40**(10): 3196-3201.
8. Phae, C.G., Shoda, M., Kita, N., Nakano, M., Ushiyama, K. Biological control of crown and root rot and bacterial wilt of tomato by *Bacillus subtilis* NB22. *Ann. Phytopathol. Soc. Jpn.*, 1992; **58**(3): 329–339.
9. Huang, H.Y., Wu, Z.Q., Tian, C.M., Liang, Y.M., You, C.J., Chen, L. Identification and characterization of the endophytic bacterium *Bacillus atrophaeus* XW2, antagonistic towards *Colletotrichumgloeosporioides*. *Ann. Microbiol.*, 2014; doi: 10.1007/s13213-014-0974-0.
10. Wang, Y., Fang, X., An, F., Wang, G., Zhang, X. Improvement of antibiotic activity of *Xenorhabdusbovienii* by medium optimization using response surface methodology. *Microb. Cell Fact.*, 2011; **10**(98): 1-15.
11. Kim, B., Kim, J. Optimization of Culture Conditions for the Production of Biosurfactant by *Bacillus subtilis* JK-1 Using Response Surface Methodology. J. Korean Soc. *Appl. Biol. Chem.*, 2013; **56**(3): 279-287.
12. Mizumoto, S., Shoda, M. Medium optimization of antifungal lipopeptide, iturinA, production by *Bacillus subtilis* in solid-state fermentation by response surface methodology. *Appl. Microbiol. Biot.*, 2007; **76**(1): 101-108.
13. Zhao, X., Han, Y., Tan, X.Q., Wang, J., Zhou, Z.J. Optimization of Antifungal Lipopeptide Production from *Bacillus* sp. BH072 by Response Surface Methodology. *J. Microbiol.*, 2014; **52**(4): 324-332.
14. El-Sersy, N.A., Ebrahim, H.A.H., Abou-Elela, G.M. Response Surface Methodology as a Tool for Optimizing the Production of Antimicrobial Agents from *Bacillus licheniformis* SN2. *Curr. Res. Bacteriol.*, 2009; **3**(1): 1-14.
15. Zhang, N., Qi, Z., Duan, H., Xie, Y., Yu, J., Lu, C., Liu, X. Optimization of medium composition for production of antifungal active substance from *Streptomyces hygroscopicus* BS-112. *Afr. J. Microbiol. Res.*, 2012; **6**(1): 71-80.
16. Song, Q., Huang, Y., Yang, H. Optimization of fermentation conditions for antibiotic production by *Actinomycetes* YJ1 strain against *Sclerotiniasclerotiorum*. *J. Agr. Sci.*, 2012; **4**(7): 95-102.
17. Yang, C.J., Zhang, X.G., Shi, G.Y., Zhao, H.Y., Chen, L., Tao, K., Hou, T.P. Isolation and identification of endophytic bacterium W4 against tomato *Botrytis cinerea* and antagonistic

activity stability. *Afr. J. Microbiol. Res.*, 2011; **5**(2): 131-136.

18. Tang, X.J., He, G.Q., Chen, Q.H., Zhang, X.Y., Ali, M.A. Medium optimization for the production of thermal stable ²-glucanase by *Bacillus subtilis* ZJF-1A5 using response surface methodology. *Bioresource Technol.*, 2004; **93**(2): 175-181.
19. Li, Z.X., Yin, G.M., Chen, T. Optimization of Riboflavin Production by Recombinant *Bacillus Subtilis* X42 Using Statistical Designs. *Adv. Mater. Res.*, 2013; **634**: 1031-1036.
20. Zhao, X., Han, Y., Tan, X.Q., Wang, J., Zhou, Z.J. Optimization of Antifungal Lipopeptide Production from *Bacillus* sp. BH072 by Response Surface Methodology. *J. Microbiol.*, 2014; **52**(4): 324-332.
21. Zhang, Y.P., Chen, B.C., Chu, C. Medium optimization for meilingmcin production by Streptomyces nanchangensis using response surface methodology. *Process Biochem.*, 2006; **41**(2): 405-409.
22. Anthony, T., Rajesh, T., Kayalvizhi, N., Gunasekaran, P. Influence of medium components and fermentation conditions on the production of bacteriocin (s) by *Bacillus licheniformis* AnBa9. *Bioresource Technol.*, 2009; **100**(2): 872-877.
23. Todorova, S., Kozhuharova, L. Characteristics and antimicrobial activity of *Bacillus subtilis* strains isolated from soil. *World J. Microbiol.Biotechnol.*, 2010; **26**(7): 1207-1216.
24. Foldes T., Banhegyi I., Herpai Z., Varga L., Szigeti J. Isolation of *Bacillus* strains from the rhizosphere of cereals and in vitro screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage micro-organisms. *J. Appl. Microbiol.*, 2000; **89**(5):840–846.
25. McKeen C., Reily C., Pusey P. Production and partial characterization of antifungal substances antagonistic to *Moniliafructicola* from *Bacillus subtilis*. *Phytopathology*, 1986; **76**(2): 136–139.