Journal of Microbiology (2014) Vol. 52, No. 4, pp. 324–332
DOI 10.1007/s12275-014-3354-3

Optimization of Antifungal Lipopeptide Production from *Bacillus* sp. BH072 by Response Surface Methodology

Xin Zhao, Ye Han, Xi-qian Tan, Jin Wang, and Zhi-jiang Zhou*

School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, P. R. China

(Received Jul 5, 2013 / Revised Sep 9, 2013 / Accepted Oct 14, 2013)

Antifungal lipopeptide produced by *Bacillus* sp. BH072 was extracted from fermentation liquor and determined as iturin A by liquid chromatography-mass spectrometry (LC-MS). For industrial-scale production, the yield of iturin A was improved by optimizing medium components and fermentation conditions. A one-factor test was conducted; fermentation conditions were then optimized by response surface methodology (RSM) to obtain the following: temperature, 29.5°C; pH 6.45; inoculation quantity, 6.7%; loading volume, 100 ml (in 500 ml flasks); and rotary speed, 150 rpm. Under these conditions, the mass concentration of iturin A was increased from 45.30 mg/ml to 47.87 mg/ml. The following components of the medium were determined: carbon sources (glucose, fructose, sucrose, xylose, rhamnose, and soluble starch); nitrogen sources (peptone, soybean meal, NH_4Cl, urea, and ammonium citrate); and metal ions (Zn^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, and K^{+}). The effects of these components on iturin A production were observed in LB medium. We selected sucrose, soybean meal, and Mg^{2+} for RSM to optimize the conditions because of several advantages, including maximum iturin A production, high antifungal activity, and low cost. The optimum concentrations of these components were 0.98% sucrose, 0.94% soybean meal, and 0.93% Mg^{2+}. After iturin A production was optimized by RSM, the mass concentration reached 52.21 mg/ml. The antifungal specific activity was enhanced from 350.11 AU/mg to 513.92 AU/mg, which was 46.8% higher than the previous result. The present study provides an important experimental basis for the industrial-scale production of iturin A and the agricultural applications of *Bacillus* sp. BH072.

***Keywords*:** Bacillus, antifungal peptide, optimization

Introduction

Lipopeptides, also known as acyl peptides, are products of microbial metabolism; these peptides contain an aliphatic chain with an amphiphilic structure produced as secondary metabolites of microorganisms (Katz and Demain, 1977). Lipopeptides exhibit good surface and antiviral activities. They can also remove heavy metal ions and degrade pesticide pollutants. Furthermore, these peptides can be developed to produce vaccines. Thus, lipopeptides, as microbial secondary metabolites, have potentially important applications in drugs, foods, cosmetic products, pesticides, and other materials. Studies have focused on the application of lipopeptides in the prevention and control of pathogenic bacteria (Bonmatin *et al.*, 2003; Toure *et al.*, 2004; Ongena and Jacques, 2008).

The biological control of plant pathogens is an important aspect of sustainable agriculture. Several *Bacillus* strains produce biologically active compounds, including lipopeptides, with an evident effect on plant disease control (Asaka and Shoda, 1996; Romero *et al.*, 2007; Zhao *et al.*, 2010). Lipopeptides, including the iturin (Maget-Dana and Peypoux, 1994), surfactin (Peypoux *et al.*, 1999), and fengycin (Ongena *et al.*, 2005) families, are produced or catalyzed by ribosomal peptide synthesis or polyketide synthases. Among these lipopeptides, iturins, and fengycins exhibit strong antifungal activities against various plant pathogens and are considered to be key factors of antagonism (Tenoux *et al.*, 1991; Eshita *et al.*, 1995; Kim *et al.*, 2010).

The iturin family consists of cyclic lipopeptides containing seven amino acids. Iturin A is a small cyclic lipopeptide containing a heptapeptide cyclized with a β-amino fatty acid that exhibits strong antifungal activity. By contrast, surfactin contains a heptapeptide cyclized with a β-hydroxy fatty acid and shows weak antibiotic activity. The strong antifungal efficacy of iturin A against various phytopathogenic fungi is comparable to available chemical pesticides (Phae and Shoda, 1990, 1991; Phae *et al.*, 1992). Iturin A has low toxicity, low allergenicity in humans and other animals (Delcambe *et al.*, 1977), and high biodegradability, along with a wide spectrum of antibiotic and surface activities. Because of these characteristics, iturin A is a potential candidate for an environmentally safe biological pesticide (Phae *et al.*, 1992). The antifungal mechanism of iturin A involves the insertion of its hydrophobic tails into the plasma membrane of indicator cells; these hydrophobic groups spontaneously assemble to form an ion channel, thereby causing cytoplasm leakage. Iturin A can also release electrolyte and polymer aggregates as well as increase the electrical conductivity and permeability of the cell membrane, causing surface tension effects on the cell membrane and inhibiting pathogenic spore formation. Chen *et al.* (2008) isolated an active substance from *B. subtilis* JA by reverse-phase high-performance liquid chromatography (HPLC) separation and identified two iturin A homologs by electrospray ionization and collision-induced

*For correspondence. E-mail: zzj@tju.edu.cn; Tel.: +86-15510969668; Fax: +86-022-27403389

dissociation mass spectrometry analysis. The molecular weights of these homologues are 1042 and 1056 Da. Antifungal activity tests have further shown that *B. subtilis* JA can inhibit wheat scab (*Fusarium graminearum*), rice sheath blight (*Rhizoctonia solani*), watermelon fusarium wilt (*Fusarium oxysporum*), *Pythium irregulare*, *Botrytis cinerea*, and various other plant pathogenic fungi.

Previous studies on iturin fermentation were conducted using a one-factor-at-a-time method. However, this method frequently fails to locate the region of optimum response because the combined effects of factors on the response have not been considered. Response surface methodology (RSM) has been increasingly used in various phases of fermentation optimization (Buchanan and Philips, 1990; Prapulla *et al.*, 1992; Haltrich *et al.*, 1993; Shih *et al.*, 2002; Shih and Shen, 2006). For instance, RSM is a powerful technique used to test multiple process variables because fewer experimental trials are needed compared with one variable used at a time. In addition, interactions between variables can be identified and quantified by this technique (Box and Wilson, 1951).

Bacillus sp. BH072, a novel bacterium isolated from a honey sample, showed antifungal activities against mold. The antifungal substance was identified as iturin A (Zhao *et al.*, 2013b). The yield and antifungal activity of iturin A were higher than those of other iturins. The amount of iturin A produced by *Bacillus* sp. BH072 was also tenfold higher than the production yield in a previous optimization study (Yang *et al.*, 2012). RSM has been employed to optimize the components of a medium and the fermentation conditions for cyclic lipopeptide production in shake-flask fermentation. The results indicated that RSM was applicable for optimizing a lipopeptide culture medium (Gu *et al.*, 2005). However, studies to optimize the fermentation conditions and components of a medium by directly measuring antifungal activity, to reveal the effect of optimizing these parameters, have not yet been reported.

In the present study, RSM was applied to optimize the culture conditions and media components so as to enhance iturin A production by *Bacillus* sp. BH072 using shake-flask fermentation. After the optimum conditions were obtained in the shake-flask fermentation, iturin A produced by *Bacillus* sp. BH072 increased. One-factor, five-level experiments were conducted to determine the optimal concentrations of each component that would result in the maximum production of iturin A. Considering the level of antifungal activity and cost of the medium, we performed RSM and developed a quadratic predictive model. The production yield of iturin A was improved.

Materials and Methods

Chemicals, microorganisms, and culture media

The bacterium BH072 used in this study was isolated from a honey sample and identified as a species of *Bacillus* (Zhao *et al.*, 2013b). The indicators *Aspergillus niger* CGMCC 3.03928, *Botrytis cinerea* CGMCC 3.4584, and *Fusarium oxysorum* CGMCC 3.2830 were purchased from the China General Microbiological Culture Collection Center. All of the components used in this study were pure reagent grade (Jiangtian Chemical Technology Co., Ltd., China). The solid media used in the antifungal experiment contained potato dextrose agar for fungi (PDA: potato, 200 g; glucose, 20 g; agar, 18 g; and distilled water, 1 L) and Luria-Bertani agar for bacteria (LBA: peptone, 10 g; yeast extract, 5 g; NaCl, 10 g; agar, 18 g; and distilled water, 1 L). The liquid medium (LB medium prepared with the same components as LBA but without the agar) was used for the fermentation test as the basic culture medium prior to optimization. The strain BH072 was activated by transferring single colonies of the strain from plates to 10 ml liquid LB activation medium in 50 ml flasks as the seed culture. The flasks were incubated with shaking at 150 rpm for 18 h at 37°C.

Growth curve and fermentation time vs antifungal activity curve

Bacillus sp. strain BH072 was initially grown on an LB agar slant and then transferred to 500 ml of LB medium with shaking at 150 rpm for 24 h at 37°C. A 5 ml aliquot of the culture was collected at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 36, 48, 60, and 72 h. Optical density (OD) was read at each time point. Afterwards, 1 ml of the culture was centrifuged at 12,000 rpm for 1 min to obtain the cell-free supernatant. A 100 µl aliquot of the cell-free supernatant was used to conduct the antifungal test by using the Oxford cup method (Zhang *et al.*, 2012). In brief, 1 ml of the fungal spore suspension was uniformly dispersed in 100 ml of PDA medium at 40°C to 50°C; the resulting mixture was coated on a solid beef extract peptone plate containing the Oxford cup. Afterwards, 100 µl of cell-free supernatant was placed at the midpoint of the Oxford cup. This test culture was incubated for 48 h at 30°C. The inhibition zone diameter was measured. The optimum levels of seed fluid and fermentation period were determined after the growth curve and the fermentation time vs antifungal activity curve were obtained.

Extraction and mass concentration calculation of iturin A

Bacillus sp. BH072 from a seed culture was incubated in a 500 ml shake flask containing 200 ml of LB medium with shaking at 150 rpm for 60 h at 30°C. After cultivation, the culture was centrifuged at 4,200 rpm for 20 min. To adjust the pH to 2.0, we added 6 M HCl to the cell-free supernatant and stored the resulting solution at 4°C overnight (Yao *et al.*, 2012) for precipitation. The precipitate was collected by centrifugation at 4,200 rpm for 20 min at 4°C and freeze dried. The residue was then extracted with 200 ml of methanol under shaking for 24 h at room temperature. The crude product was obtained by centrifugation at 4,200 rpm for 20 min and examined by LC-MS (Thermo Fisher Corporate, USA). The iturin A in the filtrate was identified by MS at m/z 600 to m/z 1300; the molecular weight of iturin A ranged from 1000 Da to 1100 Da (Chen *et al.*, 2008; Zhao *et al.*, 2013b). The methanol extraction liquid was evaporated in an oven at 60°C (Yang *et al.*, 2012). The residue was weighed and used to calculate the mass concentration.

Determination of the antifungal activity of the extracted iturin A

The antifungal activity of iturin A was assessed using the Oxford cup method (Zhang *et al.*, 2012) after iturin A was obtained by methanol extraction. The extracted iturin A was subjected to a series of 2-fold dilutions. Afterward, 5 µl of iturin A diluent was placed on the midpoint of the Oxford cup and the test culture was incubated for 48 h at 30°C. The arbitrary activity (AU/ml) was defined as the highest dilution multiple with an evident inhibition zone multiplied by 200 (1 ml/5 µl) (Motta and Brandelli, 2002), using *A. niger* CGMCC 3.03928, *B. cinerea* CGMCC 3.4584, and *F. oxysorum* CGMCC 3.2830 as indicator organisms. The antifungal specific activity (AU/mg) was equal to the arbitrary activity (AU/ml) divided by the mass concentration of iturin A mg/ml).

Analytical methods for optimizing fermentation conditions

One-factor tests: The main factors influencing microbial fermentation are temperature, initial pH, loading volume of culture medium, inoculum size, and rotary speed. Considering the culture conditions reported in previous studies, we set the main factors in this study as follows: temperatures of 10, 20, 30, 40, and 50°C; initial pH of 4.0, 5.0, 6.0, 7.0, and 8.0; loading volumes of 50, 100, 200, 300, and 400 ml in 500 ml flasks; inoculation quantities were 3%, 5%, 7%, 9%, and 11%; and rotary speeds of 0, 50, 100, 150, and 200 rpm. A single-factor experiment was performed to investigate the factors influencing iturin A production and determine the response surface test factors and levels. Each setup was conducted in three parallel tests, each with three replicates.

RSM experimental design: In preliminary experiments (Kuo, 2006), various environmental factors were investigated using the one-factor-at-a-time method to optimize the production of iturin A by *Bacillus* sp. BH072. Preliminary data indicated that the major variables affecting the performance of the culture in terms of iturin A yields were temperature, initial pH, and inoculation quantity. Therefore, these three factors were chosen for further optimization by RSM.

The central composite design (CCD) experiment was conducted in the optimum range to obtain accurate optimum temperature, initial pH, and inoculation quantity for iturin A production. For these three factors, this trial was essentially a 2^3 factorial design increased by six axial points (also called star points) and two replications of the center point (all of the factors were at level 0), resulting in a total of 20 experiments (Box and Wilson, 1951). The distance of the star points from the center point was $\alpha = 2^{(n/4)} = 1.682$ (for three factors, $n = 3$). The variables were coded according to Equation 1:

Table 1. Range of fermentation condition variables at different levels for the central composite design

Variable quantity	Parameter	Level				
		-1.68	-1	0	1	1.68
Temperature (°C)	X_1	10	20	30	40	50
Inoculation quantity (%)	X_2	3	5	7	9	11
Initial pH	X_3	4.0	5.0	6.0	7.0	8.0

Table 2. Design and results of central composite design (fermentation condition)

Trial no.	Coded level of fermentation condition			Concentration of iturin A (mg/ml)
	X_1	X_2	X_3	
1	0	0	0	45.5
2	0	0	0	43.6
3	-1	-1	1	26.7
4	-1	-1	-1	18.9
5	0	0	0	51.2
6	0	0	0	47.2
7	0	0	-1.68	8.3
8	1	1	1	18.9
9	0	1.68	0	15.5
10	0	0	0	45.3
11	1.68	0	0	1.2
12	-1.68	0	0	4.3
13	-1	1	1	17.1
14	1	-1	1	20.2
15	1	1	-1	6.5
16	1	-1	-1	4.7
17	-1	1	-1	15.6
18	0	0	1.68	40.4
19	0	-1.68	0	26.7
20	0	0	0	48.3

$$x_i = \frac{X_i - X_0}{\Delta Xi}$$

where x_i is the coded variable of A factor, X_i is the natural variable of the factor, X_0 is the value of the natural variable at the center point, and ΔX_i is the step change value.

The variables and levels for the CCD are shown in Table 1. The matrix corresponding to the CCD is shown in Table 2, together with the observed experimental data. The experimental results of the CCD were fitted with a second-order polynomial equation by a multiple regression technique. The quadratic model for predicting the optimal point was expressed according to Equation 2:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i x_j + \sum_{i<j} \sum \beta_{ij} x_i x_j$$

where Y is the predicted response; β_0, β_i, β_{ii}, β_{ij} are constant coefficients, and x_i, x_j are the coded independent variables or factors.

Analytical methods for optimizing medium components

Selection of carbon and nitrogen sources and metal ions: Several potential carbon sources were tested, including glucose and fructose as monosaccharides, sucrose as disaccharide, as well as xylose, rhamnose, and soluble starch as polysaccharides. After adding 1.0% of each carbon source to LB medium, optimized fermentation conditions obtained from the previous experiments were used. Iturin A production and antifungal activity were measured, and the results were compared among the different carbon sources. The antifun-

Table 3. Range of medium component variables at different levels for the central composite design

Variable quantity	Parameter	Level				
		-1.68	-1	0	1	1.68
Sucrose (%)	X_1	0	0.5	1	3	5
Soybean meal (%)	X_2	0	0.5	1	3	5
$MgCl_2$(%)	X_3	0	0.5	1	3	5

gal activity was measured through the Oxford cup method. Similarly, several nitrogen sources were tested, including peptone, soybean meal, urea, NH_4Cl, and ammonium citrate. Each nitrogen source was added to a flask containing 1.0% of liquid medium and 0.1% of glucose to make a C/N ratio of 10, a value shown to be the most appropriate for the maximum iturin A production in preliminary experiments (data not shown). Different metal ions, including Zn^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, and K^{+} were added to a flask to a concentration of 1.0% in LB medium to determine which metal ion best increased the production of iturin A, taking into consideration the antifungal activity and cost. Two flasks were prepared for each condition, and each experiment was repeated twice.

One-factor tests: Based on the iturin A production and antifungal activity experiments with each medium component, three components including sucrose, soybean meal, and $MgCl_2$ were selected to do one-factor, experiments, with five levels of each component in 500 ml medium to find their optimal concentrations for maximum production of iturin A. Each variable was tested at 0%, 0.5%, 1.0%, 3.0%, and 5.0% in the liquid medium while keeping the other two components at 1.0%.

RSM experimental design: CCD with five levels coded in duplicate was used to determine the optimum conditions for the production of iturin A. Preliminary data indicated that the major variables affecting the performance of the culture in terms of iturin A yield were the levels of sucrose, soybean meal, and $MgCl_2$. Therefore, these three factors were chosen for further optimization through RSM. The ranges and the levels of variables investigated in this study are given in Table 3. The matrix corresponding to the CCD is shown in Table 4, together with the observed experimental data. The experimental results of the CCD were fitted with a second-order polynomial equation (Equation 2) by a multiple regression technique. The antifungal activity of iturin A was simultaneously measured through the Oxford cup method.

Table 4. Design and results of central composite design (medium component)

Trial no.	Coded level of fermentation condition			Concentration of iturin A (mg/ml)
	X_1	X_2	X_3	
1	0	0	0	55.8
2	0	0	0	53.5
3	-1	1	1	1.5
4	0	-0	1.68	5.8
5	1	1	-1	2.1
6	0	0	0	52.1
7	-1	-1	1	6.9
8	-1	-1	-1	25.2
9	1	-1	1	3.4
10	0	1.68	0	4.4
11	1	-1	-1	18.3
12	1	1	1	3.9
13	0	0	-1.68	12.8
14	0	0	0	49.2
15	-1.68	0	0	5.8
16	1.68	0	0	2.3
17	0	0	0	50.2
18	0	0	0	48.7
19	-1	1	-1	1.7
20	0	-1.68	0	6.2

Statistical analysis

All experiments were done in triplicate, and the average concentration of iturin A was taken as the response. The statistical analysis of the data from one-factor tests were done with the SAS software package (version 9.0, SAS Institute Inc., USA), whereas the CCD and statistical analysis of the data were done with the Design Expert software package (version 7.0.0, State-Ease Inc., USA). Statistical analysis of the models was used to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equations was assessed statistically by the coefficient of determination R^2, and its statistical significance was measured by the *F*-test. The significance of the regression coefficients was determined by the *P*-value (Gangadharan *et al.*, 2008).

Results

Generation of growth curve and fermentation time vs antifungal activity curve

The growth curve of *Bacillus* sp. BH072 is shown in Fig. 1. The bacteria grew well in LB medium, with the logarithmic phase appearing at 12 h to 18 h. Using the same LB medium, the antifungal activity at different time points in the culture was measured and the relevant curve was generated (Fig. 1). The highest antifungal activity was reached at 60 h, which was in the stationary phase of the culture. Based on the generated curve, 60 h cultures were used to detect antifungal activity in the following optimization experiments.

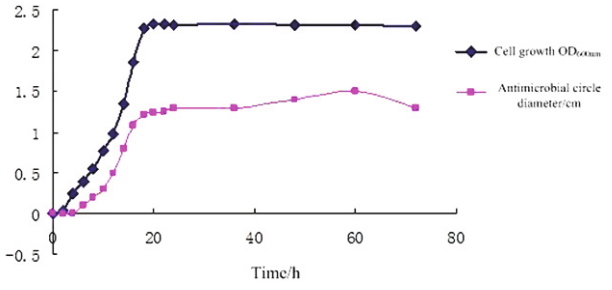

Fig. 1. The growth curve and fermentation time-antifungal activity curve of *Bacillus* sp. BH072.

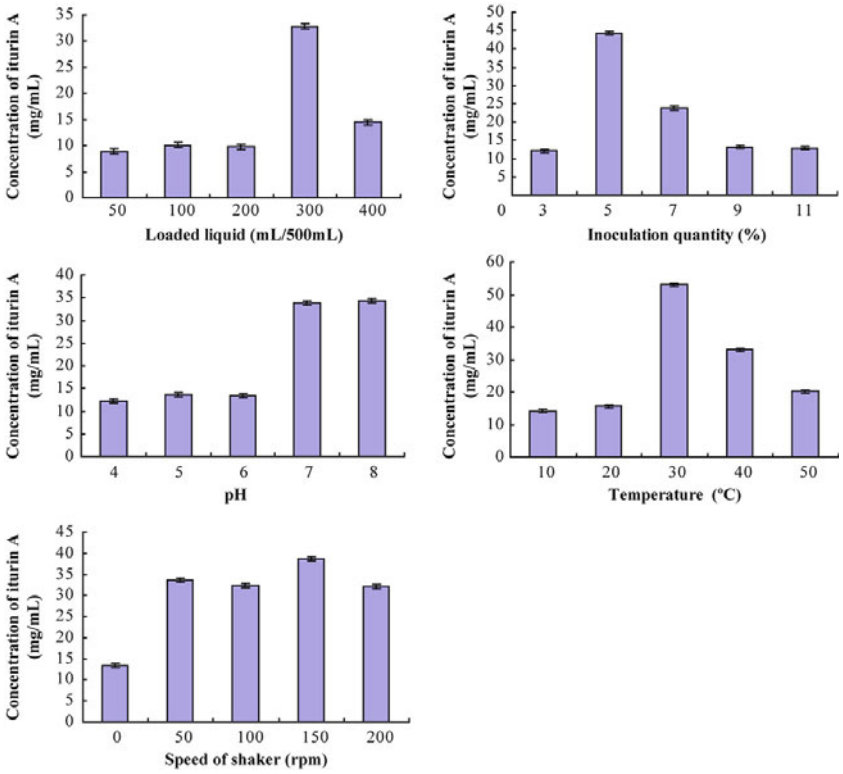

Fig. 2. Relative iturin A production after 60 h of incubation under the various culture conditions. The dark gray bar indicates the basal condition.

Analytical results for optimizing fermentation conditions

One-factor tests: Prior to optimizing medium components using a statistical experimental design, fermentation conditions affecting the production of iturin A were determined experimentally. Effects of culture conditions, including initial pH, temperature, loading volume, rotary speed, and inoculation quantity on iturin A production in LB medium were investigated. Changes in rotary speed had no significant effect on iturin A production (Fig. 2). A loading volume of 300 ml in 500 ml flasks had a higher performance than the other loading volumes and thus was used for the succeeding experiments. Iturin A production was significantly affected by changes in temperature, inoculation quantity, and initial pH, with maximum production at 30°C, 5% inoculation, and initial pH 7.0 to 8.0, respectively.

Central composite design: To fully explore the subregion of the response surface in the vicinity of the optimum, an experimental design with more than two levels of each factor is required, so that a second-order approximation to the response surface can be developed. A CCD with five coded levels was used for this purpose. The levels of the variables (Table 1) for the CCD experiments were selected according to the results of the previous experiments. The CCD design and the corresponding experimental data are presented in Table 2. By applying multiple regression analysis on the experimental data described in Table 2, the experimental results of the CCD design were fitted with a second-order polynomial equation (Equation 2), and the second-order polynomial equation obtained for iturin A production is shown in Equation 3:

$$R_1=46.79-2.43x_1-2.29x_2+6.68x_3+1.68x_1x_2+2.33x_1x_3 -1.17x_2x_3-15.20x_1^2-8.71x_2^2-7.56x_3^2$$

where x_1, x_2, and x_3 are the code values corresponding to the values of temperature, inoculation quantity, and initial pH, respectively.

The *P*-values were used as a tool to check the significance of the interaction effects, which in turn may indicate the patterns of the interactions among the variables (Fábio *et al.*, 2006). A larger magnitude for the t-test and smaller p-value correspond to a more significant coefficient. According to the ANOVA, the F-values for the overall regression is significant at the upper 5% level, which further supported the second-order model in approximating the response surface of the experimental design. From the regression coefficients

Table 5. Results of regression analysis and corresponding F and *P*-value of second-order model for optimization of iturin production of *Bacillus* sp. BH072 (fermentation condition)

Factor	Coefficient estimate	Standard error	F value	*P*-value
Intercept	46.79	1.63	37.06	< 0.0001*
x_1	-2.43	1.08	5.04	0.0486*
x_2	-2.29	1.08	4.46	0.0609
x_3	6.68	1.08	37.98	0.0001*
x_1x_2	1.68	1.42	1.40	0.2641
x_1x_3	2.33	1.42	2.70	0.1315
x_2x_3	-1.17	1.42	0.69	0.4259
x_1^2	-15.20	1.05	207.57	< 0.0001*
x_2^2	-8.71	1.05	68.16	< 0.0001*
x_3^2	-7.56	1.05	51.36	< 0.0001*

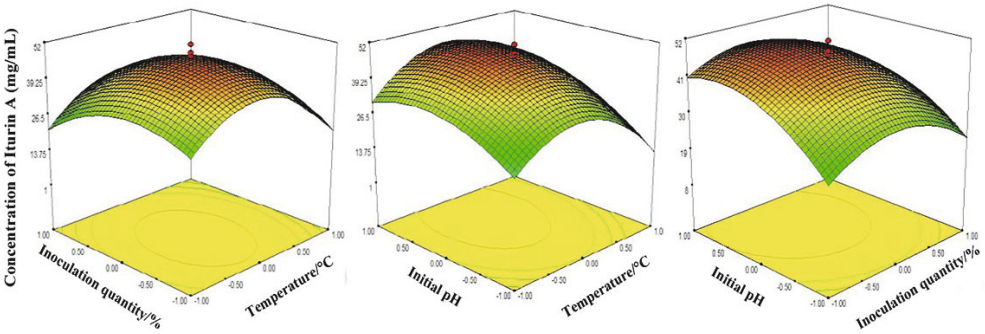

Fig. 3. Response surface and contour plots of iturin A produced by *Bacillus* sp. BH072 showing the effect of two fermentation condition variables.

and corresponding p-values in Table 5, the linear term of temperature and initial pH had significant effects on iturin A production ($P<0.05$), whereas the linear term of all other factors did not. The lack of fit item was used to represent the degree of fit between the model and experiment. In this study, the P-value was 0.0996 > 0.05, which was good for the model, without lack of fit factors. The fit of this model was also checked by the coefficient of determination R^2, which was calculated to be 0.9709, indicating that 97.09% of the variability in the response could be explained by the model. Therefore the regression equation can be used instead of real test point analysis of the experimental results.

When one of these variable factors was zero, the concentration of iturin A initially increased but later decreased with the other two factors (Fig. 3). After transforming Equation 3 to its canonical form, the optimum combination for the iturin A production was found to be the following: temperature (X_1) 29.5°C, inoculation quantity (X_2) 6.7%, and initial pH (X_3) 6.45. The model predicted a maximum response of iturin A production at 48.5365 mg/ml. Verification of the calculated maximum was conducted with experiments using the optimum combination, and an iturin A production of 47.87 mg/ml (average of three repeats) was obtained. The excellent correlation between predicted and experimental values supported the validity of the response model.

Analytical results for optimizing medium components

Influence of carbon sources, nitrogen sources, and metal ions on production and antifungal activity of iturin A: To optimize the iturin A production medium, the medium components were classified into carbon sources, nitrogen sources, and metal ions. The effects of different carbon sources, nitrogen sources, and metal ions on iturin A production of strain BH072 in LB liquid medium were examined after incubation at 150 rpm for 60 h at 30°C (Fig. 4). The addition of sucrose to LB medium showed the highest level of iturin A production enhancement, increasing by 20% over that without additional carbon sources. In concert, the antifungal specific activity of iturin A extracted from the culture containing sucrose was higher than that without addition of other carbon sources, increasing from 350.11 AU/mg (with no addition) to 489.67 AU/mg. Although inorganic nitrogen sources had negative effects on iturin A production, the organic nitrogen source, soybean meal, enhanced iturin A production. The metal ion Mg^{2+} promoted the production of iturin A. However, Fe^{3+} and Zn^{2+} had an adverse effect on iturin A production. Considering the yield of iturin A and antifungal specific activity, sucrose, soybean meal, and $MgCl_2$ were the most effective components affecting iturin A production.

One-factor tests: Based on the results obtained in the previous experiments, sucrose, soybean meal, and Mg^{2+} were found to be the determining variables for iturin A production. Hence, these factors were combined at different concentrations for optimizing iturin A production. For this purpose, a one-factor design was followed. Sucrose, soybean meal, and Mg^{2+} were used as the three factors and varied at five levels. The results show that changes in sucrose, soybean meal, and Mg^{2+} had a significant effect on iturin A production (Fig. 5). Therefore, these three factors were studied in the CCD experiments.

Central composite design: RSM was introduced to determine

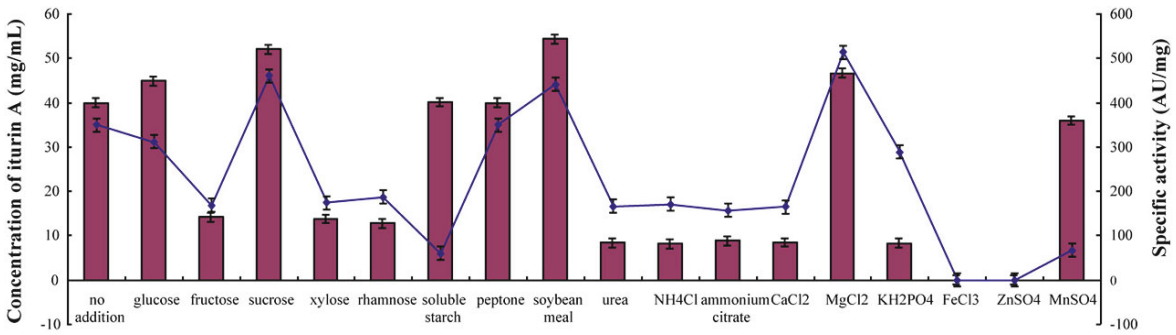

Fig. 4. Iturin A production by *Bacillus* sp. BH072 cultured with different kinds of medium components.

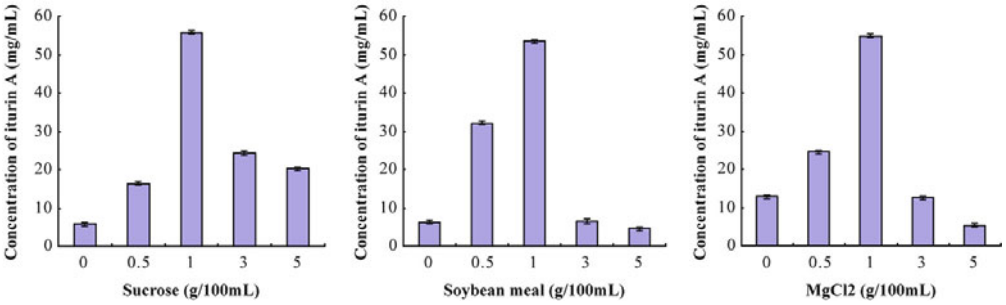

Fig. 5. Relative iturin A production after 60 h of incubation under the various concentration of sucrose, soybean meal and $MgCl_2$. The dark gray bar indicates the basal condition.

the optimal concentrations of sucrose, soybean meal, and $MgCl_2$ for maximizing iturin A production with 60 h cultures. Based on the results shown in Table 2, temperature, initial pH, and inoculation quantity were set at 29.5°C, pH 6.45 and 6.7%, respectively. The CCD experiment was performed under the same conditions as the optimization of fermentation. The design of the experiment and results are presented in Tables 4 and 6, respectively.

Regression analysis was performed to fit the response function with the experimental data. As shown in Table 6, the F and *P*-values were 49.71 and < 0.0001, respectively. The statistical significance of the second-order model equation was verified, and the coefficient of determination (R^2) of the model was calculated to be 0.9781, indicating that 97.81% of the variability in the response can be explained by the model. Thus, the response equation provided a suitable model for the response surface of the experiment examining iturin A production. The results suggest that the linear term of concentration of soybean meal and Mg^{2+} displayed a significant effect on the iturin A production ($P<0.05$), whereas the linear term of all other factors were not significant. In addition, significant interactions were noted between soybean meal (X_2) and $MgCl_2$ (X_3).The response equation obtained was as follows (Equation 4):

$$R_2=51.44-0.99x_1-3.49x_2-3.18x_3+1.65x_1x_2+0.68x_1x_3 -4.35x_2x_3-15.87x_1^2-15.43x_2^2-14.02x_3^2$$

where x_1, x_2, and x_3 are the code values corresponding to the amount of sucrose, soybean meal, and $MgCl_2$, respectively.

The yield of iturin A initially increased but later decreased, changing with the other two factors (Fig. 6). This trend was the same as that for the fermentation conditions. After transforming Equation 4 to its canonical form, the optimum combination for the iturin A production was the following: sucrose (X_1) 0.98%, soybean meal (X_2) 0.94%, and Mg^{2+} (X_3) 0.93%. The model predicted a maximum response of iturin A production at 51.9097 mg/ml. The calculated maximum was verified by repeated trials using the optimum medium components, with a production of 52.21 mg/ml (average of three repeats). The antifungal specific activity was tested simultaneously, increasing from 350.11 AU/mg to 513.92 AU/mg. The excellent correlation between predicted and experimental values confirmed the validity of the response model.

Discussion

The efficient production of lipopeptides is critical for their applications in the food, agricultural, environmental, and pharmaceutical fields (Nihorimbere *et al.*, 2012). Consequently, more attention is being paid to the qualitative and quantitative analysis of lipopeptides.

Members of the iturin family include iturin A, iturin C, iturin D, and iturin E (Roongsawang *et al.*, 2011). Iturin A is a heptapeptide (L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser) cyclized with a β-amino fatty acid, displaying strong antifungal activity. Iturin C (FA-β-NH_2-L-Asp-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser) is a novel metabolite proven to control cotton verticillium wilt. For iturin D and iturin E, the acid hydrolysates contain alpha-amino acids Asp3, Glu1, Pro1, Ser1, Tyr1, and a mixture of n-C14, iso-C15, anteiso-C15, iso-C16, and n-C16 β-amino acids. They differ from iturin A by the presence of a free carboxyl group in iturin D and a carboxymethyl group in iturin E (Besson and Michel, 1987). In our previous studies, the iturin A gene (*ituA*) of *Bacillus* sp. BH072 was detected by

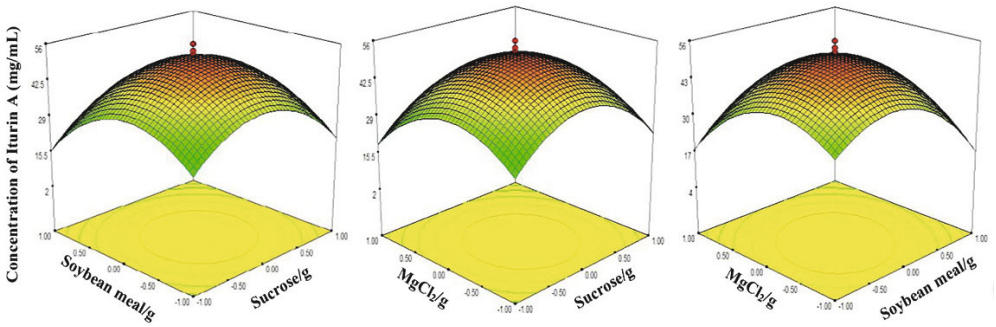

Fig. 6. Response surface and contour plots of iturin A produced by *Bacillus* sp. BH072 showing the effect of two medium component variables.

Table 6. Results of regression analysis and corresponding F and *P*-value of second-order model for optimization of iturin production of *Bacillus* sp. BH072 (medium component)

Factor	Coefficient estimate	Standard error	F value	*P*-value
Intercept	51.44	1.80	49.71	< 0.0001*
x_1	-0.99	1.20	0.68	0.4287
x_2	-3.49	1.20	8.48	0.0155*
x_3	-3.18	1.20	7.04	0.0242*
x_1x_2	1.65	1.56	1.11	0.3163
x_1x_3	0.68	1.56	0.19	0.6753
x_2x_3	4.35	1.56	7.73	0.0194*
x_1^2	-15.87	1.17	185.52	< 0.0001
x_2^2	-15.43	1.17	175.34	< 0.0001*
x_3^2	-14.02	1.17	144.67	< 0.0001*

PCR assays, and the antifungal substance extracted from the strain was identified as iturin A by an LC-MS method (Zhao *et al.*, 2013b). Based on the results of gene sequence detection, LC-MS analysis, and the amino acid sequence homology alignment, the antifungal activity of *Bacillus* sp. BH072 is caused by iturin A.

In this study, both fermentation conditions and medium components were optimized by RSM methods to effectively enhance the yield of antifungal lipopeptides of *Bacillus* sp. BH072. The optimum fermentation conditions for iturin A production were the following: temperature (X_1) 29.5°C, inoculation quantity (X_2) 6.7%, and initial pH (X_3) 6.45. Effects of different carbon sources, nitrogen sources, and metal ions on the production of iturin A cultured using the optimized fermentation conditions were studied and then further optimized by RSM. The results revealed that the combination of 0.98% sucrose, 0.94% soybean meal, and 0.93% Mg^{2+} enhances antifungal activity against pathogenic fungi. It demonstrated that the production and antifungal activity of iturin A were influenced by different medium components. The maximum iturin A production was 52.21 mg/ml, with an antifungal specific activity of 513.92 AU/mg. The iturin A production of wild type strain BH072 before optimization was higher at 45.30 mg/ml, with an antifungal specific activity of 350.11 AU/mg, than that of other reported strains. Moreover, the optimization result was much better than that of similar studies conducted by other researchers. A lipopeptide antibiotic produced by *Bacillus subtilis* RB14-CS was used to predict the optimum amounts of the carbon and nitrogen sources in the medium through RSM (Mizumoto and Shoda, 2007). The maximum iturin A concentration was 5.591 μg/g initial wet okara under optimized conditions. Zhao *et al.* (2013b) reported the influence of three critical parameters including nitrogen sources, initial pH, and metal ions on the production of antifungal lipopeptides from *Bacillus amyloliquefaciens* Q-426. The optimal conditions for iturin A production by *B. subtilis* S3 obtained from RSM were pH 6.0, 0.93% maltodextrin, 1.11% glucose, 0.72% corn steep powder, 1.5 mM $MgSO_4$, 0.75 mM KH_2PO_4, rotation speed 180 rpm, and area of aeration 4.35 cm^2. A 180% increase of iturin A production (from 47.19 mg/L to 132.23 mg/L) was observed after optimization with RSM.

Several lipopeptides exhibited excellent antifungal activity against plant pathogens. The detection of antifungal activity was a measure of the optimization of lipopeptide production. During the extraction of iturin A, its antifungal activity may decrease. Therefore, the determination of antifungal activity was also necessary. There have been many previous RSM optimization studies on the antifungal effect of lipopeptides. Huang *et al.* (2010) demonstrated that *Penicillium notatum* was sensitive to surfactin and iturin, whose minimal inhibitory concentrations were 62.5 μg/ml and 31.25 μg/ml, respectively. To guarantee the validity of the optimization, the yield and the antifungal activity were simultaneously measured in our study, something that rarely has been reported previously.

RSM is a powerful tool for optimizing production conditions. The significant improvement in the lipopeptide yield of *Bacillus* sp. BH072 suggests that RSM is a rapid, valuable, and simple method. When the response function was fitted to the raw experimental data, the coefficient of determination (R^2) of the model was very close to 1, indicating that the statistical method overcame the difficulty of approximation owing to experimental error. This result confirms the validity of the statistical method (Mullai *et al.*, 2013). Optimizing the process using RSM is critical for industrial-scale production of iturin A.

Iturin A inhibits wheat scab (*F. graminearum*), rice sheath blight (*R. solani*), watermelon fusarium wilt (*F. oxysporum*), *P. irregulare*, *B. cinerea*, and various other plant pathogenic fungi. After the optimization of fermentation conditions and medium components by RSM, the concentration was increased from 45.30 mg/ml to 52.21 mg/ml, the antifungal specific activity was enhanced from 350.11 AU/mg to 513.92 AU/mg. Considering its strong antifungal activity and its potential for industrial-scale production, we expect that iturin A produced by *Bacillus* sp. BH072 will contribute to the bio-control of agricultural pathogens and be used in the field of food safety.

Acknowledgements

Xin Zhao is grateful for the technical contributions of Mr. Bin Qiao and would like to thank Mr. Jinjin Diao, Miss Xiaodi Hong, Mr. Jiping Wei, Miss Jie Fan and Miss Qian Peng for their advice and help during this study.

References

Asaka, O. and Shoda, M. 1996. Biocontrol of *Rhizoctonia solani* Damping-Off of Tomato with *Bacillus subtilis* RB14. *Appl. Environ. Microbiol.* **62**, 4081–4085.

Besson, F. and Michel, G. 1987. Isolation and characterization of new iturins: iturin D and iturin E. *J. Antibiot.* **40**, 437–442.

Bonmatin, J.M., Laprevote, O., Peypoux, F. 2003. Diversity among microbial cyclic lipopeptides: Iturins and surfactins. Activity-structure relationships to design new bioactive agents. *Comb. Chem. High Throughput Screen* **6**, 541–556.

Box, G.E.P. and Wilson, K.B. 1951. On the experimental attainment of optimum conditions. *J. Royal. Statist. Soc. (Ser. B).* **13**, 1–5.

Buchanan, R.L. and Philips, J.G. 1990. Response surface model for predicting the effects of temperature, pH, sodium chloride

content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocutogenes*. *J. Food Protect.* **53**, 370–374.

Chen, H., Wang, L., Su, C.X., Gong, G.H., Wang, P., and Yu, Z.L. 2008. Isolation and characterization of lipopeptide antibiotics produced by *Bacillus subtilis*. *Lett. Appl. Microbiol.* **47**, 180–186.

Delcambe, L., Peypoux, F., and Besson, F. 1977. Structure of iturin and iturin-like substances [proceedings]. *Biochem. Soc. T.* **5**, 1122–1124.

Eshita, S.M., Roberto, N.H., Beale, J.M., Mamiya, B.M., and Workman, R.F. 1995. Bacillomycin L(c), a new antibiotic of the iturin group-isolations, structures, and antifungal activities of the congeners. *J. Antibiot*. **48**, 1240–1247.

Fábio, C.S., Danilo, D.F., Hilario, C.M., Flávia, M.L.P., Patrizia, P., and Attilio, C. 2006. Use of response surface methodology for optimization of xylitol production by the new yeast strain *Debaryomyces hansenii* UFV-170. *J. Food Eng.* **76**, 376–386.

Gangadharan, R., Anandan, V., and Zhang, G. 2008. Optimizing the functionalization process for nanopillar enhanced electrodes with GOx/PPY for glucose detection. *Nanotechnology* **19**, 1–7.

Gu, X.B., Zheng, Z.M., Yu, H.Q., Wang, J., Liang, F.L., and Liu, R.L. 2005. Optimization of medium constituents for a novel lipopeptide production by *Bacillus subtilis* MO-01 by a response surface method. *Process Biochem*. **40**, 3196–3201.

Haltrich, D., Press, M., and Steiner, W. 1993. Optimization of a culture medium for increased xylanase production by a wild strain of *Schizophyllum commune*. *Enzyme Microbiol. Technol.* **15**, 854–859.

Huang, X.Q., Wang, Y.F., Cui, Y.H., and Hua, X. 2010. Optimization of antifungal effect of surfactin and iturin to *Penicillium notatum* in syrup of peach by RSM. *Int. J. Pept. Res. Ther.* **16**, 63–69.

Katz, E. and Demain, A.C. 1977. The peptide antibiotics of *Bacillus*: chemistry, biogenesis, and possible functions. *Bacteriol. Rev.* **41**, 449–474.

Kim, P.I., Ryu, J., Kim, Y.H., and Chi, Y.T. 2010. Production of biosurfactant lipopeptides Iturin A Fengycin and Surfactin A from *Bacillus subtilis* CMB32 for control of *Colletotrichum gloeosporioides*. *J. Microbiol. Biotechnol*. **20**, 138–145.

Kuo, C.Y. 2006. Optimization of cultivation conditions for iturin A production by *Bacillus subtilis* using solid state fermentation. *Master Thesis*. Da-Yeh University, Taiwan.

Maget-Dana, R. and Peypoux, F. 1994. Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. *Toxicol.* **87**, 151–174.

Mizumoto, S. and Shoda, M. 2007. Medium optimization of antifungal lipopeptide, iturin A, production by *Bacillus subtilis* in solid-state fermentation by response surface methodology. *Appl. Microbiol. Biotechnol.* **76**, 101–108.

Motta, A.S. and Brandelli, A. 2002. Characterization of an antibacterial peptide produced by *Brevibacterium linens*. *J. Appl. Microbiol.* **92**, 63–70.

Mullai, P., Yogeswari, M.K., and Sridevi, K. 2013. Optimisation and enhancement of biohydrogen production using nickel nanoparticles-A novel approach. *Bioresour. Technol.* **141**, 212–219.

Nihorimbere, V., Cawoy, H., Seyer, A., Brunelle, A., Thonart, P., and Ongena, M. 2012. Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain *Bacillus amyloliquefaciens* S499. *FEMS Microbiol. Ecol.* **79**, 176–191.

Ongena, M. and Jacques, P. 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* **16**, 115–125.

Ongena, M., Jacques, P., Toure, Y., Destain, J., Jabrane, A., and Thonart, P. 2005. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. *Appl. Microbiol. Biotechnol*. **69**, 29–38.

Peypoux, F., Bonmatin, J.M., and Wallach, J. 1999. Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol*. **51**, 553–563.

Phae, C.G. and Shoda, M. 1990. Investigation of optimal conditions for foam separation of iturin, and antifungal peptide produced by *Bacillus subtilis*. *J. Ferment. Bioeng.* **71**, 118–121.

Phae, C.G. and Shoda, M. 1991. A new fungus which degrades hydrogen sulfide, methanethiol, dimethyl sulfide and dimethyl disulfide. *Biotechnol. Lett.* **13**, 375–380.

Phae, C.G., Shoda, M., and Kita, N. 1992. Biological control of crown and root rot and bacterial wilt of tomato by *Bacillus subtilis* NB22. *Annal. Phytopathol. Society Japan* **58**, 329–339.

Prapulla, S.G., Jacob, S., Chand, N., Rajalakshmi, D., and Karanth, N.G. 1992. Maximization of lipid production by rhodotroula gracilis CFR-1 using response surface methodology. *Biotechnol. Bioeng*. **40**, 965–969.

Romero, D., de Vicentev, A., Rakotoalyv, R.H., Dufour, S.E., Veening, J.W., Arrebola, E., Cazorla, F.M., Kuipers, O.P., Paquot, M., and Perez-Garcia, A. 2007. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. *Mol. Plant-Microbe Interact.* **20**, 430–440.

Roongsawang, N., Washio, K., and Morikawa, M. 2011. Diversity of nonribosomal peptide synthetases involved in the biosynthesis of lipopeptide biosurfactants. *Int. J. Mol. Sci.* **12**, 141–172.

Shih, I.L. and Shen, M.H. 2006. Application of response surface methodology to optimize production of poly(e-lysine) by streptomyces albulus IFO14147. *Enzym. Microb. Technol.* **39**, 15–21.

Shih, I.L., Van, Y.T., and Chang, Y.N. 2002. Application of statistical experimental methods to optimize production of poly(g-glutamic acid) by *Bacillus licheniformis* CCRC 12826. *Enzym. Microb. Technol.* **31**, 213–220.

Tenoux, I., Besson, F., and Michel, G. 1991. Studies on the antifungal antibiotics: bacillomycin D and bacillomycin D methylester. *Microbios* **67**, 187–193.

Toure, Y., Ongena, M., Jacques, P., Guiro, A., and Thonart, P.J. 2004. Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. *Appl. Microbiol.* **96**, 1151–1160.

Yang, J., Ji, J.Y., Kang, Z.S., and Huang, L.L. 2012. Optimization of fermentation conditions of antifungal lipopeptide produced by *Bacillus subtilis* E1R-j. *Acta Agriculturae Boreali-occidentalis Sinia.* **21**, 54–60.

Yao, D.H., Ji, Z.X., Wang, C.J., Qi, G.F., Zhang, L.L., Ma, X., and Chen, S.W. 2012. Co-producing iturin A and poly-c-glutamic acid from rapeseed meal under solid state fermentation by the newly isolated *Bacillus subtilis* strain 3-10. *World J. Microbiol. Biotechnol.* **28**, 985–991.

Zhang, Y., Zhang, Q.J., Feng, X.H., Li, S., Xia, J., and Xu, H. 2012. A novel agar diffusion assay for qualitative and quantitative estimation of epsilon-polylysine in fermentation broths and foods. *Food Res. Int.* **48**, 49–56.

Zhao, P.C., Quan, C.S., Jin, L.M., Wang, L.N., Wang, J.H., and Fan, S.D. 2013a. Effects of critical medium components on the production of antifungal lipopeptides from *Bacillus amyloliquefaciens* Q-426 exhibiting excellent biosurfactant properties. *World J. Microb. Biotechnol.* **29**, 401–409.

Zhao, X., Zhou, Z.J., Han, Y., Wang, Z.Z., Fan, J., and Xiao, H.Z. 2013b. Isolation and identification of antifungal peptides from *Bacillus* BH072, a novel bacterium isolated from honey. *Microbiol. Res*. **168**, 598–606.

Zhao, Z., Wang, Q., Wang, K., Brian, K., Liu, C., and Gu, Y. 2010. Study of the antifungal activity of *Bacillus vallismortis* ZZ185 in vitro and identification of its antifungal components. *Bioresour. Technol.* **101**, 292–297.