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Optimization of *Bacillus aerius* strain JS-786 cell dry mass and its antifungal activity against *Botrytis cinerea* using response surface methodology

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Abstract: The optimization of fermentation conditions is necessary for field application of biological control agents. The present study was designed to optimize the fermentation conditions for the *Bacillus aerius* strain, JS-786 in terms of cell dry mass and its antifungal activity against *Botrytis cinerea* with response surface methodology. A strain of bacteria with strong antifungal activity was isolated from the phyllosphere of tomato plant and identified as *B. aerius* JS-786 based on the sequence homology of its 16S rRNA gene. After the success of preliminary antifungal activity tests, response surface methodology was used to optimize the fermentation conditions (medium pH, gelatin percentage, incubation period, rotatory speed and incubation temperature) to maximize the cell dry mass and antifungal activity against *B. cinerea*. A 2^5 factorial central composite design was employed and multiple response optimization was used to determine the desirability of the operation. The results of regression analysis showed that at the individual level, all of the experimental parameters were significant for cell dry mass; significant results were obtained for antifungal activity pH, incubation period, rotatory speed and incubation temperature. The interactive effect of the incubation period, rotatory speed and incubation temperature was significant. Maximum cell dry mass (8.7 g/L) and inhibition zone (30.4 mm) were obtained at pH 6.4, gelatin 3.2%, incubation period 36.92 h, rotatory speed 163 rpm, and temperature 33.5°C. This study should help to formulate a more rational and cost-effective biological product both in terms of bacterial growth and antifungal activity.

Key words: antifungal activity; *Bacillus aerius*; *Botrytis cinerea*; cell dry mass; response surface methodology

INTRODUCTION

Botrytis cinerea is an important pathogenic fungus that infects at least 235 plant species, including herbaceous annual and perennial plants. It can survive both parasitically and saprophytically on the same wide range of host plants [1-3]. The fungus is especially fatal for the plants grown in a greenhouse due to the humid environment, and it causes high yield losses [1,3]. Global losses caused by this fungus account for 20% of the total yield of affected crops and this cost was estimated to be about 10-100 billion Euro yearly. Other crop losses were recorded as 20% of cupflowers in Holland, 20-25% of strawberry in Spain, 15-40% of vineyards in France and 10-20% of vegetables in China [4-6]. Fungicides are commonly applied to manage this fungus, but the extensive use of synthetic fungicides has created several environmental and economic complications. In addition, it is the main cause of development of resistant strains of plant pathogens against traditional synthetic pesticides [2,7]. In this situation, biological control is the most sustainable approach for the management of plant pathogens. The use of biological agents has gained an important position for integrated plant-disease management at the commercial level [8], and the genus *Bacillus* has showed a broad spectrum of biological control [9,10], with several important attributes, which include crop growth promotion, nitrogen fixation and stimulation of plant nutrient uptake. Furthermore, they control plant diseases by suppressing disease-causing agents, producing antimicrobial secondary metabolites and competing for nutrients (phosphate and iron) with different pathogens [11-13].

Despite the discovery of a broad range of potentially effective biological control agents, only a few are

available as commercial products [14], being not more than 5% of total agricultural pesticides used [8]. This might be due to complications during fermentation and formulation of these agents [14]. The high cost of production and low yield are the main limitations for the commercialization of *Bacillus*-based products for the control of plant pathogens [15]. For industrial scale fermentation, culture conditions are of key importance because they play a significant role in final yield and quality of the product [16]. Therefore the development of cost- and yield-effective culture media for biological control agents is crucial [14].

Fermentation is a multivariate process that makes its optimization a tedious process. The main objectives of process optimization are to maximize quantity and quality, and minimize cost and developmental time by the use of suitable statistical techniques. Selection of critical factors is initially done, and those that prove to be important are then optimized by suitable statistical techniques [17]. Experimental designs based on study of the effect of a single factor while holding others constant are unable to illustrate the interactive effects of all the factors involved; moreover, these are time consuming, costly and require multiple trials to find the optimum levels.

The response surface methodology (RSM) is a factorial design that helps us to find out the relationship between one or two dependent variables with a number of independent variables. RSM is suitable for multifactor experiments and requires fewer trials, and it is very useful for determining the most suitable conditions for any process and forecasting of responses. The method is commonly used in the optimization of microbial culture conditions [18,19].

This study is a very important starting point for the development of a biological control agent for the control of *B. cinerea*, its identification, and optimization of its culture conditions for maximizing biomass and metabolites activity against *B. cinerea* by using RSM.

MATERIALS AND METHODS

Microbial isolation and selection of the biocontrol agent

The phylloplane bacteria were isolated from leaves of tomato plant from Liaoning (China). For isolation purposes, tomato plant leaves were collected from different cities in Liaoning Province of P.R. China. Leaf samples were cut into small pieces and shaken with sterilized phosphate buffer saline for 5 min; 100. One hundred µL of the saline buffer solution from each sample were transferred to nutrient agar (NA) plates and incubated at 28°C for 24 h. A total of 215 bacterial isolates were selected based on differences in colony morphology and pigment production by visual observations. The dual culture technique was used to test the inhibitory activity of isolates on the growth of *B. cinerea*. Pure cultures of the fungus were initially grown in Petri dishes containing standard PDA (20% potato extract, 2% dextrose and 1.5% agar) medium and incubated at 28°C for 5 days. After this period, 8-mm disks were cut from the edge of actively growing colonies of fungus with the aid of a cork borer. Two plugs were placed at opposite edges of each dish. The bacterial isolates were streaked on the center of the PDA plate at the time of fungi transplanting. After incubation for 5 days at room temperature, the radii of growth inhibition zones of the fungus were measured in two perpendicular directions by a vernier caliper. Out of 215 isolates screened, only one strain showed strong antifungal activity and was selected for further experiments in this study. For long-term storage, the isolate was preserved at -80°C using glycerol.

Identification of the selected antifungal bacterium

The strain JS-786 with strong antifungal activity was selected and identified on the basis of 16S ribosomal RNA (rRNA) gene sequencing analysis and biochemical characterization. Genomic DNA of the selected strain was isolated using the bacterial DNA mini kit (Sangon Biotech Co. Ltd, Shanghai, China) according to manufacturer's instructions. The 16S rRNA gene was amplified by PCR with Taq DNA polymerase and primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- TACGGCTACCTTGTTACGACTT-3'). The conditions for thermal cycling were

as follows: denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was kept at 72°C for 10 min and then cooled to 4°C [20]. The PCR product obtained was sequenced by an automated sequencer ((using an ABI automated sequencer). The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database using the NCBI-BLAST tool. The neighbor joining method was employed to construct a phylogenetic tree using MEGA 5.0 software [21] on the basis of 16S rRNA gene sequence.

Morphology, growth and biochemical studies on viable colonies of the tested bacterium were also performed. Gram staining, motility, endospore formation were tested according to standard methods. The Voges-Proskauer (VP) test was performed using MR-VP medium (0.5% glucose, 0.5% peptone and 0.5% dipotassium hydrogen phosphate in distilled water) and Barritt's reagents A (6% solution of a-naphthol in 100 mL of 95% ethyl alcohol) and B (16% solution of potassium hydroxide in distilled water). Arginine dihydrolase, lysine decarboxylase, urea hydrolysis and indole production tests were performed by using arginine dihydrolase, lysine decarboxylase, Christensen's urea and tryptophan broths, respectively. A methyl red (MR) test was carried out by inoculating the test bacteria on MR-VP broth and the results were determined by adding methyl red solution. An oxidase test was performed by placing a colony of test bacteria on filter paper soaked with tetramethyl-p-phenylenediamine dihydrochloride. The ONPG (O-Nitrophenyl-β-D-galactopyranoside) test for β-galactosidase was performed as described by Flores et al. [22]. Hydrolysis of esculin was checked by culturing the bacteria on bile-esculin agar slant. Gelatin hydrolysis, nitrate reduction and citrate utilization ability of the test bacteria were determined by culturing the bacteria on nutrient gelatin medium, nitrate broth and Simmons citrate agar slant, respectively. Carbohydrate fermentation ability was tested using different carbohydrate (D- or L-arabinose, D-mannitol, D-mannose, D-xylose, fructose, galactose, lactose and sucrose) semisolid media tubes.

Preparation of bacterial cell suspension and cell-free supernatant

The bacterium test culture was grown in LB liquid medium, shaken at 200 rpm for 72 h at 30°C, and then centrifuged at 10000 rpm for 15 min to separate the bacterial cells and the supernatant. The bacterial cells were washed twice in 0.9% NaCl by centrifugation (15min at 10000 rpm and 4°C) and resuspended in sterile distilled water to give a final concentration of 1×10^9 CFU/mL before application. The cell-free supernatant was filtered through 0.45-μm pore size membrane and stored at 4°C until further use.

Detached leaf assay

Fully expanded top leaves were detached from 7-week-old tomato plants grown in the greenhouse. The detached portion of the midrib of each leaf was scratched with a moist cotton plug and placed in a Petri dish (150 mm) on moist filter paper. The bacterial cell suspension and cell-free supernatant of JS-786 were sprayed on leaf petioles as separate treatments. Soon after bacterial spraying, tomato leaf petioles were challenged with a pathogen by placing a cultural disk of *B. cinerea* in the center of each leaf petiole. The Petri dish was placed in a growth chamber with a 14 h light and 10 h darkness cycle at 22°C. The leaf petioles sprayed with distilled water served as non-treated control. Treatments were arranged in a randomized complete block with six replications and 10 leaf petioles in each replication. After one week, the lesion diameter on each leaf petiole was measured in two perpendicular directions and expressed in mm.

Antifungal activity in greenhouse pot trials

Tomato plants were grown for 5 weeks in 10-cm plastic pots containing growth medium. After this period, the cell suspension (10^9 CFU/mL) and cell-free supernatant of strain JS-786 were applied separately as foliar spray. At the same time after treatment with bacterial cell suspension and cell-free supernatants, 15 leaf petioles (5 top, 5 central and 5 lower) from each tomato plant were challenged with *B. cinerea* by placing a culture disk in the center of each selected leaf petiole. The plants sprayed with distilled water served

Table 1. Experimental design and results for the optimization of cell dry mass and inhibition zone for strain JS-786.

						Cell dry mass (g/L)			Inhibition zone (mm)		
Run	X_1	X_2	X3	X_4	X_5	observed	predicted	residual	observed	predicted	residual
1	6.5	3.0	35.0	150.0	34.0	8.45	9.19	-0.74	30.20	30.80	-0.60
2	6.5	3.0	35.0	150.0	34.0	8.95	9.19	-0.24	28.60	30.80	-2.20
3	6.5	3.0	35.0	150.0	34.0	9.25	9.19	0.06	30.80	30.80	0.00
4	7.0	2.0	40.0	100.0	40.0	3.80	4.08	-0.28	10.70	11.29	-0.59
5	7.0	4.0	40.0	200.0	40.0	5.30	5.22	0.08	12.60	12.81	-0.21
6	6.0	2.0	30.0	100.0	40.0	4.95	4.69	0.26	12.50	12.70	-0.20
7	5.5	3.0	35.0	150.0	34.0	6.65	6.45	0.20	18.50	17.62	0.88
8	7.0	4.0	40.0	100.0	28.0	5.10	5.34	-0.24	16.00	16.14	-0.14
9	6.5	3.0	35.0	150.0	34.0	9.55	9.19	0.36	31.50	30.80	0.70
10	6.0	4.0	30.0	200.0	40.0	6.20	6.37	-0.17	19.60	20.29	-0.69
11	7.0	4.0	30.0	100.0	40.0	3.30	3.98	-0.68	8.30	8.71	-0.41
12	6.0	4.0	40.0	100.0	40.0	4.75	4.86	-0.11	13.10	12.52	0.58
13	6.5	3.0	25.0	150.0	34.0	6.60	6.71	-0.11	20.30	19.46	0.84
14	6.5	3.0	35.0	150.0	46.0	6.10	5.88	0.22	18.60	17.82	0.78
15	6.0	2.0	40.0	200.0	40.0	6.10	5.93	0.17	12.60	13.38	-0.78
16	7.5	3.0	35.0	150.0	34.0	4.75	4.96	-0.21	12.70	12.39	0.31
17	6.0	2.0	30.0	200.0	28.0	5.40	5.42	-0.02	14.50	15.48	-0.98
18	6.0	4.0	40.0	200.0	28.0	6.75	6.68	0.07	20.30	21.66	-1.36
19	6.5	3.0	35.0	150.0	34.0	9.90	9.19	0.71	33.20	30.80	2.40
20	6.5	5.0	35.0	150.0	34.0	5.25	5.10	0.15	13.50	13.14	0.36
21	7.0	4.0	30.0	200.0	28.0	5.05	4.70	0.35	14.80	14.92	-0.12
22	6.5	3.0	45.0	150.0	34.0	8.10	8.07	0.03	26.50	24.79	1.71
23	7.0	2.0	30.0	100.0	28.0	3.40	3.54	-0.14	6.40	7.33	-0.93
24	6.5	3.0	35.0	250.0	34.0	6.25	6.80	-0.55	24.10	22.66	1.44
25	6.5	3.0	35.0	150.0	22.0	5.80	6.18	-0.38	22.30	21.30	1.00
26	6.0	2.0	40.0	100.0	28.0	5.70	6.05	-0.35	20.60	20.14	0.46
27	6.5	3.0	35.0	150.0	34.0	9.35	9.19	0.16	29.70	30.80	-1.10
28	6.5	1.0	35.0	150.0	34.0	5.25	5.03	0.22	12.30	12.48	-0.18
29	6.0	4.0	30.0	100.0	28.0	4.25	4.32	-0.07	8.90	8.56	0.34
30	6.5	3.0	35.0	50.00	34.0	5.40	4.56	0.84	15.60	14.22	1.38
31	7.0	2.0	30.0	200.0	40.0	5.90	5.59	0.31	17.20	15.63	1.57
32	7.0	2.0	40.0	200.0	28.0	5.90	5.90	0.00	17.20	17.00	0.20

X_1 – pH of medium pH; X_2 – gelatin (%); X_3 – incubation period (h); X_4 – rotatory speed (rpm); X_5 – incubation temperature (°C)

as non-treated control. Treatments were arranged in a randomized complete block with six replications of each treatment and five plants per replication.

Culture medium and fermentation conditions

The initial inoculum of strain JS-786 was prepared by transferring a single colony to a 250-mL conical flask containing 100 mL of basal medium (CIB medium) composed of $MnSO_4.4H_2O$, 0.042 g/L; $CaCl_2$, 0.031 g/L; KH_2PO_4, 0.5 g/L; yeast extract, 8.0 g/L; $MgSO_4.7H_2O$, 4.0 g/L; glucose, 8.0 g/L; K_2HPO_4, 0.5 g/L, and $(NH_4)_2SO_4$, 1.0 g/L, and shaken for 12 h at 28°C [23-25]. Then, 10% (v/v) of the seed culture was added to 250-mL conical flasks containing 100 mL of the experimental medium consisting of the basal medium adjusted according to experimental design (Table 1). The cultures were incubated at different temperatures, using different rotatory speeds for different incubation periods, as determined by experimental design (Table 1).

Optimization of fermentation conditions

The single variable approach was used to identify parameters with significant effects on cell dry mass and

antifungal activity of the selected strain. Temperature, initial pH, incubation period, rotatory speed and gelatin percentage had significant effects on dry cell mass and antifungal activity of the isolated strain. These factors were further studied according to the central composite tool of RSM to fit a second order polynomial model using MINITAB17 software (Minitab Inc., State College, PA, USA). The crucial factors involved in the study and their levels are given in Table 1. A total of 32 triplicated experiments were carried out simultaneously (Table 1).

Estimation of cell dry mass

Cells were harvested by centrifugation at 10000 rpm for 20 min. The cell pellet was washed with phosphate buffer and recentrifuged. After that the cell pellet was dried to constant mass in an oven at 80°C for 24 h.

Estimation of antifungal activity

The cultures were centrifuged at 10000 rpm for 20 min and the supernatants were used to evaluate antifungal activity. The spores of *B. cinerea* were washed from the surface of PDA with sterile saline containing 0.01% Tween 20 (v/v). The spores were separated from hyphal fragments by passing the solution obtained after washing through 4 consecutive sterile absorbent cotton wool plugs. Antifungal activity was determined by the Oxford cup plate assay system [26]. The PDA medium (70 mL) was heated until completely melted, slowly cooled to 50°C, and rapidly mixed with 8 mL of a spore suspension of *B. cinerea* (1×10^7). After the medium in the Petri dishes solidified, Oxford cups were put onto the medium surface and the supernatant (150 µL) was added into each Oxford cup. The antifungal activity was determined by measuring the diameter of inhibition zones after incubating the dishes for 5 days at 28°C.

Data analysis

The central values selected for the experimental design were medium pH, 6.5; gelatin, 3%; incubation period of 35 h; rotatory speed 150 rpm and incubation temperature, 34°C. For computing the regression equation, the experimental variables were coded according to equation 1:

$$xi = \frac{Xi - \bar{X}}{\Delta xi} \qquad i = 1, 2, 3, \ldots, k$$

where xi is the independent variable coded value, Xi the independent variable real value, $\bar{X}$ the independent variable real value on the central point and Δxi the step change value.

The response variables (cell dry mass and inhibition zone) were fitted by a second order model in order to correlate the response variables to the independent variables [24]. The general form of the second degree polynomial equation is

$$Y = b_\circ + \sum_i bixi + \sum_i \sum_j bijxixj + \sum biixi^2$$

where Y is the measured response, $b_\circ$ is the intercept, bi, bij and bii are interaction coefficients of linear, second order and quadratic terms, respectively, and xi and xj are variables. The variable xixj represents the first order interaction between xi and xj (i<j).

Statistical analysis of the model was performed using analysis of variance (ANOVA). This analysis included the coefficient of determination R^2 that measures the goodness of fit of regression model, correlation coefficient R, Fisher's F-test (overall model significance), and its associated probability P(F). The analysis also included the Student's t-value for the estimated coefficients and associated probabilities, P(t) [24]. For each variable, quadratic models were represented as surface plots (cell dry mass) and contour plots (inhibition zone).

RESULTS

Identification of the isolate

The isolate JS-786 was identified as *Bacillus aerius* based on physiological, biochemical and 16S rRNA sequence comparison with the sequences deposited in the NCBI database. The phylogenetic tree (Fig. 1) revealed that isolate JS-786 belonged to the genus *Bacillus* and it has highest similarity (99%) with *B. aerius*. This is in agreement with the physiological and biochemical characteristics of isolate JS-786 (Table 2).

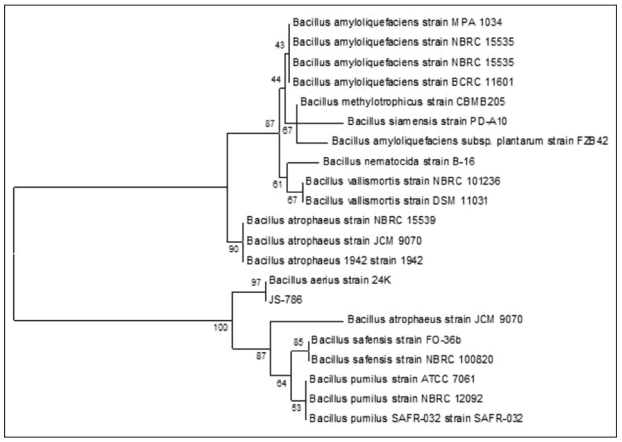

Fig. 1. Phylogenetic tree showing relationships of taxa related to strain JS-786 built by comparative analysis of 16S rRNA gene sequences following the UPGMA method. The tree was built by the neighbor-joining method based on a bootstrap analysis of 1000 replicates. The tree is drawn to scale, with the branch length in the same units as those of the evolutionary distances used to surmise the phylogenetic dendrogram. Bootstrap values (stated as percentages of 1000 repetitions) >90% are specified at nodes. 16S rRNA sequence of strain was deposited to NCBI and waiting for the accession number.

Table 2. Results of physiological and biochemical tests of strain JS-786.

Test	Results	Test	Results
Gram staining	+	Esculin hydrolysis	+
Motility	+	Gelatin hydrolysis	+
Endospore formation	+	Nitrate reduction	+
pH rang	4-10	Starch hydrolysis	+
Optimum pH	6.5	Voges-Proskauer test	+
Temperature range	8-40°C	Citrate utilization	+
Optimum temperature	32°C	Acid production from:	
Arginine dihydrolase	-	D-or L-arabinose	+
Lysine decarboxylase	-	D-mannitol	+
Urea hydrolysis	-	D-mannose	+
Indole production	-	D-xylose	+
Methyl red test	-	Fructose	+
Oxidase	+	Galactose	+
Catalase	+	Lactose	+
Beta galactosidase	+	Sucrose	+

Antifungal activity

The antifungal activity of the strain JS-786 was first tested by dual-culture technique where it showed a strong inhibitory effect against *B. cinerea* (Figs. 2 A and 3 A). The strain was subsequently evaluated for its antifungal activity against *B. cinerea* on detached tomato leaves under laboratory conditions and on

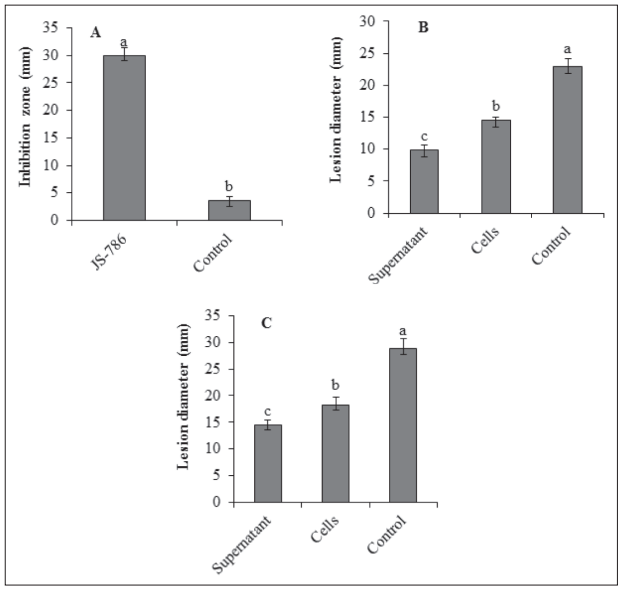

Fig. 2. Results for antifungal activity of strain JS-786 against *B. cinerea*. **A** – The results for antifungal activity in dual culture technique, inhibitions zones for JS-786 and sterilized distilled water as negative control were recorded as 30.03 and 3.57 mm, respectively. **B** – The results for antifungal activity on detached tomato leaves minimum diameter were recorded in cell-free supernatant followed by bacterial cell suspensions and sterilized distilled water as negative control (9.87, 14.53 and 22.86 mm, respectively). **C** – Lesion diameter under greenhouse conditions, the minimum lesion diameter was recorded in cell-free supernatant (14.5 mm) followed by bacterial cell suspension and sterilized distilled water as negative control (18.30 and 28.8 mm, respectively). Data corresponding to treatments labelled with different letters are significantly different (P=0.05; Fisher's least significant difference test); the bars show the standard error.

plants under greenhouse conditions. The strain JS-786 showed strong antifungal activity both on detached leaves (Figs. 2 B and 4 D-F) and under greenhouse conditions (Figs. 2 C and 4 A-C).

Optimization of culture conditions for cell dry mass and antifungal activity

Cell dry mass and antifungal activity was optimized by central composite design. Five factors (medium pH, gelatin percentage, incubation period, rotatory speed and incubation temperature) were optimized using central composite design. Thirty two experiments for the above mentioned variables were performed with 16, 6, and 10 replications at cube, center and axial points, respectively. All experiments were conducted in triplicate and their means are given in Table 1. The interactive effect of culture parameters on

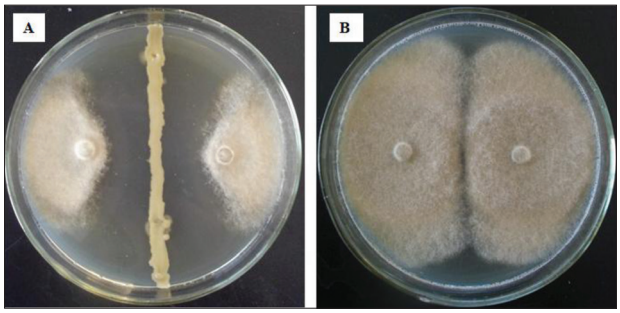

Fig. 3. Antagonistic activity of strain JS-786 against *B. cinerea* by dual-culture technique. **A** – Strain JS-786. **B** – Sterilized distilled water (control).

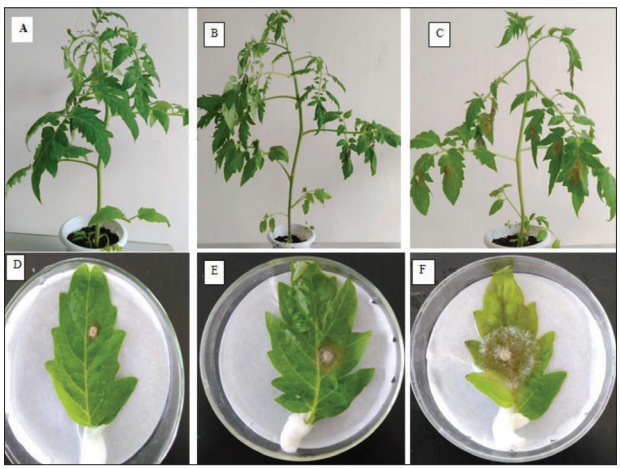

Fig. 4. Antifungal activity of strain JS-786 against *B. cinerea*. Antifungal activity under greenhouse conditions on tomato plants (**A-C**) and on detached tomato leaves under laboratory conditions (**D-F**). **A** and **D** – treatment of the cell free supernatant; **B** and **E** – treatment of the bacterial cell suspension. **C** and **F** – treatment of sterilized distilled water (control).

cell dry mass and antifungal activity against *B. cinerea* was studied by RSM. Multiple regression was employed for further analysis of all the experimental parameters. The F test was used to test the statistical significance of models, and showed that the regression models for both cell dry mass and antifungal activity were significant at 95% confidence level as exhibited by very high F values ($102.26_{\text{cell dry mass}}$ and $323.88_{\text{inhibition zone}}$) and low P values ($0.000_{\text{cell dry mass}}$ and $0.000_{\text{inhibition zone}}$). This shows that the interactive effects of all the parameters significantly contributed to the enhancement of the cell dry mass and antifungal activity. The linear, square and quadratic coefficients of most of the variables were considered significant at P>0.05. As individual factors, medium pH, temperature, gelatin percentages, rotatory speed and incubation period showed significant

Table 3. Full second order polynomial regression analysis results of cell dry mass of strain JS-786.

Model term	Coeff.	SE Coeff.	T-Value	P-Value
X_1	-0.3729	0.0506	-7.37	0.000
X_2	0.0174	0.0506	0.34	0.732
X_3	0.3410	0.0506	6.74	0.000
X_4	0.5590	0.0506	11.05	0.000
X_5	-0.0771	0.0506	-1.52	0.132
X_1^2	-0.8722	0.0458	-19.05	0.000
X_2^2	-1.0326	0.0458	-22.55	0.000
X_3^2	-0.4513	0.0458	-9.86	0.000
X_4^2	-0.8784	0.0458	-19.19	0.000
X_5^2	-0.7909	0.0458	-17.28	0.000
X_3X_4	-0.1344	0.0620	-2.17	0.033
X_3X_5	-0.4094	0.0620	-6.60	0.000
X_4X_5	0.1281	0.0620	2.07	0.042

X_1 – pH of medium pH; X_2 – gelatin (%); X_3 – incubation period (h); X_4 – rotatory speed (rpm); X_5 – incubation temperature (°C)

Table 4. Full second order polynomial regression analysis results of inhibition zone from strain JS-786.

Model term	Coeff.	SE Coeff.	T-Value	P-Value
X_1	-1.306	0.128	-10.23	0.000
X_2	0.165	0.128	1.29	0.201
X_3	1.333	0.128	10.44	0.000
X_4	2.110	0.128	16.53	0.000
X_5	-0.870	0.128	-6.81	0.000
X_1^2	-3.948	0.116	-34.18	0.000
X_2^2	-4.498	0.116	-38.94	0.000
X_3^2	-2.168	0.116	-18.77	0.000
X_4^2	-3.089	0.116	-26.74	0.000
X_5^2	-2.810	0.116	-24.33	0.000
X_2X_4	0.857	0.156	5.48	0.000
X_3X_4	-1.516	0.156	-9.69	0.000
X_3X_5	-2.249	0.156	-14.38	0.000

X_1 – pH of medium pH; X_2 – gelatin (%); X_3 – incubation period (h); X_4 – rotatory speed (rpm); X_5 – incubation temperature (°C)

effects on both cell dry mass and antifungal activity (Tables 3 and 4). The interaction between gelatin percentage and rotatory speed showed a significant positive effect on antifungal activity. Similarly, the interaction between rotatory speed and temperature was positively significant for cell dry mass. The interactive effect between incubation period and rotatory

speed, and incubation period and temperature was significantly negative for both cell dry mass and antifungal activity. This indicates that cell dry mass and antifungal activity increased with the initial increase in incubation period, rotatory speed and incubation temperature, but after a certain level any further increase in these factors reduced the responses. The experimental results of optimization of cell dry mass and antifungal activity were fit to second order polynomial equations (Equation 3 and 4).

$$Y_1 = -4.373 + 0.8921X_1 + 0.12426X_2 + 0.03753X_3 + 0.002418X_4 + 0.03789X_5 - 0.06977X_1^2 - 0.020652X_2^2 - 0.000361X_3^2 - 0.000007X_4^2 - 0.000439X_5^2 - 0.000011X_3X_4 - 0.000273X_3X_5 + 0.000009X_4X_5 \quad (1)$$

$$Y_2 = -1008.7 + 202.66X_1 + 24.5786X_2 + 9.796X_3 + 0.5737X_4 + 7.787X_5 - 15.790X_1^2 - 4.498X_2^2 - 0.08673X_3^2 - 0.001236X_4^2 - 0.07806X_5^2 - 0.01715X_2X_4 - 0.006062X_3X_4 + 0.07497X_3X_5 \quad (2)$$

where Y_1 and Y_2 are predicted values for cell dry mass and inhibition zone, respectively, while X_1, X_2, X_3, X_4 and X_5 are medium pH, gelatin percentage, incubation period, rotatory speed and incubation temperature, respectively.

The goodness of the models was evaluated by coefficients of determination (R_1^2>0.95, R_2^2>0.98), which showed that the model for antifungal activity explained more than 95% of the total variability in antifungal activity, and the model for cell dry mass explained more than 98% of total variability in cell dry mass. The adjusted coefficients of determinations were also considerably high (adjusted R_1^2>0.94, and adjusted R_2^2>0.97) to support the soundness of both the models.

Further, response surface and contour plots based on predictive models were constructed to explain the independent and interactive effects of each variable on response variables. 3D response surface graphs of cell dry mass and the 2D contour graphs of inhibition zone were plotted for each pair of variables while remaining fixed at other central point(s) (Figs. 5 and 6). From these plots, the optimum levels of different independent variables and the corresponding responses could be forecasted. It is apparent that cell dry mass and inhibition zone initially increases as the levels of independent variables (medium pH, gelatin percentage, incubation period, rotatory speed, and incubation temperature) increase, while after a certain level any further increase in their values resulted in decreased cell dry mass and inhibition zone.

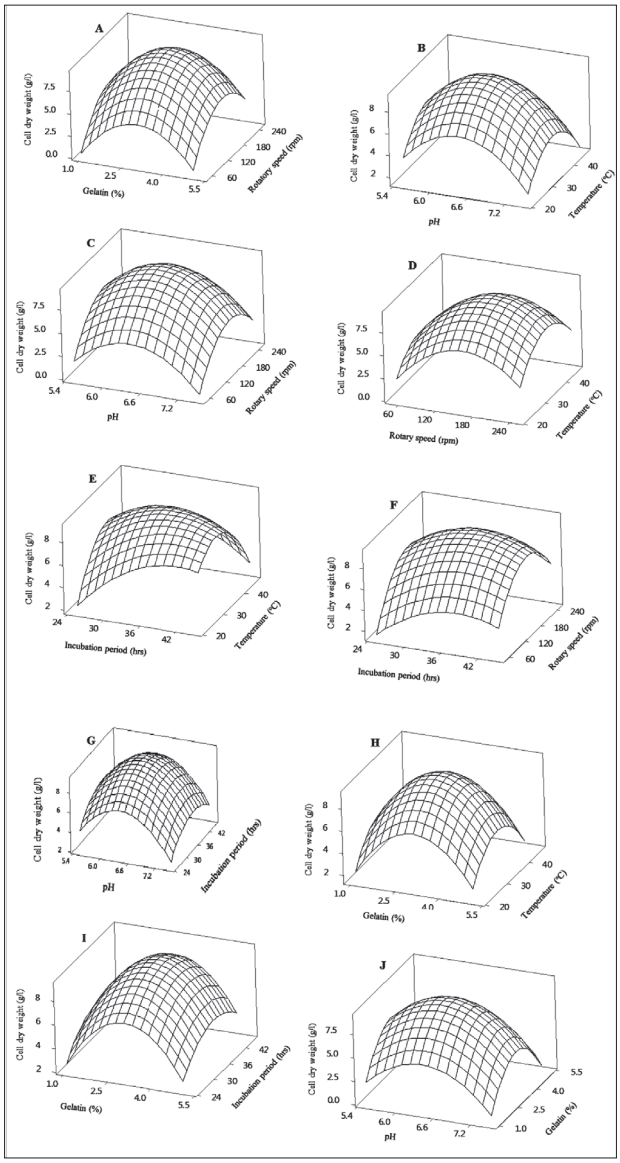

Fig. 5. Response surface plots, defined by the model Y_1 fitted from the results of the central composite design (CCD) representing interactive effect of selected variables on cell dry mass. **A** – Effect of gelatin percentage and rotatory speed. **B** – Effect of medium pH and incubation temperature. **C** – Effect of medium pH and rotatory speed. **D** – Effect of rotatory speed and incubation temperature. **E** – Eeffect of incubation period and temperature. **F** – Effect of incubation period and rotatory speed. **G** – Effect of medium pH and incubation period. **H** – Effect of gelatin percentage and incubation temperature. **I** – Effect of gelatin percentage and incubation period. **J** –Effect of medium pH and gelatin percentage.

After validating independent models, the response optimization by means of desirability function in MINITAB 17 software was used to find the optimum levels of all the parameters to produce the highest val-

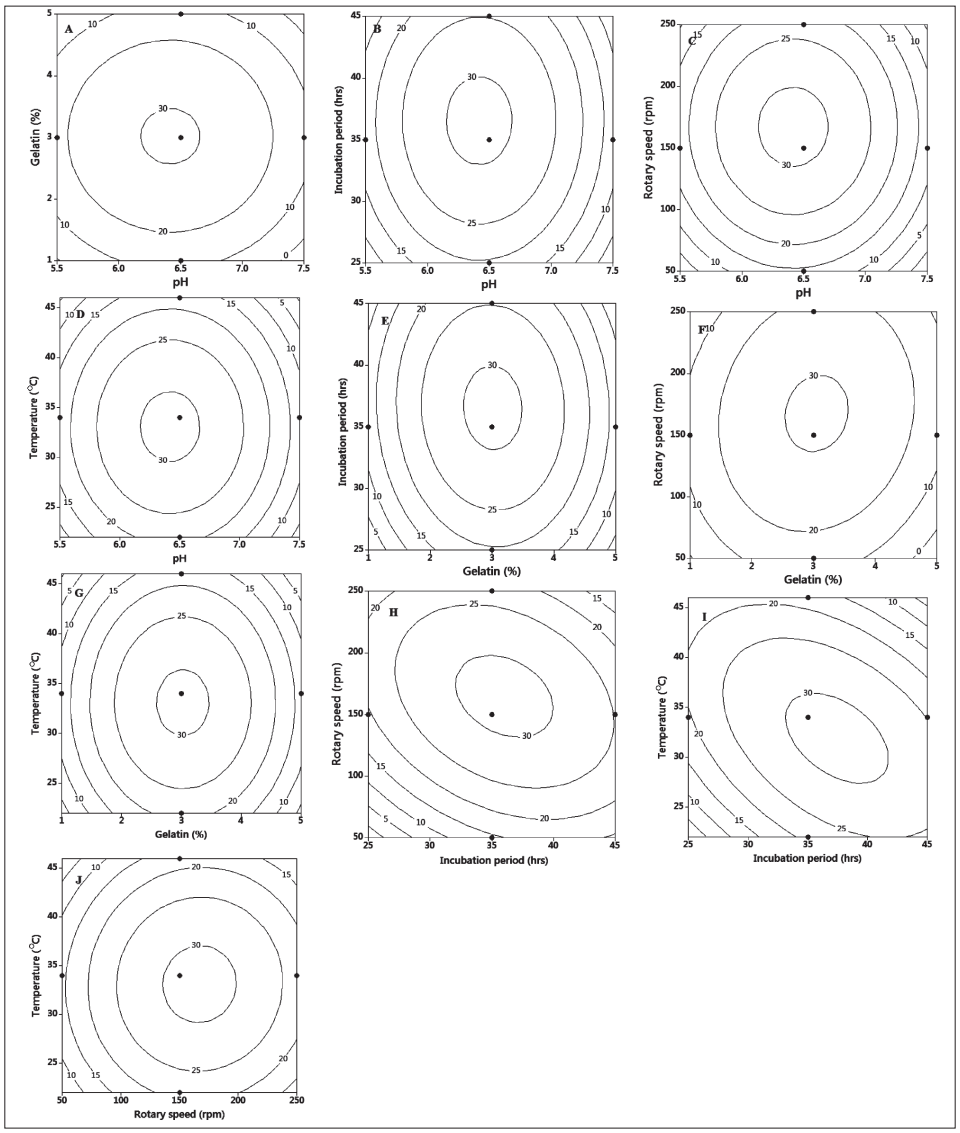

Fig. 6. Contour plots of inhibition zone (mm) of strain JS-786 against *Botrytis cinerea*. **A** – Effect of medium pH and gelatin percentage. **B** – Effect of medium pH and incubation period. **C** – Effect of medium pH and rotatory speed. **D** – Effect of medium pH and incubation temperature. **E** – Effect of gelatin percentage and incubation period. **F** – Effect of gelatin percentage and rotatory speed. **G** – Effect of gelatin percentage and incubation temperature. **H** – Effect of incubation period and rotatory speed. I – Effect of incubation period and temperature. **J** – Effect of rotatory speed and incubation temperature.

ues for both cell dry mass and antifungal activity. The optimization analysis predicted a maximum 9.3824 g/L of cell dry mass and 31.55 mm inhibition zone at pH 6.4, gelatin 3.2%, incubation period 36.92 h, rotatory speed 163 rpm and temperature 33.5°C, with the desirability of more than 96%. The models were verified by conducting experiments using optimized conditions, and the maximum obtained values for cell dry mass and inhibition zone were 8.7 g/L and 30.4 mm, respectively (Fig. 7). This was in close agreement with the predicted values.

DISCUSSION

In recent years, many studies have demonstrated problems arising from the presence of pesticide residues in the environment, foods and feeds [27]. This has led to restrictions and the reduction of availability of some

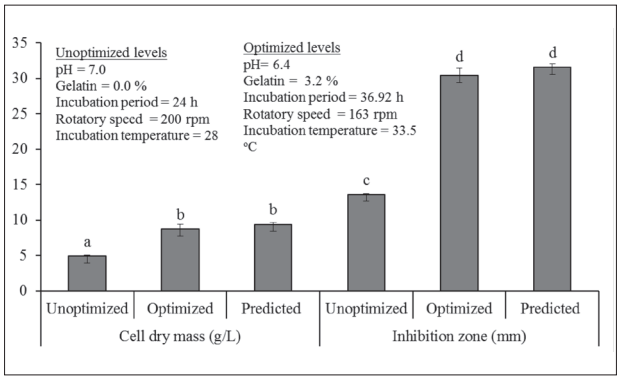

Fig. 7. Experimental confirmation of optimized conditions for cell dry mass and inhibition zone of strain JS-786.

chemical fungicides previously used to control disease in plants and spoilage of their products used for food. Alternative methods to control these pathogens and spoilage organisms are now being investigated. This study was mainly designed to identify the biological control agent from the tomato phyllosphere and to provide optimal fermentation conditions for mass production. A total of 215 bacterial strains were isolated from tomato plant leaves collected from different locations in Liaoning, China. After an initial fungal test, one bacterial isolate showed very strong antifungal activity and was identified as *B. aerius*. Furthermore, this study focused on optimization of fermentation conditions of the isolated biocontrol agent through the central composite design and response surface methodology. Culture conditions play a key role and could also be optimized to gain the maximum benefit in terms of quality and quantity. RSM permitted the determination of the best culture conditions with the highest responses, and it is cost-effective and time-saving. In the present investigation, appropriate models were developed to define the response relating to cell dry mass and inhibition zone. The validations of both models were confirmed by comparing the experimental values and predicted values at the optimum points. The optimization technique allowed a significant enhancement in cell dry mass and inhibition zone produced by the strain JS-786 against *B. cinerea*.

The growth of aerobic bacteria, such as *B. aerius*, as well as the multiplication and production of antifungal substances are thought to be oxygen-dependent [28,29]. Therefore, the amount of dissolved oxygen is an important fermentation parameter [30]. The cell dry mass and inhibition zone produced by strain JS-786 were significantly influenced by the concentration of dissolved oxygen in the culture medium. Previous findings suggested that maintaining the dissolved oxygen concentration above 30% improved cell dry mass and antifungal activity by 24% and 7%, respectively, as compared with culture medium without a dissolved oxygen-maintaining arrangement [31]. Fermentation of bacteria in flasks is strongly correlated with the speed of flask rotation. Increasing the speed could enhance the level of dissolved oxygen in the fermentation medium. The cell dry mass and inhibition zone increased as the flask rotation increased, showing that a higher level of dissolved oxygen favored cell dry mass and antifungal activity, and these results are in line with previous findings [25]. The maximum cell dry mass and inhibition zone of the strain JS-786 were at 3.2% of gelatin. Further increase in gelatin percentage caused an increase in the duration of the lag phase of the bacteria. These results are in agreement with previous findings [32].

Both higher and lower pH have a negative influence on cell dry mass and inhibition zone. Results of the present investigation are in complete agreement with previous findings suggesting that the pH value has a critical role in the production of secondary metabolites, and that its maintenance at medium levels is optimum for neutrophils, such as *B. aerius* [25,31,33,34]. One of the probable reasons for reduced antifungal activity was that the basic enzymes that are crucial for antibiotic production become degenerated or inactivated. Furthermore, it is broadly recognized that low temperature caused the stiffening of membrane lipids, decreased enzyme activities and solubility of solute molecules, while at high temperature, enzymes were denatured, leading to a reduction in bacterial growth, and hence reduced antifungal activity [35-37]. Neither high nor low temperature was favorable to cell dry mass and inhibition zone. These results are in full agreement with previous investigations [25,29]. The temperature influence differs from strain to strain, so it could be rationally speculated that the optimum level of temperature might activate the vital enzymes or induce related gene expression that determine the cell dry mass and antifungal activity of strain JS-786.

To conclude, an RSM modeling technique was employed to optimize fermentation conditions (medium pH, gelatin, incubation period and rotatory speed) to obtain maximum cell dry mass and inhibition zone.

RSM provided accurate predictions for both cell dry mass and inhibition zone with considerably high R^2 values. The cell dry mass and inhibition zone obtained at optimal conditions were significantly higher than under unoptimized conditions. The methods used in the present investigation proved to be powerful tools for optimization and prediction of bacterial fermentation conditions and antifungal activity.

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