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Medium optimization for production of antifungal active substances from antagonistic *Brevibacillus laterosporus* and its antifungal activity analysis

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The antagonist bacterium *Brevibacillus laterosporus* ZQ2 with broad-spectrum antifungal activity was isolated from an apple rhizosphere at Mount Tai in China. The fermentation medium for the production of antifungal substances from ZQ2 was statistically optimized. In one-factor-at-a-time experiments, five components, including carbon sources, nitrogen sources, and inorganic salt, were selected. Two factors were determined as critical based on the Plackett-Burman design. Steepest ascent experiments were then used to determine the design space containing the optimum point, followed by application of the response surface methodology. A quadratic model fitting the actual production was established using a central composite design. The obtained optimum medium for the antifungal substances production consisted of the following (in g/L): glucose, 15.6; soybean meal, 8.2; starch, 20; ammonium sulfate, 2; and monopotassium phosphate, 1. Furthermore, the inhibition rate of the substances against *Rhizoctonia solani* increased by more than 132% compared with the control. *B. laterosporus* ZQ2 strongly inhibited the growth of many apple phytopathogens *in vitro*. The inhibition rates against different fungi ranged from 55.26 to 79.02%. As well, the strain significantly reduced the disease damage caused by *R. solani* in the *in vivo* tests. Maximum disease protection (46.89%) was recorded when the bacterium applied 3 days before pathogen inoculation.

Key words: *Brevibacillus laterosporus*, antifungal activity, fermentation optimization.

INTRODUCTION

Rhizoctonia solani, *Penicillium expansum*, and *Botrytis cinerea* are common and destructive phytopathogenic fungi with a wide range of hosts and are major constraints to apple production (Sharma et al., 2009). At present, chemical control remains the primary method for preventing the diseases caused by these fungi. Numerous synthetic chemicals comprise the majority of the market share of fungicides (Imre et al., 2009; Jönssson et al., 2010). However, because of the increasing number of resistant mutants and environmental pollution, the demand for more effective and safer fungicides with novel modes of action is increasing (Zhenzhen et al., 2010). The use of antagonistic microorganisms for biological control is considered to be one such alternative method.

Over the last few years, various antifungal bacteria

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have been investigated as potential biocontrol agents. Scientists have focused on the use of antagonistic bacteria and their active substances. Members of the *Brevibacillus* clade are well-known biocontrol microorganisms that produce structurally diverse secondary metabolites with broad-spectrum antibiotic activity. Some of these metabolites, such as chitinase and gramicidin S, have been extensively studied (Tatsushi and Kiyoshi, 2009). Numerous *Brevibacillus* species can potentially be used as biocontrol agents in agricultural production, and some of these strains have become research hotspots (Saikia et al., 2011; Sharma et al., 2012).

A suitable fermentation medium for the production of antifungal substances is the basis of further study. Traditionally, a non-statistical approach (e.g., one-factor-at-a-time experiments) is frequently used in medium optimization. However, this method ignores the interactions among the variables and is time-consuming (Yaqian et al., 2008). Under such circumstances, statistically based experimental designs, such as the Plackett-Burman (PB) design and response surface methodology (RSM), are more suitable for medium optimization. These technologies can be applied in medium optimization to obtain better results and high yields of the products of interest using a small number of experiments (Quan et al., 2010). Along with the development of statistical analysis software, computer-aided design (CAD) also plays a very important role in the optimization of fermentation.

Antagonist *B. laterosporus* strain ZQ2 (NCBI GenBank Accession No. EU471747), which strongly inhibits numerous phytopathogenic fungi on apple, was investigated in the previous research (Zhen et al., 2011). In the present study, the fermentation medium for the antifungal substances production was optimized using a combination of traditional non-statistical technology and statistically based experiments, and the antifungal activities of this strain were investigated both *in vitro* and *in vivo*. The aim of this study was to provide theoretical basis for the further study and application of strain ZQ2 and its active metabolites.

MATERIALS AND METHODS

Determination of antifungal activities

The antifungal activities of ZQ2 were determined on a potato dextrose agar (PDA; 200 g of potato, 20 g of glucose, 10 g of beef extract, 2 g of ammonium sulfate, 1 g of sodium chloride, and 20 g of agar in 1 L distilled water) plate using *R. solani* as the fungal indicator (Dake et al., 2007). The bacterium was inoculated into a potato dextrose broth (PDB) medium (200 g of potato, 20 g of glucose, 10 g of beef extract, 2 g of ammonium sulfate, and 1 g of sodium chloride in 1 L distilled water) and incubated for 48 h at 30°C. After centrifugation, the filtrate was diluted with a melted PDA medium at a 1:20 (v/v) ratio and poured into Petri dishes. Six-millimeter disks of the tested fungi were placed at the center of the mixed PDA plate. Inhibition of fungal growth was evaluated after a 3-day incubation period as the percent reduction of mycelial growth compared with that in the control plates (without the bacterial cell-free filtrate in the medium). The antifungal activities were calculated using the following equation (mycelia length was measured by cross transposition method):

$$\text{Growth inhibition (\%)} = \frac{\text{mycelia length in the control plate} - \text{mycelia length in the treated plate}}{\text{mycelia length in the control plate.}} \times 100 \quad (1)$$

Medium optimization for production of antifungal substances from ZQ2

The fermentation medium of ZQ2 was optimized by combining non-statistical and statistical methods to obtain a higher yield of antifungal substances for further study. Growth inhibition was measured as the evaluation index of metabolite production.

One-factor-at-a-time experiments

Medium optimization based on the initial PDB medium was conducted to maximize the production of the antifungal substances. Different inorganic carbon sources (e.g., glucose, sucrose, lactose, and maltose, concentration 2%), organic carbon sources (e.g., starch, corn flour, potatoes, and mannitol, concentration 2%), inorganic nitrogen sources (e.g., ammonium sulfate, ammonium nitrate, sodium nitrate, and ammonium chloride, concentration 0.2%), organic nitrogen sources (e.g., peptone, yeast extract, beef extract, and soybean meal, concentration 1%), and inorganic salts (e.g., calcium carbonate, magnesium sulfate, monopotassium phosphate, and sodium chloride, concentration 0.1%) were tested by replacing the corresponding components of the PDB medium with an equivalent concentration of the nutrient substance. The production of active substances in each treatment was estimated by measuring the antifungal activity against *R. solani*.

PB design

The components that exhibited significant effects on the antifungal metabolite production were selected as the critical factors in PB design. Each independent variable was designated as low level (-1, initial concentration in the fermentation medium) or high level (+1, 1.25-fold of the initial concentration) (Table 1). Both the random designations of the experiments and the analysis of linear regression of the data were conducted using Minitab software. If the differences were significant between the mean of the center points and that of the variable points ($P<0.05$), the optimum would be within the designated space; otherwise, it would be outside the space, and steepest ascent experiments should be applied (Zhiwen and Xunli, 2008). The direction of the ascent is decided by the coefficients of the variables in the regression equation and is parallel to the normal of the contour line of the response curve. Experiments should be performed along the path until the increase in the response value stops. The optimal point obtained from the steepest ascent can be used as the center point of the central composite design (CCD).

Table 1. Designations of the variables in PB experiments.

Factor (%)	Level	
	1	1
Glucose	2.0	2.5
Starch	2.0	2.5
$(NH_4)_2SO_4$	0.2	0.25
Soybean meal	1.0	1.25
KH_2PO_4	0.1	0.125

Central composite design (CCD)

Based on the above experiments, CCD was used to obtain a quadratic regression model for the production of the antifungal substances by optimizing the proportion of the significant variables in the medium. The central points of the significant variables were chosen according to steepest ascent experiments, and other factors were maintained at a low level in the medium. SAS 9.0 software was used in designing experiments and obtaining the regression equation. Each variable was designated 5 levels (-2, -1, 0, 1, and 2), and 13 experiments were required according to the software, including 5 replications at the center point. The data were analyzed using the RSM program of SAS 9.0 to establish a model; inhibition rate was used as the response value (Y).

Validation experiments

Fermentation was conducted in the optimum medium obtained from RSM. All values representing the means of the triplicate experiments were analyzed to verify the significance of the model and validate the correspondence between the predicted value and the actual yield.

Antifungal activity and biocontrol efficacy of strain ZQ2

In vitro experiments

Seven pathogenic fungi, namely, *Fusarium oxysporum*, *F. solani*, *Physalospora piricola*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *P. expansum*, and *B. cinerea*, were obtained from the Plant Protection College of the Shandong Agriculture University (Taian, China) and maintained on PDA plates at 4°C prior to use. The inhibitory activities of the antifungal substances produced by ZQ2 were determined as the percent reduction in mycelial growth using the optimum medium obtained above. For the control, the target fungi were grown on PDA plates without the culture filtrate. The results were recorded after 72 h of incubation at 30°C. The morphology along the edges of the inhibited *R. solani* mycelia was then observed under a light microscope (Nikon Eclipse E200, Japan).

In vivo tests

Soil was randomly collected 20 cm deep from the ground surface in an apple orchard, and 6 kg of soil was loaded into individual earthen pots. Apple seeds were surface-sterilized with 1% sodium hypochlorite for 20 s, rinsed with sterilized distilled water, and then dried in an aseptic air stream. Six seeds per pot were sown and cultured for 40 days under room temperature with a 12 h photoperiod photoperiod. Seedlings with five to six expanded leaves were separated into four groups and treated as follows: (I) pathogen alone, (II) ZQ2 alone, (III) pre-inoculation with ZQ2 and pathogen inoculation 3 days later, (IV) ZQ2 and pathogen inoculation at the same time, (V) post-inoculation with ZQ2 after 3 days of pathogen inoculation, and (VI) control treatment (sterilized distilled water). The strain ZQ2 and pathogen spores were inoculated into PDB medium and incubated for 24 h at 30°C to prepare the inocula for the infection tests, following the technique described by Yazici et al. (2011). Disease severity was evaluated as the percentage of lesion areas on leaves. Each treatment was carried out in triplicate. Data were recorded 10 days later and analyzed using SAS 9.0 software.

RESULTS

One-factor-at-a-time experiments

Different carbon sources, nitrogen sources, and inorganic salts were investigated. By measuring the antifungal activity under different media, glucose (A), starch (B), ammonium sulfate (C), soybean meal (D), and monopotassium phosphate (E) were chosen as the optimum inorganic carbon, organic carbon, inorganic nitrogen, organic nitrogen, and inorganic salt sources, respectively (data not shown). The PDB medium was modified by mixing 20 g/L of glucose, 20 g/L of starch, 2 g/L of ammonium sulfate, 10 g/L of soybean meal, and 1 g/L of monopotassium phosphate and used as the basis for the PB design.

Optimization using the PB design and steepest ascent experiments

The aim of the PB experiments was to screen for factors that significantly influence the production of antifungal substances. The effects of each variable on the response value were digitally analyzed (Table 2) and the following linear regression equation was established:

$$Y = 55.66–1.4A–0.99B+1.95C–7.76D–1.23E \quad (2)$$

Based on the results, glucose and soybean meal exhibited significant negative effects ($P<0.05$) on production. Therefore, these effects should be appropriately reduced to obtain high yields, and other insignificant variables should be maintained at low levels. The differences between the mean of the center points and that of the variable points were not significant ($P>0.05$), and steepest ascent experiments were applied using the technique described earlier Table 3. The direction of the path was determined by Equation 2, indicating that decreasing the glucose and soybean meal concentrations could enhance the production of active substances. The center points of the PB design were considered as the origin and used in steepest ascent experiments. The inhibition rate reached 66.52% when glucose and soybean meal concentrations were at 1.60 and 0.86%,

Table 2. Results of PB experiments.

A	Dummy	B	Dummy	C	Dummy	D	E	Inhibition rate (%)
1	-1	1	-1	-1	-1	1	1	52.25±0.87
1	1	-1	1	-1	-1	-1	1	60.02±1.02
-1	1	1	-1	1	-1	-1	-1	53.52±0.56
1	-1	1	1	-1	1	-1	-1	48.15±0.43
1	1	-1	1	1	-1	1	-1	57.74±0.92
1	1	1	-1	1	1	-1	1	55.22±0.76
-1	1	1	1	-1	1	1	-1	71.12±1.15
-1	-1	1	1	1	-1	1	1	70.33±1.04
-1	-1	-1	1	1	1	-1	1	69.03±1.13
1	-1	-1	-1	1	1	1	-1	63.46±0.72
-1	1	-1	-1	-1	1	1	1	69.05±0.98
-1	-1	-1	-1	-1	-1	-1	-1	58.01±0.64

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

Run	Standard variable		Actual variable		Inhibition rate (%)
	A	D	A	D	
Step length Δ	-1.00	-0.71	-0.20	-0.07	
Original point	0	0	2.00	1.00	52.26±1.02
Original point＋Δ	-1.00	-0.71	1.80	0.93	58.17±0.93
Original point＋2Δ	-2.00	-1.42	1.60	0.86	66.52±1.05
Original point＋3Δ	-3.00	-2.13	1.40	0.79	63.78±1.12
Original point＋4Δ	-4.00	-2.84	1.20	0.72	52.65±0.87
Original point＋5Δ	-5.00	-3.55	1.00	0.65	42.12±0.58
Original point＋6Δ	-6.00	-4.26	0.80	0.58	39.43±0.67

respectively; these values were determined as the center point of CCD (Table 5).

Optimization of the medium via RSM

In recent years, RSM has become a popular tool for optimization techniques; it is used extensively to achieve optimum results using fewer experiments. The design and results of the central composite method used in the current study are shown in Tables 4 and 5. The following regression equation of the model was obtained based on the results obtained by SAS 9.0:

$$Y = 75.21+1.09A+0.69D–2.33A^2+0.32A*D–1.47D^2 \quad (3)$$

Where Y represents the inhibition rate, A represents the glucose content, and D represents the soybean meal content. The reliability of the model was determined using ANOVA (Table 6). The *P*-value of the equation was less than 0.01, indicating the extreme significance of the model; R^2 was 0.9657, implying that more than 96% of the experimental data can be explained by the model. The *P*-value of the lack-of-fit test was 0.4378, which is ideally insignificant. Therefore, the model can be used to predict the actual yield of the antifungal substances with high accuracy. The quadratic term A*D, with a *P*-value less than 0.5, is not significant. After discarding that term, the equation was modified as:

$$Y = 75.21+1.09A+0.69D–2.33A^2–1.47D^2 \quad (4)$$

The matrix was solved using the software, and the optimum concentrations of each variable were calculated. The maximum inhibition rate can theoretically reach 70.28% when glucose and soybean meal concentrations are at 1.56 and 0.82%, respectively. The three-dimensional response surface shown in Figure 1 intuitively reflects the correlation between the two main factors.

Validation experiments

Verification experiments were conducted using the optimum compositions obtained by the SAS system, with A=1.56 and D=0.82, and the results were compared with

Table 4. Designations of the variables in central composite design.

Factors (%)	Level of factor				
	-1.414	-1	0	1	1.414
Glucose	1.32	1.40	1.60	1.80	1.88
Soybean meal	0.76	0.79	0.86	0.93	0.96

Table 5. Central composite design of the two variables and the response values.

Run	Factor		Inhibition rate (%)
	Glucose	Soybean powder	
1	-1	-1	49.52±0.81
2	-1	1	54.53±0.79
3	1	-1	46.92±0.65
4	1	1	40.34±0.87
5	-1.414	0	52.82±1.02
6	1.414	0	66.64±0.97
7	0	-1.414	60.10±0.83
8	0	1.414	63.09±1.12
9	0	0	64.76±1.15
10	0	0	58.28±1.01
11	0	0	66.05±0.73
12	0	0	61.57±0.88
13	0	0	63.15±0.96

Table 6. Analysis of variances (ANOVA) for the model.

Source	Sum of squares	DF	Mean square	F-Value	*P* > F
Model	6023.99	5	1204.87	35.04	< 0.0001
Residual	240.26	7	34.89		
Lack of Fit	110.44	3	36.15	1.13	0.4378
Pure Error	130.82	4	32.96		
Corrected Total	6265.78	12			

R^2=0.9657, Adj R^2= 0.9522, Predicted R^2=0.9257.

those of the initial fermentation medium. The experiment was repeated three times. The average value of the actual inhibition rate was 69.75%, which is basically the same as the predicted value. The mean inhibition rate of the non-optimized media was 30.07%, showing that a 2.32-fold increase was obtained by optimization. These results prove that the model is reliable and feasible and can be used to accurately predict actual response values.

Antifungal activity and biocontrol efficacy of strain ZQ2

In vitro experiments

The antifungal substances produced by ZQ2 displayed strong *in vitro* inhibition against *F. oxysporum*, *F. solani*, *P. piricola*, *A. alternata*, *C. gloeosporioides*, *P. expansum*, and *B. cinerea* after the three-day incubation period (Table 7). The maximum inhibition rate was observed against *A. alternata* (79.02%), followed by *C. gloeosporioides* (66.83%) and *F. solani* (66.41%). Microscopic obser-vations revealed that the mycelia of *R. solani* along the edges of the interaction zone appeared thick and opaque, their cytoplasmic contents accumulated, and parts of the mycelia ruptured. No spores were observed within the field of vision. However, the fungal mycelia on the control plate showed normal, clear, thin radial growth with visible spores.

In vivo tests

The biocontrol efficacy of ZQ2 on different pathogens is summarized in Table 8, which shows that inoculation with pathogens resulted in severe disease (64.44%). However,

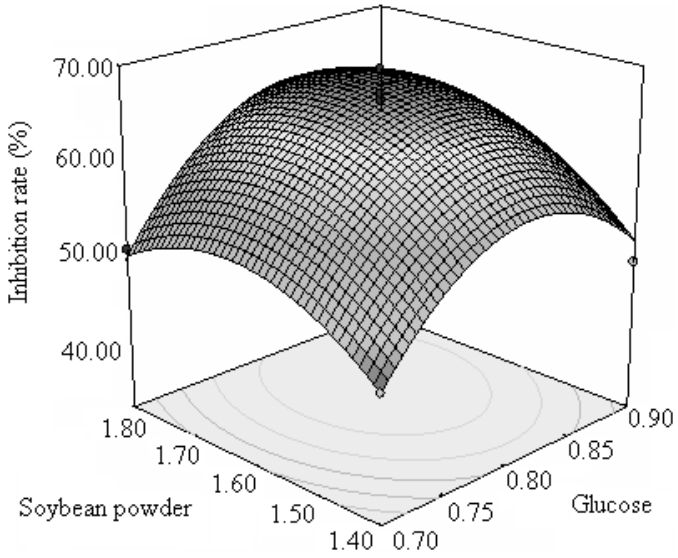

Figure 1. 3D response surface curve of the two variables predicted by the model.

Table 7. Inhibition of phytopathogens using *B. laterosporus* strain ZQ2 antifungal metabolites.

Pathogenic fungi	*F. oxysporum*	*F. solani*	*P. piricola*	*A. alternata*	*C. gloeosporioides*	*P. expansum*	*B. cinerea*
Inhibition rates (%)	64.70	66.41	55.26	79.02	66.83	55.87	61.95

Table 8. Biocontrol efficacy of strain ZQ2 on the infection of R. solani.

Treatment	Disease severity (%)
I	64.44±2.05
II	0.00
III	17.55±0.32
IV	37.69±0.96
V	50.92±1.33
VI	0.00

the application of ZQ2 significantly reduced the degree of damage caused by these pathogens. The results show that ZQ2 effectively protects apple from being infected by *R. solani*. Maximum protection was observed in treatment III. Compared with the control, the percentage of lesion areas on leaves was reduced by more than 46% when inoculation with the bacterium was performed three days before that with the pathogen.

DISCUSSION

Most of the current antibiotic products are already generated by microorganisms; therefore, fermentation is an essential tool in the antibiotic industry, and optimization of the fermentation medium is of great importance (Jinjiang et al., 2010; Nan et al., 2012). In the present work, one-factor-at-a-time experiments were conducted, and five components affecting the antifungal substance production of *B. laterosporus* ZQ2 were preliminarily determined. Combined technologies were then used in medium optimization. Using the PB experiments, medium components with significant effects on the production of antifungal substances were screened and insignificant variables were discarded. The center points of the two main variables for CCD were then determined using a steepest ascent design. The optimum medium with the highest production was finally obtained via RSM. Using the validation experiments, the model constructed by the software was proven to be reliable and feasible in production prediction. Thus, the application of statistical optimization was successful and greatly improved the fermentation medium for *B. laterosporus* ZQ2.

Brevibacillus spp. can produce a wide variety of metabolites with antifungal activity, which can control plant diseases as biocontrol agents (Sunita et al., 2010; Hassi et al., 2012; Prasanna et al., 2013). A number of these active metabolites are fungicidal or fungistatic peptides that are non-ribosomally synthesized by multi-enzyme-catalyzed systems (Jing et al., 2012). The me-

chanisms of action of many antifungal peptides remain largely undetermined, despite the fact that numerous investigations on such have been conducted over the last few decades. Daniel et al. (2010) found that many short cationic peptides accumulate on the cell membrane of fungal hyphae and disturb sterol-rich membrane domains. Thicker hyphae and depolarized cells were observed in micrographs, and most spores did not germinate after antifungal peptide treatment. These findings are very similar to the results of the present study, suggesting that *B. laterosporus* strain ZQ2 may also secrete peptides with similar inhibitory mechanisms against fungi.

The strain ZQ2 significantly inhibited many apple phytopathogens both *in vitro* and *in vivo*, thus showing that it has great potential in agricultural applications. Future works could aim to provide an alternative resource for controlling fungal diseases in apples using *B. laterosporus* ZQ2 as a novel biocontrol agent.

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