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OPTIMIZE THE CULTURE AND FERMENTATION CONDITION OF BIOCONTROL STRAIN PH-120 AGAINST *SCLEROTINIA SCLEROTIORUM*

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Abstract

Sclerotinia sclerotiorum is the main disease in cucumber cultivation in northern China. In the early stage of laboratory, a biocontrol strain *Penicillium halotolerans* (Ph-120) with high-efficiency antagonistic effect on the pathogen was screened out from cucumber rhizosphere soil. In order to improve the antifungal activity of the fermentation broth of Ph-120 strain against *Sclerotinia sclerotiorum*, the culture conditions and fermentation conditions of Ph-120 strain were optimized by single factor test, orthogonal test and response surface methodology. The results showed that the hyphae of Ph-120 strain were affected by different single factors in the following order: temperature > nitrogen source > carbon source > pH; The optimal fermentation conditions were as follows: carbon source amount 24.0g/L, nitrogen source amount 2.2g/L, fermentation temperature 25℃, and pH value 6. The antifungal rate of the fermentation broth against pathogen was 94.6% after optimization in model verification experiment, which was 16.7% higher than that under the optimization condition. The experiment provide a theoretical basis for that development of biopesticide.

Key words: *Sclerotinia sclerotiorumt*; Biocontrol fungi; *Penicillium halotolerans*; Response surface method; Inhibition rate.

Introduction

Cucumber *Sclerotinia sclerotiorum* is one of the destructive diseases in cucumber cultivation in northern China (Pan *et al.,* 2006), and it is caused by *Sclerotinia sclerotiorum* (LIB) debary (Ju, 2001), which can infect cucumber from seedling stage to adult stage (Li, 2020). As the primary infection source of disease cycle and the main form of pathogen survival, *Sclerotinia* can survive for as long as 8 years in adverse environment (Clarkson *et al.,* 2007; Willetts & Wong, 1980). Since *Sclerotinia sclerotiorum* can be transmitted not only through the air but also through the soil, it is very difficult to control it, causing significant economic losses in agricultural production (Qu, 2020). At present, *sclerotinia sclerotiorum* is mainly controlled by chemicals and integrated with agricultural control. With the limited use of chemical fungicides, environmental-friendly microbial fungicides have become a hot research and development, in which fungal fungicides is an effective method that can not be ignored (Li *et al.,* 2006; Zhang *et al.,* 2022). *Penicillium*, as a biocontrol fungus, has the advantages of rapid propagation, strong sporulation ability, low price, wide source of culture medium raw materials, and so on, which provides convenience for the industrial production of biocontrol fungi (Pan *et al.,* 2007). The *Penicillium striatisporum* (Chen *et al.,* 2014) and *Penicillium oxalicum* (Shi *et al.,* 2007) have been shown to have a good inhibition effect on the *Sclerotinia sclerotiorum*.

At present, there is a lack of biocontrol agents that can be promoted and applied in production, and biocontrol fungi for cucumber *Sclerotinia sclerotiorum* are not reported (Campbell, 1947). The biocontrol fungi used for the prevention and treatment of *Sclerotinia sclerotiorum* on other plant are reported most frequently by *Coniothyrium minitans*, *Penicillium*, *Aspergillus* and *Trichoderma* spp., (Li *et al.,* 2006; Kang *et al.,* 2014; Wei *et al.,* 2016). In 1996, when studying the biological control mechanism of *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in Taiwan, it was found that *Bacillus subtilis* and *Pseudomonas cepacia* had strong inhibitory effects (Ma *et al.,* 2006). In 2002, a Swedish company developed a biological agent of *Pseudomonas chlororaphis* to control this pathogen, which was approved by the European Union Plant Science Committee (SCP) (Yang *et al.,* 2014; Alderson & Goodfellow, 1979). Therefore, the fungal inhibitors against cucumber *Sclerotinia sclerotiorum* need to be further studied in order to safely control the disease and protect the environment.

In addition, environmental factors had a significant effect on biocontrol bacteria against *Sclerotinia sclerotiorum*. These environmental factors were mainly concentrated on single factor changes such as single temperature, single pH, and single carbon or nitrogen source.However, different environmental factors may have a comprehensive effect on the inhibition rate of biocontrol bacteria. Appropriate environmental factors can further improve the antifungal effect of microorganisms against *Sclerotinia sclerotiorum* (Costa *et al.,* 2002). Therefore, *Sclerotinia sclerotiorum* was taken as the target disease in this experiment, the culture condition of Ph-120 strain was optimized by single factor test and orthogonal test, and the optimal fermentation parameters of liquid medium was determined by response surface methodology to improve the inhibition effect of the fermentation broth on pathogen. The research on the regulatory effect and biological control mechanism of cucumber *Sclerotinia sclerotiorum* promotes the large-scale application of biological control and chemical or agricultural control methods and provides good data support for establishing a comprehensive control system.

Material and Methods

Test materials: Test strains: *Penicillium halotolerans* Ph-120 and *Sclerotinia sclerotiorum*, all of which were isolated and provided by the Pathological Laboratory of Jilin Agricultural University.

Test medium: PDA medium: 200.0g potato, 20.0g glucose, 15.0g agar and 1.0L distilled water.

PD medium: 200.0g potato, 20.0g glucose, 1.0L distilled water.

Carbon source test medium: 3.0g potassium dihydrogen phosphate, 2.0g yeast extract, 2.0g peptone, 1.5g magnesium sulfate, 15.0g agar, 20.0g carbon source (glucose, sucrose, soluble starch, dextrin powder, and maltose), and 1.0L distilled water.

Nitrogen source test medium: 3.0g potassium phosphate monobasic, 1.5g magnesium sulfate, 20.0g glucose, 15.0g agar, 20.0g nitrogen source (urea, yeast extract powder, peptone, ammonium chloride, ammonium dihydrogen phosphate), 1.0L distilled water.

Experimental method

Optimization of culture conditions of biocontrol strains: The culture condition of the biocontrol strain *Penicillium halotolerans* with good antifungal effect was optimized.

Screening of optimum carbon source, nitrogen source and pH for growth of biocontrol strain: The carbon source, nitrogen source and PDA medium with different pH were respectively poured into culture plates and inoculated with fungal cakes, and three replicates were set for each group, and the plates were cultured in a constant temperature box at 25℃. The hyphal length was measured by "cross method" and the experimental data were recorded.

Screening of optimum temperature for growth of biocontrol strain: The PDA medium was poured into a petri dish and inoculated with fungal cakes. Three replicates were set for each group, and the samples were cultured in thermostatic ovens at 20℃, 25℃, 30℃ and 35℃, respectively, and the test data were recorded.

Orthogonal test: Carbon source, nitrogen source, pH and temperature were taken as single factor, and the specific scheme was shown in table 1 according to the orthogonal experimental principle of four factors and three levels. The optimal combination was obtained by range analysis.

Table 1. Orthogonal experimental design.

Level	Factors			
	Carbon source	Nitrogen source	pH	Temperature (℃)
1	Glucose	Peptone	6	20
2	Sucrose	Yeast extract powder	8	25
3	Dextrin	Urea	9	30

Optimization of fermentation condition of *Penicillium halotolerans*

Screening of fermentation medium: Five groups of culture conditions suitable for the growth of *Penicillium halotolerans* were screened out by orthogonal test, and the fermentation conditions were optimized according to the levels of the selected factors. After seven days, the antifungal activity of the fermentation broth of each combination was measured by growth rate method and the optimal liquid medium was screened out.

Single factor test: The three most important factors affecting hypha growth rate were selected by orthogonal test. With the inhibition rate as the response index, the effects of fermentation temperature, nitrogen source addition and carbon source addition were investigated in sequence by using the single factor rotation method.

Response surface test: On the basis of single factor experiment, Box-Behnken experimental design and analysis were used to optimize the design by three factors and three levels of response surface methodology (Shu *et al.*, 2016).

Results

Optimization of biocontrol fungi culture conditions

Effects of different carbon and nitrogen sources, pH and temperature on hypha growth rate: The growth rate of Ph-120 varied significantly under different carbon and nitrogen sources, pH, and temperature (Fig. 1), the mycelial growth rate in different carbon source media was as follows:glucose > sucrose > blank control > dextrin > soluble starch > maltose,the growth rate of hyphae in different nitrogen sources is as follows:peptone > yeast extract > urea > blank control > diammonium hydrogen phosphate > ammonium chloride, the growth rate of hyphae in different pH media was as follows: 6.0 > 7.0 > 5.0 > 8.0 > 9.0. The hyphal growth rate at different temperatures ranged from high to low: 25℃> 20℃ > 30℃ > 15℃ > 35℃.

Intuitive analysis of the results of orthogonal test for hypha growth: The optimal 3 levels were selected from the above single factor experiments of nitrogen source, carbon source, temperature and pH, and the orthogonal table of 4 factors and 3 levels was established. The experimental analysis showed that the range of temperature was the largest, 0.11, indicating that temperature was the most important factor affecting the hypha growth, followed by nitrogen source, carbon source and pH. The mycelial growth rate of P-120 was analyzed based on the four factors and it was found that the optimal carbon source was dextrin powder, nitrogen source was peptone, pH 7.0, and temperature was 25℃ (Table 2).

Optimization of biocontrol strain fermentation conditions

Screening of fermentation medium: According to the orthogonal results, the hypha growth rates and growth vigor were better under test numbers 1, 2, 4, 7 and 8. The liquid medium was prepared for the antifungal test and the antifungal rate was calculated (Table 3). The results showed that No.2 had the best inhibitory rate of 89.5%, so No. 2 was chosen as the fermentation medium for the experiment.

Single factor experiment of fermentation condition optimization: As shown in Fig. 2, carbon source addition in the medium and fermentation temperature had a greater effect on the inhibition rate of *Sclerotinia sclerotiorum*, while nitrogen source addition had a smaller effect. When the amount of carbon source was 25g/L, the amount of nitrogen source was 2g/L, and the fermentation temperature was 25℃, the respective inhibition rates were the highest.

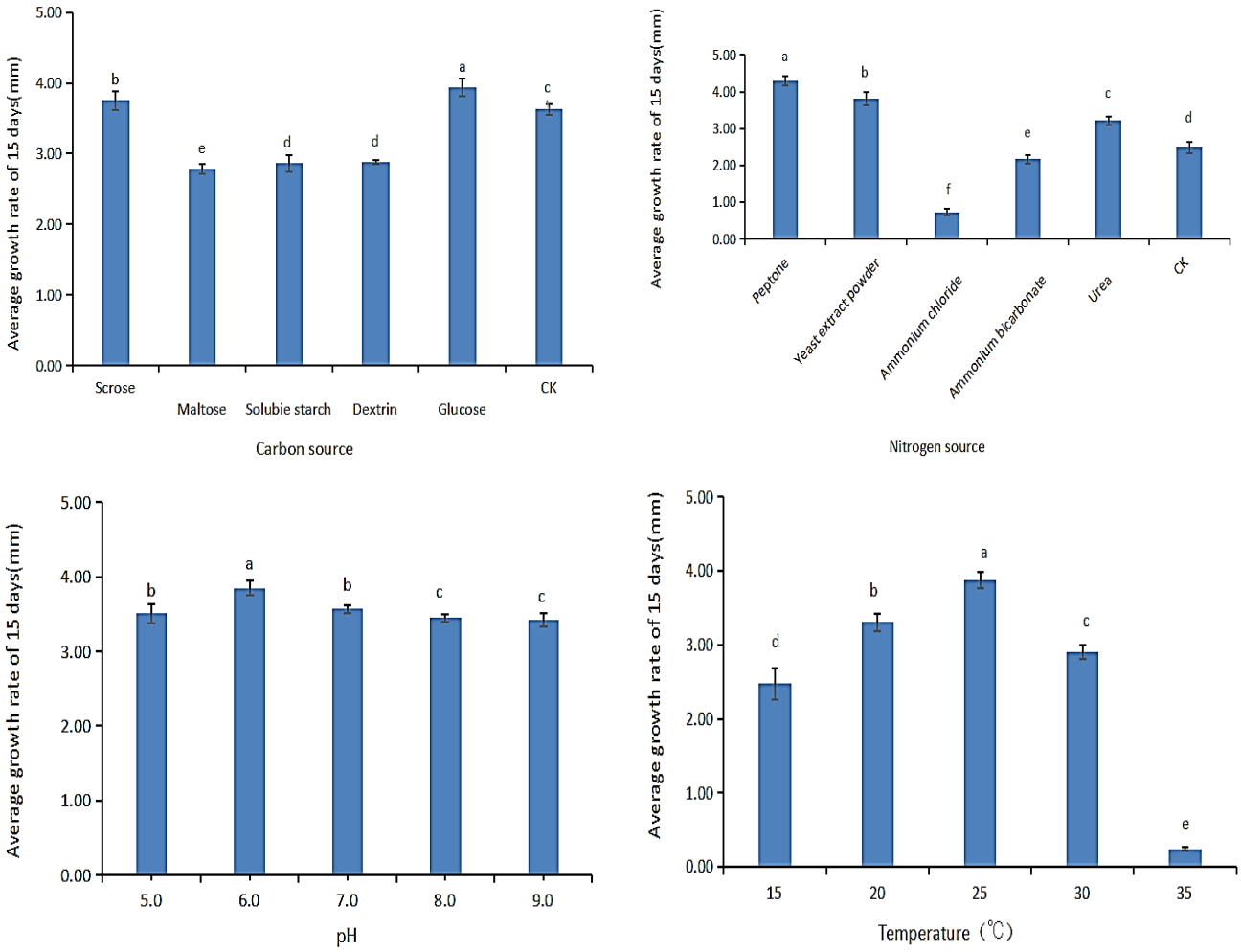

Fig. 1. Effects of different carbon and nitrogen sources, pH and temperature on hypha growth rate of Ph-120 strain.

Table 2. The results of direct-viewing analysis of mycelial growth.

Test number	Carbon source	Nitrogen source	pH	Temperature (℃)	Growth rate (mm/d)	Growth vigor
1	Glucose	Peptone	5.0	20	3.25 ± 0.213	++
2	Glucose	Yeast extract powder	6.0	25	4.04 ± 0.347	+++
3	Glucose	Urea	7.0	30	2.93 ± 0.294	+
4	Sucrose	Peptone	6.0	30	3.37 ± 0.623	++
5	Sucrose	Yeast extract powder	7.0	20	2.61 ± 0.428	+
6	Sucrose	Urea	5.0	25	3.04 ± 1.003	++
7	Dextrin powder	Peptone	7.0	25	4.73 ± 0.541	+++
8	Dextrin powder	Yeast extract powder	5.0	30	3.35 ± 0.622	++
9	Dextrin powder	Urea	6.0	20	2.71 ± 0.084	+
T1	3.03	3.36	2.85	2.55	-	-
T2	2.67	2.97	3.00	3.51	-	-
T3	3.21	2.58	3.06	2.85	-	-
X1	0.34	0.37	0.32	0.28	-	-
X2	0.29	0.33	0.33	0.39	-	-
X3	0.36	0.29	0.34	0.32	-	-
R	0.07	0.08	0.02	0.11	-	-

Note: X represents the average value of the sum of factor test results; ++: Hypha grows stronger; ++: Indicates that the hypha grows normally; +: Hypha growth is weak

Table 3. Inhibition effect of five groups of liquid medium.

Test number	Inhibition rate (%)
1	28.6d
2	89.5a
4	77.2b
7	56.5c
8	19.7d

Response surface methodology for fermentation condition optimization

Model establishment and significance test: On the basis of single factor experiment (Dertli *et al.*, 2016; Chen *et al.*, 2018), Medium No. 2 was selected with antifungal rate (Y) as the response value and carbon source addition (X_1),

nitrogen source addition (X_2) and fermentation temperature (X_3), the three factors that significantly affected the mycelial growth rate, as the investigation factors (Tables 4 and 5).

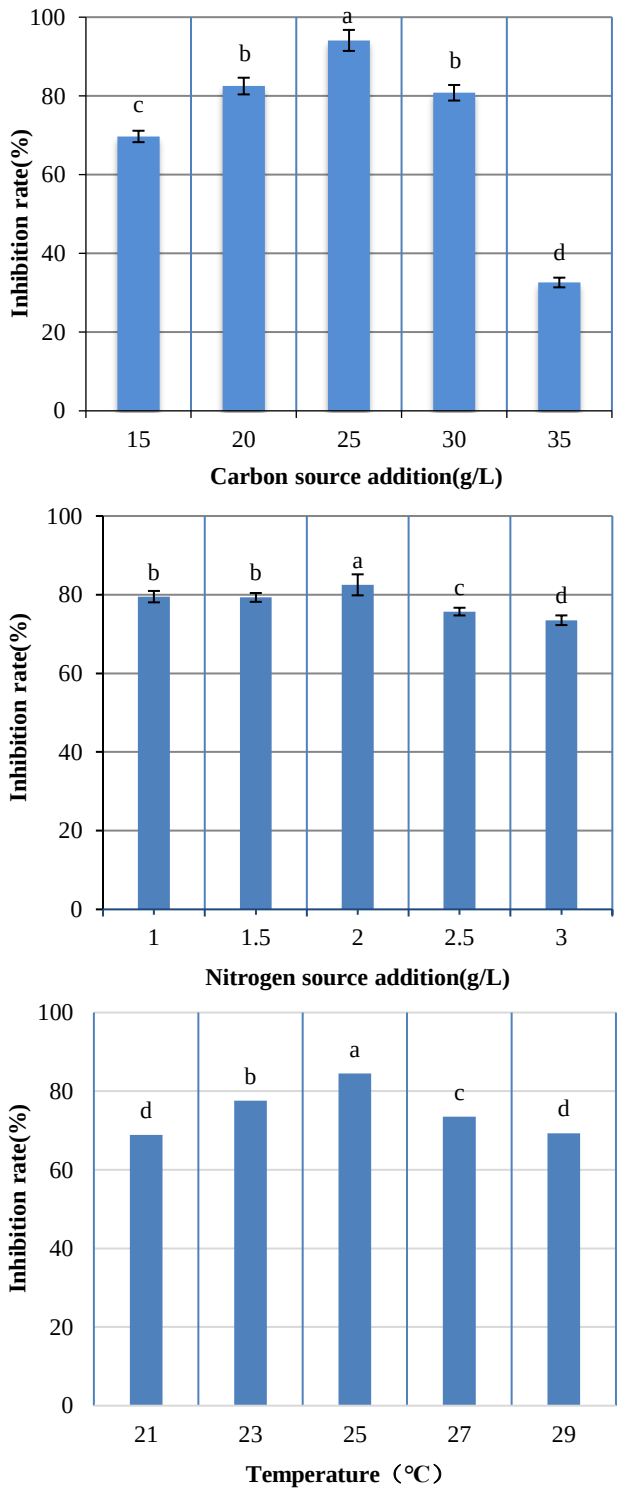

Fig. 2. Effects of different carbon (left), nitrogen (middle) and fermentation temperature (right) on the antibacterial rate.

Table 4. Variables and level of Box-Behnken design.

Variable (factor)	Unit	Code	Level -1	0	1
Carbon source addition	g/L	X_1	20	25	30
Nitrogen source addition	g/L	X_2	1.5	2	2.5
Fermentation temperature	℃	X_3	23	25	27

Table 5. Results and analysis of Box-Behnken experiments fermentation conditions optimization of strain 120

Code	X_1	X_2	X_3	Y Bacteriostatic rate (%)
1	20	1.5	25	84.3
2	30	1.5	25	71.4
3	20	2.5	25	87.6
4	30	2.5	25	81.5
5	20	2.0	23	85.8
6	30	2.0	23	79.0
7	20	2.0	27	77.4
8	30	2.0	27	64.2
9	25	1.5	23	85.9
10	25	2.5	23	89.5
11	25	1.5	27	67.4
12	25	2.5	27	86.1
13	25	2.0	25	96.2
14	25	2.0	25	94.0
15	25	2.0	25	93.0
16	25	2.0	25	92.8
17	25	2.0	25	96.6

The scores in Table 5 were fitted with Design expert V8.0.5 software, and the regression equation was obtained:

$$\text{Inhibition rate} = 94.52-4.87X_1+4.46X_2-5.64X_3+1.70X_1X_2-1.60X_1X_3+3.77X_2X_3-9.47X_1^2-3.85X_2^2-8.45X_3^2$$

Response surface analysis: The response surface curves and contour lines of the interaction between carbon source addition, nitrogen source addition, and fermentation temperature on the inhibition rate are shown in Fig. 3 (Wang *et al.*, 2018; Morita *et al.*, 1985).

The interaction between carbon source addition and nitrogen source addition of *Sclerotinia sclerotiorum* when the fermentation temperature is at the central level, that is, 25℃ (Figs. 3a and 3d). The interaction between carbon source addition and fermentation temperature at a central level of nitrogen source addition, 2.5 g/L (Figs. 3b and 3e). The interaction between nitrogen source addition and fermentation temperature when the carbon source addition is at the central level, that is, 20 g/L (Figs. 3c and 3f). All three figures indicated that the inhibition rate increased first and then decreased with the increase of carbon and nitrogen source addition and fermentation temperature.

The maximum response value was predicted by software. The combination of the factors in the model was the amount of carbon source 24.00g/L, the amount of nitrogen source 2.21g/L, the fermentation temperature 24.56℃, and the predicted maximum inhibitory rate was 96.6%.

Verification test: In order to facilitate the practical operation, the optimal combination of fermentation medium factors was revised as follows: the amount of carbon source added 24.0g/L, the amount of nitrogen source added 2.2g/L, the fermentation temperature of 25℃ and repeated three times to verify the reliability of the response surface design results. Meanwhile, the fermentation results in the original medium were used as the control. The results showed that under the optimal conditions, the inhibition rate was 94.6%, which was 16.7% higher than the 77.3% before optimization.

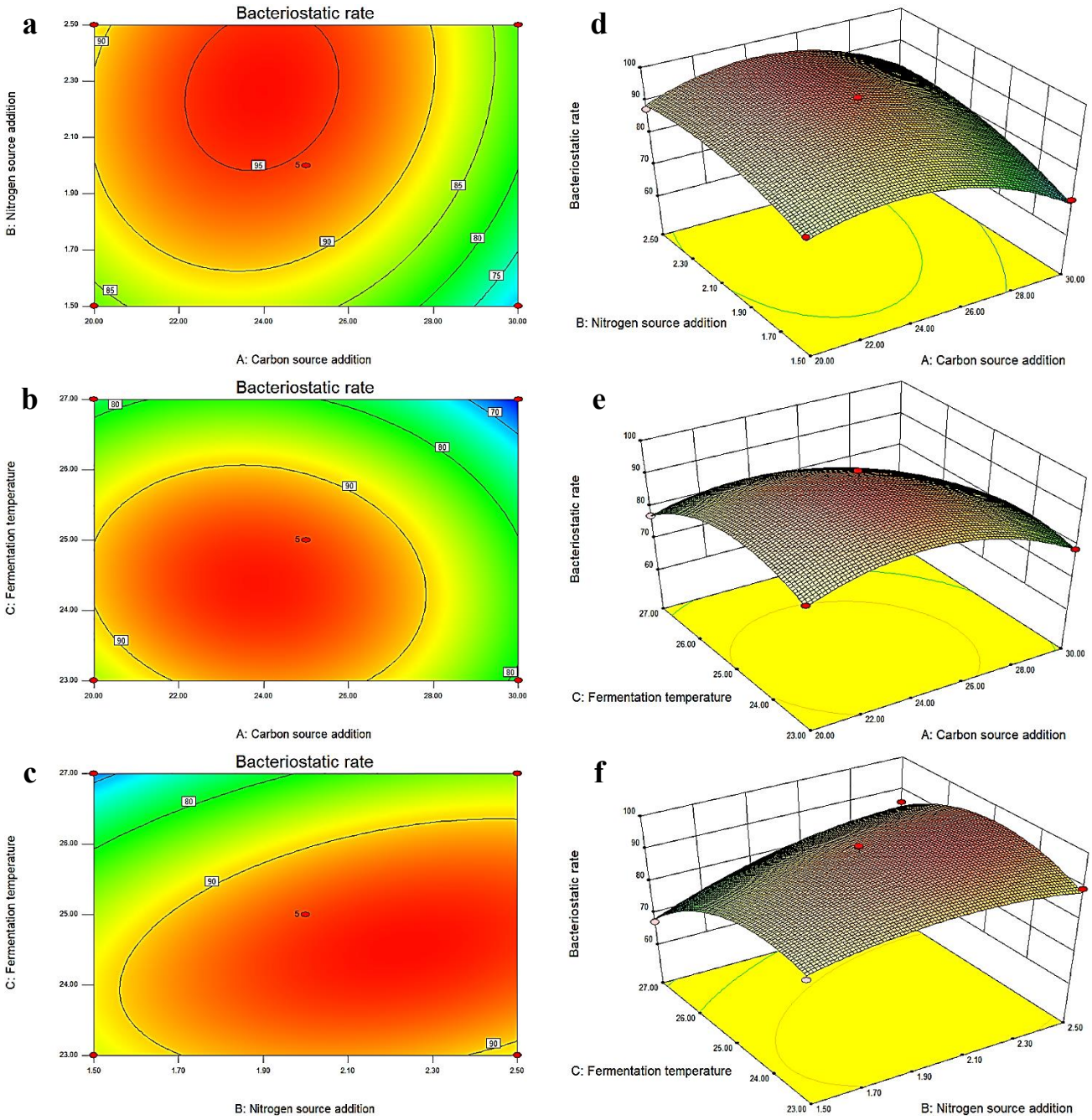

Fig. 3. Contour line and response surface diagram of interaction of various factors on inhibition rate of cucumber *Sclerotinia sclerotiorum*.

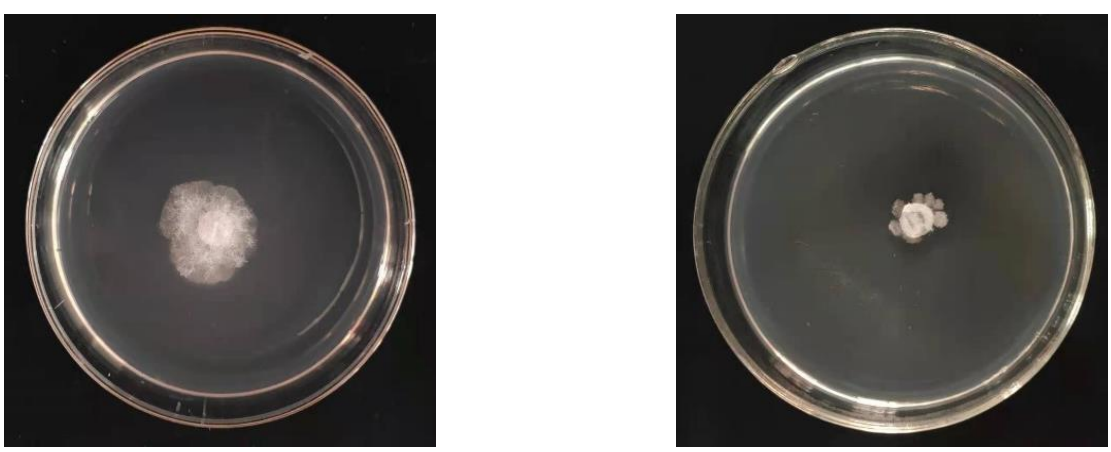

Fig. 4. Comparison of bacteriostatic rate between initial condition (left) and optimized condition (right).

Discussion

At present, chemical control is the main method for preventing and controlling cucumber sclerotinia disease in production. However, with the long-term use of a single variety of chemical agents, the resistance of harmful organisms to pesticides has developed rapidly, and problems such as excessive pesticide residues and serious environmental pollution still exist (Yuan *et al.*, 2023), Biological control, as an important means of developing green agricultural production in the new era, has become a hot research topic today (Deb *et al.*, 2024). As one of the commonly found fungi in nature, Penicillium has enormous potential for biological control. The active metabolites of *Penicillium* fungi have many types and rich functions, from which natural antibacterial, anti-insect or anti-virus substances can be developed and applied to the field of biological control (Gao *et al.*, 2020). Scholars have found that Penicillium citrinum has significant inhibitory effects on various pathogens, including Sclerotinia sclerotiorum (Sharma *et al.*, 2021). The greenhouse experiment conducted by Wang *et al.*, (2016) proved that the fermentation filtrate of *Penicillium* QMYCS-2 could effectively control tobacco black shank with the control efficiency of 73.25%. Yan (2016) found that the fermentation broth of *Penicillium chrysogenum* had a good antagonistic effect against *Rhizoctonia solani*, and it could promote plant growth and induce systemic disease resistance. The *Penicillium halophila* screened in this study could be used as a potential candidate strain for the production of linoleic acid (Sharma *et al.*, 2017), but no application in the field of biological control has been reported. Therefore, we need to actively study the effect and mechanism of *Penicillium halophila* on the prevention and control of cucumber *Sclerotinia sclerotiorum*, promote the comprehensive combination of biological control strategies and chemical or agricultural control methods, and lay the foundation for the establishment of a good comprehensive prevention and management technology.

In the early stage of the experiment, a strain of Ph-120 was screened and identified as *Penicillium halophilic*. This experiment aims to improve the antifungal activity of the strain by optimizing its cultivation and fermentation conditions. The results showed that using Design Expert software to model and predict glucose, yeast extract, and different temperatures, the highest inhibition rate was 96.60% at the optimal addition level of glucose, yeast extract, and temperature of 24.00 g/L, 2.21 g/L, and 24.56℃, respectively. The validation experiment showed that the inhibition rate was 94.60%, close to the predicted value, and increased by 16.7% compared to before optimization. Because of some characteristics of *Sclerotinia sclerotiorum* itself, such as large genetic variation, strong fecundity and adaptability, it is easy to produce drug resistance to the drug. Therefore, the rational use of pesticides in vegetable production has become a major problem to be solved urgently (Li *et al.*, 2021). On the basis of the research in this paper, we need to continue to analyze the main active substances that inhibit *Sclerotinia sclerotiorum* in *Penicillium halotolerans*. Ph-120, and prepare the biological pesticides that can be used by large-scale fermentation and other methods to enable them to be applied to production practice.

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