

Medium Optimization for Antifungal Active Substance Production from *Streptomyces Lydicus* Using Response Surface Methodology

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Abstract Response surface methodology was used to optimize the medium for antifungal active substance production from *Streptomyces lydicus* E12 in flask cultivation. Initially, the component factors, which influence antifungal substance production, were studied by varying one factor at a time. Starch, soybean cake powder, $K_2HPO_4 \cdot 3H_2O$ and $MgSO_4 \cdot 7H_2O$ were found to have a significant effect on the production of antifungal substances by the traditional design. Then, a Box–Behnken design was applied for further optimization. A quadratic model was found to fit antifungal active substance production. The analysis revealed that the optimum values of the tested variable were starch 84.96 g/L, soybean cake powder 4.13 g/L, glucose 5 g/L, $MgSO_4 \cdot 7H_2O$ 1.23 g/L, $K_2HPO_4 \cdot 3H_2O$ 2.14 g/L and NaCl 0.5 g/L. The test result of 67.44% antifungal inhibition agreed with the prediction and increased by 14.28% in comparison with the basal medium.

Keywords Antifungal substances · Inhibition · Medium optimization · Response surface methodology · *Streptomyces lydicus*

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Introduction

With the development of human society, the inappropriate application of antibiotics has gradually become a significant problem that affects people's lives. In spite of the fact that antibiotics are the most prescribed drugs all around the world, many aspects of their effects have posed serious challenges ever since the emergence of resistant bacteria [1]. Novel research on microorganisms to obtain new pharmacological compounds that are less toxic and that show effective activity against resistant microorganisms has been conducted under high attention due to the increased incidence of both fungal and bacterial infections and the emergence of drug resistance [2, 3]. Thus, finding novel compounds with bioactivity and strong effectiveness against the pathogenic bacterium is extremely urgent for the development of pharmaceutical technology.

A considerably large number of natural products have been studied, which have provided many possibilities for pharmaceutical and industrial use. The majority of products studied are secondary metabolites produced by microorganisms [4]. *Actinobacteria* are the most economical and useful prokaryotes, which are the major source of secondary metabolites with antifungal activity [5]. They have been the origin of the largest number of new antibiotic drug candidates with applications in many other therapeutic areas [6, 7]. Among *Actinobacteria*, particularly those of the genus *Streptomyces* are widely known for their ability to produce a variety of types of secondary metabolites, which have vast medical and industrial applications [8].

Streptomyces is a gram-positive bacterium that produces many clinically and agriculturally important secondary metabolites, including two-thirds of natural antibiotics, antitumor agents and immunosuppressors used in medicine [9]. Balachandran et al. [10] isolated *Streptomyces galbus*

ERINLG-127 and extracted an ethyl acetate extract, 2,3-dihydroxy-9,10-anthraquinone, as the principal active component. The compound showed good antimicrobial activity against the tested bacteria and fungi. They isolated 33 isolates from different forest soil samples. Among the 33 isolates, *Streptomyces* sp. ERINLG-51 was useful to identify novel molecules with potential activity towards pathogenic microorganisms and cancer [11]. At the same time, they also reported that *Streptomyces* sp. ERINLG-01 showed good antimicrobial, antioxidant and cytotoxic properties [12]. The antifungal active substances, which were produced by the streptolydigin-producing bacterium *Streptomyces lydicus*, are of great interest to the world. Cheng et al. [13] reported that significant variations in the levels of proteins played important roles in amino acid and carbohydrate metabolism, which boosted streptolydigin production in *S. lydicus*. The exogenous glutamate and proline not only directly provided precursors of streptolydigin synthesis but also potentially altered metabolic homeostasis during the improvement of streptolydigin production in *Streptomyces lydicus* E9 (E9) [14]. Metabolic analysis demonstrated that the addition of exogenous glutamate and proline and the reduction in intracellular levels of amino acids might be attributed to their faster conversion and consequently improved the accumulation of streptolydigin in strain E9 under a higher pitching ratio [15].

To conduct a further study on the antifungal substances, fermentation medium for their production was first investigated by response surface methodology (RSM). RSM is a group of statistical techniques for designing experiments, building models, evaluating the effects of factors and attempting to find optimum conditions of factors for appropriate responses [16–18]. It gave vital information for design and system optimization and was additionally useful in the analysis of a couple of responses at the same time [19]. It has been used not only to evaluate the relative significance of several variables simultaneously but also to identify and quantify the interacting effects among different variables [20]. The information attained from investigations typically carried out in an iterative manner is utilized to guide the search for better responsive variables, which makes RSM an advantageous and favourable method compared with classical methods of optimization. Velayudam and Murugan [21] combined Plackett–Burman design (PBD) with Box–Behnken design (BBD) to optimize antimicrobial compound production of *Streptomyces rochei* BKM-4. They used the predicted values from fitted equations and the observed values were in very good agreement. The yield of ϵ -PL by *Streptomyces albulus* IFO 14147 was increased significantly by 98.4%, from 0.75 to 8.13 g/L, when the strain was cultivated in the optimal medium developed by RSM, described by Shih and Shen [22].

The traditional ‘single-factor experiment’ with successive variation is frequently used in medium optimization to obtain high yields of useful metabolites in microbiology or to choose some primary factors from the various ones, but the complicated interactions among these factors are regarded to influence metabolite production. Although the block can be perfectly fulfilled by statistical methods, such as RSM and factorial design, particularly response surface analysis has been proven a useful and powerful method for optimizing target metabolite production and evaluating the significance of several factors simultaneously [20, 23–25].

In the present work, traditional single-factor methodology and statistical technology-based experimental designs were combined to optimize the medium for antifungal active substances. We started by using single-factor experiments to select the main factors that affect antifungal substance production, followed by a Box–Behnken design (BBD) using RSM to optimize the medium components.

Experimental

Microorganism

Strain E12 was induced by ultraviolet irradiation of strain E9, which was derived from *S. lydicus* AS 4.2501 (CGMCC NO. 1692), after treatment with nitrous acid solution and UV irradiation. *Rhizoctonia solani* Kühn (ACCC36076) was purchased from Beijing Beina Chuan-glian Biotechnology Institute. Strain E12 was first cultured on solid culture medium for approximately 7 days.

Growth Medium and Culture Conditions

The growth medium used for strain E12 was as follows: solid culture medium consisted of 20 g/L starch, 5 g/L glucose, 2 g/L peptone, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/L NaCl, 2 g/L corn steep liquor and 20 g/L agar. The seed medium consisted of 30 g/L starch, 5 g/L glucose, 2 g/L yeast extract, 4 g/L peptone, 1.5 g/L K_2HPO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 g/L NaCl. The basic fermentation medium consisted of 40 g/L starch, 5 g/L glucose, 2 g/L peptone, 1.0 g/L K_2HPO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 g/L NaCl.

Potato dextrose agar (PDA, medium for *R. solani* Kühn and antifungal activity test) consisted of fresh potatoes as described in Gams et al. [26] with some modifications: 200 g sliced (peeled) potatoes were homogenized with 300 mL tap water, then strained through muslin (yielding approximately 300 mL potato extract). The extract was combined with 20 g glucose and 18 g agar, mixed and diluted to a final volume of 1 L and then sterilized at 121 °C for 15 min.

The E12 strain was first cultured in solid culture medium for approximately 7 days. Subsequently, approximately 1.0 cm² strains from the solid medium were inoculated into 100 mL of seed medium for 48 h at (28 ± 0.5) °C as the first-grade seeds. Then, 10 mL first-grade seed was transferred from the seed medium into another 90 mL seed medium for 24 h at (28 ± 0.5) °C as second-grade seeds, prepared for fermentation. Fermentations were carried out in 100 mL medium (pH 6.8 ± 0.5) in Erlenmeyer flasks (250 mL) at 220 r/min and at 28 ± 0.5 °C. The strain E12 culture with initial inoculum sizes (10% vol/vol) was transferred to each flask with fermentation medium.

Analytical Methods

Samples were collected and centrifuged for 5 min at 12,000 r/min; treated broth was obtained by filtration through a 0.22 µm filter (Beijing Dingguo Changsheng Biotech Co. Ltd, Beijing, China).

An inhibition test of *R. solani* Kühn mycelial growth was carried out as follows: a total of 600 µL treated broth was sampled and mixed with melted PDA at 1:20 (v/v) or 1:40 (v/v) in Petri dishes (9.0 cm diameter). The agar block of *R. solani* was made by a puncher with an inside diameter of 6 mm and cultured on PDA incorporated with broth to test the inhibition of *R. solani* mycelial growth. Each treatment consisted of two replications. *R. solani* growing on PDA dissolved in sterile water was used as a control. The inhibition of *R. solani* was assessed as mycelial inhibition efficiency (%) at 36 h after cultivating *R. solani* at (28 ± 0.5) °C [27, 28]:

$$\text{Inhibition (\%)} = 100 - [100 \times (R_1^2 - r^2) / (R_0^2 - r^2)], \quad (1)$$

where r is the radius of the *R. solani* agar block; R_1 is the colony radius of *R. solani* on PDA incorporated with treated broth; and R_0 is the colony radius of *R. solani* on PDA incorporated with sterile water.

Experimental Design

Single Factor Experiment

The optimization of culture medium was carried out by traditional non-statistical technology, one-factor-at-a-time experiments.

The effects of different carbon sources on the antifungal activity of E12 were tested as follows, as described in Guo [29]: The carbon sources (glucose and starch) in the fermentation medium (starch 40 g/L, glucose 5 g/L, peptone 2 g/L, K₂HPO₄·3H₂O 1 g/L, NaCl 0.5 g/L, MgSO₄·7H₂O 0.5 g/L) were substituted by glucose, sucrose, glycerin, sodium citrate, sodium acetate, starch, soybean cake powder and corn syrup, each at a concentration of 40 g/L,

respectively. After inoculation for 12 h, samples were taken every 6 h for a total of 48 h. Centrifugation at 4500 r/min was used to remove the mycelia, and samples were stored under −20 °C to test together.

The effects of different nitrogen sources on the antifungal activity of E12 were tested as follows: the test methods were the same as described for carbon sources, with the nitrogen source (peptone) of the medium being replaced by 2 g/L yeast extract, soybean cake powder, urea, corn steep liquor, ammonium sulphate and beef extract.

The optimal ranges of carbon and nitrogen sources were determined as follows: the original fermentation medium was used, while the concentrations of the carbon and nitrogen sources were changed on the basis of the above experimental results. Consequently, the optimum concentration ranges of carbon and nitrogen sources for antifungal activity were determined in the response surface design.

The effect of different metal ions on E12 antifungal activity was tested as follows: different concentrations of CuSO₄·5H₂O, K₂HPO₄·3H₂O, MnSO₄·H₂O, FeSO₄·H₂O, NaCl, ZnSO₄, CaCl₂, CoCl₂·6H₂O and MgSO₄·7H₂O replaced the metal ion of the original fermentation medium (K₂HPO₄·3H₂O, NaCl, MgSO₄·7H₂O), with no other metal ions in the control medium. Antifungal activity was tested using the method above to detect important metal ions and their optimal range for E12.

Box–Behnken Design (BBD)

After critical factors were identified via screening and a significant range of variation was detected in the design space, the Box–Behnken design was employed to obtain a quadratic model. Furthermore, RSM was performed to describe the nature of the response surface in the optimum region, with antifungal active substance production as the response. The Box–Behnken design was generated by ‘Design-Expert’ software (version 8.0.6.1, Stat-Ease, Inc., Minneapolis, USA). According to this design, starch, soybean cake powder, K₂HPO₄·3H₂O and MgSO₄·7H₂O were the four most significant factors (as shown in Table 1), and 29 experiments (as shown in Table S1) were planned containing five replications to estimate the experimental

Table 1 Code and real values of factors chosen for the BBD

Variables	Level of variables		
	−1	0	1
A: starch (g/L)	60	75	90
B: soybean cake powder (g/L)	2	4	6
C: K ₂ HPO ₄ ·3H ₂ O (g/L)	0.57	1.71	2.85
D: MgSO ₄ ·7H ₂ O (g/L)	1.23	3.08	4.92

uncertainty variance. Also 'Design-Expert' was used for subsequent experimental data analyses. The performance of the model was evaluated by analysis of variance (ANOVA). The coefficient of determination R^2 was used to measure the quality of fit of the polynomial model equation.

Results and Discussion

Effects of Carbon and Nitrogen Sources on the Antifungal Activity of E12

As shown in Fig. 1, after glucose and starch were replaced in the original medium with glucose, sucrose, glycerol, sodium citrate, starch, corn steep liquor and soybean cake powder, the antifungal activity in decreasing order was with starch, soybean cake powder, corn steep liquor, glucose, sucrose, glycerol and sodium citrate. Starch as a carbon source resulted in the highest antifungal activity, reaching 78.50%, whereas the antifungal activity resulting from sodium citrate was the lowest among the carbon sources that were tested in this research. It had also been proven in previous studies [29, 30] that glucose, sucrose and glycerol as carbon sources inhibit the antifungal activity of E9 fermentation broth. The existence of such differences might be caused by the fact that the strain being used in this experiment was a product of mutagenesis, so the use of carbon sources and antifungal substance synthesis patterns were different from those of E9. The concentration of glucose, 40 g/L in this experiment, might have been too high and inhibited the growth of *Streptomyces* and the synthesis of its antifungal product. Souagui et al. [31] also demonstrated that the concentration of glucose must be decreased for the highest antifungal production to occur in *Streptomyces* sp. SY-BS5. Zhu et al. [32] found that when glucose concentration exceeded 20 g/L, it would inhibit avilamycin biosynthesis of the

Streptomyces viridochromogenes strain AS4.126. Zhang et al. [33] also confirmed that *Streptomyces coelicolor* mycelial growth was inhibited when glucose was increased to 55.3 g/L from 42.6 g/L in the medium. However, as glucose was 16.8–42.6 g/L, the secondary metabolites of blue pigment in *Streptomyces coelicolor* had already declined slowly; when it reached 42.6 g/L, the blue pigment decreased dramatically. Glucose is the most easily accessed energy and carbon source in a microbiology fermentation process and promotes microbial cell growth and division, but higher glucose concentrations will induce product inhibition. Therefore, starch was used in this study as a carbon source, considering that it is economical and readily available. The remaining glucose (5 g/L) in the original fermentation medium was available as a fast-acting carbon source, which helped *Streptomyces* to adapt to the new environment rapidly.

The same content of peptone, yeast extract, beef extract, corn steep liquor, urea, ammonium sulphate and soybean cake powder replaced the nitrogen in the original medium, and the results showed that E12 antifungal activity was greater than 80% when soybean cake powder, peptone and beef extract were used as nitrogen sources (as shown in Fig. 2). The results of previous studies [10, 31] showed that yeast extract was the essential substrate for antifungal activity production in *Streptomyces*. Inhibition of *Rhizoctonia* decreased successively as beef extract, Soybean cake powder, corn steep liquor, peptone, yeast extract, urea and ammonium sulphate were used as nitrogen sources. Therefore, the effect of the chosen organic nitrogen sources on the fermentation broth inhibition of E12 was significantly higher than that of the selected inorganic nitrogen sources in this experiment, which was consistent with other former studies [34, 35]. On the one hand, excessive ammonium ions could inhibit the growth and the process of secondary metabolism in *Streptomyces*; on the other hand, excessive urea and ammonium sulphate could increase the environmental osmotic pressure, thus inhibiting microbial growth and metabolism. The optimization of fermentation

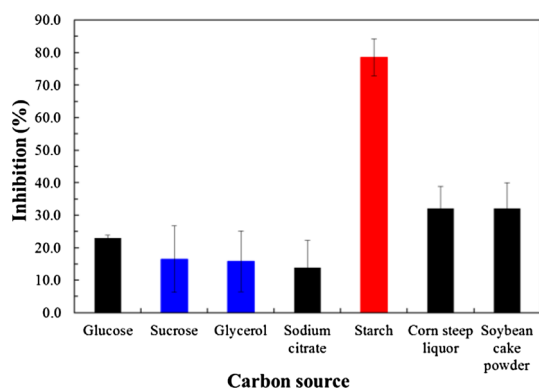


Fig. 1 Influence of different carbon sources on inhibition

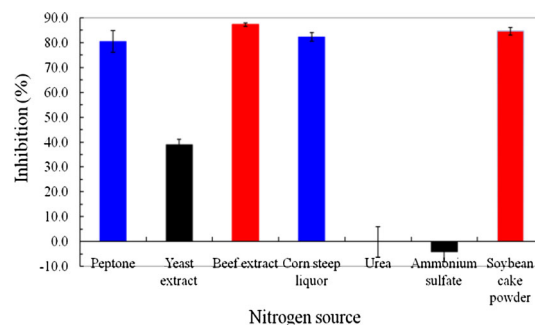


Fig. 2 Influence of different nitrogen sources on inhibition

conditions, especially the physical and chemical factors, is crucial to the target products due to their economical and practical effects. Soybean cake powder was chosen as a nitrogen source for further study due to its lower cost and convenient availability, although beef extract resulted in the maximum inhibitory rate.

Following selection of the best carbon and nitrogen sources, we sought to determine their optimal concentrations experimentally. As shown in Fig. 3a, antifungal activity of E12 against *Rhizoctonia* increased as starch content increased in the range selected. However, when the starch concentration exceeded 60 g/L, the antifungal activity reached saturation. As the starch concentration reached 80 g/L, the inhibitory activity reached a maximum of 92.00%. If the starch concentration exceeded 80 g/L, the antifungal activity of E12 began to decline, although the trend was not obvious. Therefore, the optimal range of starch in the medium was 60–90 g/L.

As shown in Fig. 3b, the antifungal activity of the fermentation broth of strain E12 reached the maximum at 24 h when the concentrations of soybean cake powder as a source of nitrogen were 2 g/L and 4 g/L. Afterward, the antifungal activity of the fermentation broth of strain E12 decreased during 48-h fermentation. However, the antifungal activity of the fermentation broth of strain E12 gradually increased during 48-h fermentation when the concentrations of soybean cake powder as a source of nitrogen were 1 and 8 g/L, respectively. Moreover, at different concentrations of soybean cake powder in the range of 1–4 g/L, there was a positive correlation between the inhibitory activity of the fermentation broth and the concentration of soybean cake powder. However, when the soybean cake powder concentration reached 8 g/L, the antifungal activity was inhibited, possibly because excessive nitrogen inhibited the biosynthesis of antibiotics, so that the antifungal activity was decreased [34, 36]. Therefore, taking the trend of the experimental results into consideration, the optimal range of soybean cake powder concentration was 3–6 g/L.

Influence of Different Metal Ions on the Antifungal Activity of E12

It was found that the antifungal activity of the fermentation broth changed when the type of metal ion was changed. Souagui et al. [31] found that antifungal production by *Streptomyces* sp. SY-BS5 was positively affected by an increased NaCl concentration. Table S2 shows that 50 mmol/L Zn^{2+} added to the medium resulted in the highest antifungal activity. However, its initial inhibition had reached 98.96%. Therefore, the inhibition was caused by Zn^{2+} acting against *Rhizoctoniasolani* rather than promoting the synthesis of antifungal substances. The same situation also applied to Co^{2+} and Cu^{2+} ions themselves employed against *Rhizoctonia* fungus at inhibitory concentrations of 20 and 50 mmol/L. Different ions at various concentrations exhibited different antifungal activities, but K^+ and Mg^{2+} produced the best antifungal activity at all the concentrations. Therefore, the K^+ and Mg^{2+} concentration ranges for BBD were preferably chosen to be 5–25 mmol/L and 5–20 mmol/L (approximately 0.57–2.85 g/L and 1.23–4.92 g/L).

BBD Experimental Design and Analysis

In the single factor experiments conducted above, starch (A), soybean cake powder (B), $K_2HPO_4 \cdot 3H_2O$ (C) and $MgSO_4 \cdot 7H_2O$ (D) were ensured as major factors, which influence the antifungal activity of E12 fermentation broth, and the experimental concentration range of each factor was determined, with glucose (5 g/L) and NaCl (0.5 g/L) values remaining the same as in the original medium. After 40-fold dilution, the diluted-broth was used to detect the inhibition efficiency with the PDA. Table S1 was designed using the software Design-Expert.V8.0.6.1-Box-Behnken design, including 29 experiments and the corresponding results.

Design-Expert.V8.0.6.1 software was used to conduct the data analysis shown in Table S1 by establishing a

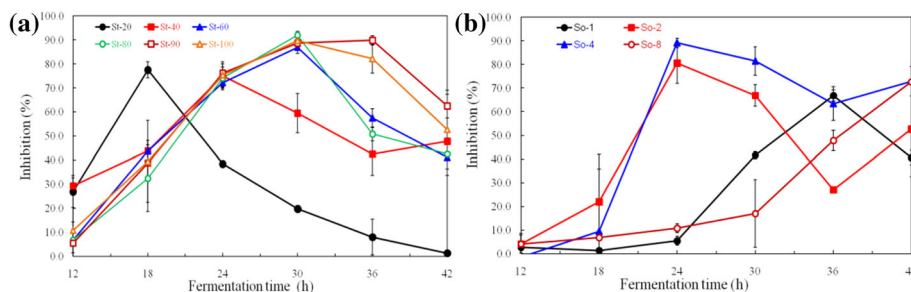


Fig. 3 Influence of different starch (A) and soybean cake powder (B) concentrations on inhibition of E12. **a** St-20: 20 g/L starch, St-40: 40 g/L starch, St-60: 60 g/L starch, St-80: 80 g/L starch, St-100:

100 g/L starch. **b** So-1: 1 g/L soybean cake powder, So-2: 2 g/L soybean cake powder, So-4: 4 g/L soybean cake powder, So-8: 8 g/L soybean cake powder

Table 2 Analysis of variance (ANOVA) for the regression equation of the inhibition model

Source	Sum of squares	Degrees of freedom	Mean square	F value	P value Prob > F	Evaluation
Model	4789.79	14	342.13	18.10	<0.0001	Significant
A	235.59	1	235.59	12.46	0.0033	
B	38.88	1	38.88	2.06	0.1735	
C	569.94	1	569.94	30.15	<0.0001	
D	566.50	1	566.50	29.96	<0.0001	
AB	8.76	1	8.76	0.46	0.5071	
AC	0.11	1	0.11	5.936×10^{-3}	0.9397	
AD	0.090	1	0.090	4.760×10^{-3}	0.9460	
BC	76.39	1	76.39	4.04	0.0641	
BD	3.03	1	3.03	0.16	0.6951	
CD	82.54	1	82.54	4.37	0.0554	
A ²	1329.90	1	1329.90	70.34	<0.0001	
B ²	1511.98	1	1511.98	79.97	<0.0001	
C ²	1430.90	1	1430.90	75.68	<0.0001	
D ²	31.57	1	31.57	1.67	0.2172	
Residual	264.69	14	18.91			Not significant
Lack of fit	243.14	10	24.31	4.51	0.0797	
Pure error	21.55	4	5.39			
Cor total	5054.49	28				

Table 3 Analysis of R² for regression equation of inhibition

Standard deviation	Mean	C.V. (%)	PRESS	R ²	Adj R ²	Pred R ²	Adeq precision
4.35	54.88	7.92	1434.18	0.9476	0.8953	0.7163	14.207

quadratic regression equation model: Inhibition = $74.18 - 4.403 \times A + 1.80 \times B + 6.89 \times C - 6.87 \times D + 1.48 \times A \times B + 0.17 \times A \times C - 0.15 \times A \times D - 4.35 \times B \times C - 0.87 \times B \times D - 4.54 \times C \times D - 14.32 \times A^2 - 15.27 \times B^2 - 14.85 \times C^2 - 2.21 \times D^2$.

Variance analysis of the secondary polynomial model above is shown in Table 2. The model F statistic was 18.33, which showed that the model displayed good predictive power. The P value of the lack-of-fit analysis was 0.0797, which is greater than 0.05, indicating that the lack of fit of the model was not significant. The model showed a good degree of fit, and the experimental error was small. Therefore, the model is suitable for use in the analysis of the effect of medium composition of fermentation broth on E12 antifungal activity and its prediction [36]. In addition, the analysis of variance results also showed that the quadratic terms have a significant explanatory power on the total variance carried by the tested regression equation. Therefore, the impact of various factors on the response of the experiment was not a simple linear relationship. As we can observe from the results of the analysis, the significant items for the value of inhibition rate are A, C, D, A², B² and C².

Table 3 shows the R² analysis of the regression equation. The table contains the R² and adjusted R² used for

evaluation of regression equation fitting. The coefficient of determination R² is 0.9476, which indicates that the non-linear relationship between the dependent and independent variables is significant. Therefore, within the selected scope, the regression model can be used to predict inhibition efficiency of fermentation broth of strain E12 against *Rhizoctoniasolani*. The adjusted R² is 0.8953, which indicates that the model can explain the response value variation of E12 fermentation broth antifungal activity by 89.53%. There is a certain degree of consistency between the predicted and adjusted values of R². In conclusion, the model fits well.

Parameter estimation and factor effect analysis of the quadratic polynomial regression are shown in Table S3. The interactions of the four factor effects in decreasing order are, K₂HPO₄·3H₂O: MgSO₄·7H₂O > soybean cake powder: K₂HPO₄·3H₂O > starch: soybean cake powder > soybean cake powder: MgSO₄·7H₂O > starch: K₂HPO₄·3H₂O > starch: MgSO₄·7H₂O. In the single factor effect, the order of inhibition within the scope tested in the study was as follows: K₂HPO₄·3H₂O, MgSO₄·7H₂O, starch, soybean cake powder.

After quadratic polynomial regression analysis, response analysis of single factors for inhibition values was also carried out. Figure 4 shows the four single factors of E12

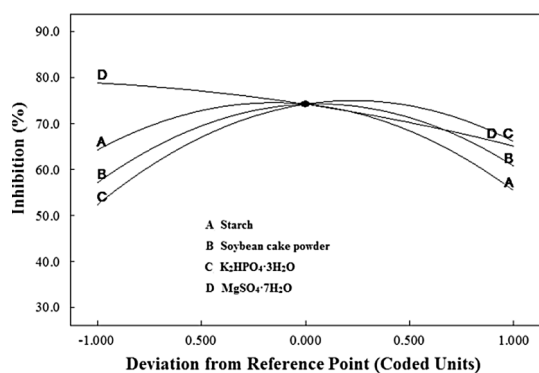


Fig. 4 Response curves of single factors

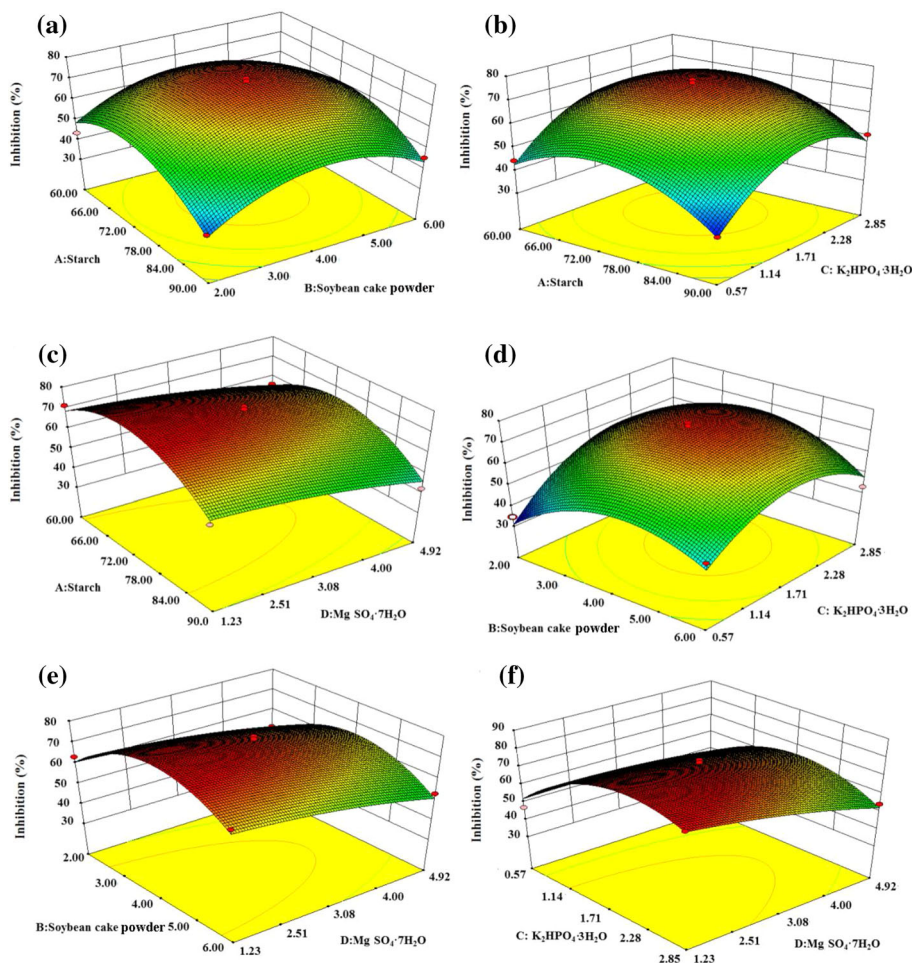
fermentation medium starch (A–A), soybean cake powder (B–B), $K_2HPO_4 \cdot 3H_2O$ (C–C) and $MgSO_4 \cdot 7H_2O$ (D–D), which affected the antifungal activity of the fermentation broth. When the other three factors were fixed at the middle level, each individual factor changed the effect on the response [37]. When the vertical coordinate was defined as the inhibition rate value and the horizontal axis was defined as ranges of four factors, coding was used to replace the

actual value of the four factors. Intermediate values of the four independent variables 75, 4, 1.71 and 3.08 were fixed for the study of any single factor change effect on inhibition. For instance, when the effect of starch on inhibition was studied, soybean cake powder, $K_2HPO_4 \cdot 3H_2O$ and $MgSO_4 \cdot 7H_2O$ concentrations were fixed at 4, 1.71 and 3.08 g/L, respectively. As the figure shows, each perturbation trend of single factor affects the response. As the values of A, B and C varied in the interval of $[-1, 1]$, the Y value increased and then decreased and eventually reached the maximum level in the vicinity of 0.000; the effect of D decreased as the concentration increased within $[-1, 1]$ and eventually reached a maximum level in the vicinity of -1.000 .

Interaction analysis of response surface for the inhibition rate model is shown in Fig. 5. In the study of the interaction of two factors, the other variables were kept constant at the intermediate value of the range studied. When it came to the interaction between starch and soybean cake powder, the concentrations of $K_2HPO_4 \cdot 3H_2O$ and $MgSO_4 \cdot 7H_2O$ were 1.71 and 3.08 g/L. Figure 5a shows the impact of the interaction between starch and

Fig. 5 3D response surface curve plot of 4 factors and their mutual effects on inhibition.

a Effects of starch and soybean cake powder and their mutual effects. **b** Effects of starch and $K_2HPO_4 \cdot 3H_2O$ and their mutual effects. **c** Effects of starch and $MgSO_4 \cdot 7H_2O$ and their mutual effects. **d** Effects of $K_2HPO_4 \cdot 3H_2O$ and soybean cake powder and their mutual effects. **e** Effects of $MgSO_4 \cdot 7H_2O$ and soybean cake powder and their mutual effects. **f** Effects of $K_2HPO_4 \cdot 3H_2O$ and $MgSO_4 \cdot 7H_2O$ and their mutual effects



soybean cake powder, as well as the reciprocal action on inhibition rate; the figure also shows that the interaction is significantly strong. With soybean cake powder concentration fixed, the inhibition of the broth increased as the starch concentration increased, then continued to increase as the starch concentration decreased. The same situation also appeared for the starch and $K_2HPO_4 \cdot 3H_2O$ interaction, which influenced the inhibitory rate value (shown in Fig. 5b) and the interactive effect between starch and $MgSO_4 \cdot 7H_2O$ on the inhibitory rate values (shown in Fig. 5c). Both starch and $MgSO_4 \cdot 7H_2O$ are the significant factors, which influence the inhibition value greatly, and their interaction is also strong as shown in Fig. 5c. In the case of soybean cake powder and $K_2HPO_4 \cdot 3H_2O$ concentration, as $K_2HPO_4 \cdot 3H_2O$ concentration increased in the experimental range, the inhibition rate was significantly reduced, which showed that high concentrations of $K_2HPO_4 \cdot 3H_2O$ are not conducive to biosynthesis of antifungal substances against *Rhizoctonia*. A similar situation also appeared for the interactive effect of $MgSO_4 \cdot 7H_2O$ and soybean cake powder on the inhibitory rate values (Fig. 5e) and the interactive effect between $K_2HPO_4 \cdot 3H_2O$ and $MgSO_4 \cdot 7H_2O$ (Fig. 5f). To summarize, there is a strong interaction between the pairs of factors discussed above.

Optimization and Validation of Analog Design-Expert System

After analysis by the Design-Expert software and with concentration of glucose and NaCl being fixed at 5 and 0.5 g/L, respectively, an optimized medium condition of broth inhibition was derived: starch concentration was 84.96 g/L, soybean cake powder concentration was 4.13 g/L, $K_2HPO_4 \cdot 3H_2O$ concentration was 2.14 g/L, $MgSO_4 \cdot 7H_2O$ concentration was 1.23 g/L and predictive inhibition value is 72.00%. To verify the predicted results, a verification test was conducted under the experimental conditions. A 14.28% increase was shown between the inhibition of original medium and the optimized fermentation medium whose values were 53.16 and 67.44%, respectively (as shown in Fig. 6). Validating and predicted values are basically the same, indicating that the model is reasonable and effective.

Conclusions

After the single-factor method experiment, starch, soybean cake powder, $K_2HPO_4 \cdot 3H_2O$ and $MgSO_4 \cdot 7H_2O$ were found to be the significant factors influencing antifungal activity of E12. When the four key factors were studied using Design-Expert software, a quadratic polynomial model showed the relationship between starch, soybean

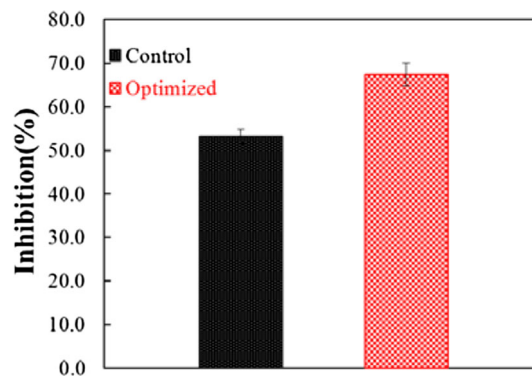


Fig. 6 Antifungal activity of E12 fermentation broth in optimized medium (Control: original medium, Optimized: optimization medium)

cake powder, $K_2HPO_4 \cdot 3H_2O$ and $MgSO_4 \cdot 7H_2O$. Also, A, C, D, A^2 , B^2 and C^2 affected the value of inhibitory rate to significant levels. A strong interaction between all factors was analysed by response surface. The optimized medium conditions for maximum inhibitory rate value were starch 84.96 g/L, soybean cake powder 4.13 g/L, $K_2HPO_4 \cdot 3H_2O$ 2.14 g/L, $MgSO_4 \cdot 7H_2O$ 1.23 g/L, glucose 5 g/L and NaCl 0.5 g/L. The predictive value was 72.00%. In this medium, the experimental result of inhibition was 67.44%, which was consistent with theoretical predictions, indicating that the model was significant and a good fit. The maximum antifungal activity of the optimized fermentation broth was improved by 14.28%.

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