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OPTIMIZATION OF FERMENTATION MEDIUM AND FERMENTATION CONDITIONS OF BIOCONTROL STRAIN L131 AGAINST *ALTERNARIA ALTERNATA*

XINYUE MA, XILIN SUN, XI ZHAO, YUFAN WANG, LINGYUE ZHU, WENXING LI AND XIAOMEI WANG*

College of Plant Protection, Jilin Agricultural University, Changchun 130118, China
**Corresponding author's email: 2232906297@qq.com; wxm820@126.com*

Abstract

Early blight is one of the most widespread and devastating diseases of tomato. Our previous research has isolated and screened a biocontrol strain L131 in vegetable rhizosphere soil, which has good inhibitory effect against *Alternaria alternata* and was identified as *Penicillium citrinum*. The fermentation medium and fermentation conditions were optimized by single-factor experiments, orthogonal experiments and response surface experiments. The results showed that the optimal fermentation medium was lactose 27.33 g·L-1, beef paste 2.39 g·L-1 and NaCl 1.70 g·L-1. A model was developed using Design Expert software and the predicted inhibition rate was 81.60% under the above conditions. The actual inhibition rate obtained after validation was 81.55%, which was close to the predicted value. It was 15.14% higher than that before optimization. Meanwhile, the optimal fermentation conditions were as follows: liquid volume 100/250 mL, 20℃, and 7 d. This experiment provides the basis for the development of large-scale fermentation and biopesticides in future production.

Key words: Tomato early blight; Biocontrol fungi; *Penicillium citrinum*; Response surface methodology.

Introduction

Tomato is one of the most common fruit and vegetable crops. China, Indian, Turkey, America and Egypt are the major tomato producing countries (Foolad, 2007). According to the statistics of the Food and Agriculture Organization of the United Nations, tomato is the largest consumption vegetable in the world, with a gross production value of about $102.6 billion. China's tomato planting area is about 1.1 million hectares, and its production is about 64.84 million tons, ranking the first in the world, accounting for 35% of the world's total production. Tomatoes are popular as the richest source of vitamin A, vitamin C and lycopene, which help protect the body from cancer and heart disease (Bohm *et al*., 2012; Breemen *et al*., 2008).

Many fungal, bacterial, viral and nematode diseases after occurred in commercial production of tomatoes of large plantings and long-term continuous cropping, among which tomato early blight caused by *A. alternata* is more severe. A variety of Alternaria spp., are related to early blight of solanaceae plants such as tomatoes and potatoes. In addition to the large-spored Alternaria pathogens, such as *A. solani*, *A. tomatophila*, *A. cretica* and *A. subcylindrica*, the small-spored Alternaria pathogens, such as *A. alternata*, *A. arborescens* and *A. tenuissima*, are often isolated from the early blight strains of Solanaceae. And the isolation rate of small-spored Alternaria pathogens were much higher than that of large-spored Alternaria pathogens, even reaching 100% (Pragya *et al*., 2017). The pathogen used in this study is *A. alternata*, which is capable of producing a variety of toxins in infected plants, such as Altenuene (ALT), Alternariol (AOH) and Alternariol methyl ether (AME) (Yelko *et al*., 2016; Andersen *et al*., 2001). Several recent studies have shown that AOH and AME can induce cell cycle arrest, apoptosis and DNA damage effects, causing health problems in humans and animals (Bensassi *et al*., 2012; Fernández-Blanco *et al*., 2016).

Tomato early blight is usually controlled cultural practices, fungicide treatment, biological control and the use of resistant varieties. The continuous use of chemicals to control disease usually brings many problems, such as environmental pollution, toxicity to non-target organisms, and causing resistance in pathogen populations. The use of biological control methods that are safer to the environment is considered to be an effective alternative (Varma *et al*., 2008). There are many kinds of antagonistic microorganisms used for biological control of plant diseases, and their sources are rich. Studies have shown that various microorganisms around leaves and roots have good inhibitory effects on the infection of pathogens. The research on the prevention and control of tomato early blight mainly focuses on *Trichoderma* spp. (Gao, 2010), *Serratia* spp. (Dong *et al*., 2015) and *Pseudomonas* spp. (Abiodun *et al*., 2017).

It is necessary to optimize the fermentation medium and fermentation conditions in order to maximize the control effect of the biocontrol strain. The nature and concentration of fermentation medium components affect the availability of nutrients and microbial cell metabolism, and thus the inhibitory effect on target pathogens (Hajjaj *et al*., 2001). Among them, carbon, nitrogen and trace elements usually play a dominant role in influencing fermentation productivity (López *et al*., 2010). In this experiment, the best combination of carbon source, nitrogen source and inorganic salt was first screened on the basis of PDB medium, using the inhibition rate of tomato early blight as the measurement index. The amount of the 3 ingredients added was then optimized by response surface experiment, after which the optimum fermentation conditions were determined. To promote the large-scale application of biological control methods, the purification and structural identification of inhibitory substances in fermentation broth, and the development of biological control preparations provide better data support.

Material and Methods

Test materials: Strain L131 (*P. citrinum*), *A. alternata*. The above two strains were isolated and provided by Plant Disease Institute of Jilin Agricultural University.

Potato dextrose agar (PDA) medium: Potato 200g·L-1, Glucose 20g·L-1, agar 15g·L-1;
Potato dextrose broth (PDB) medium: Potato 200g·L-1, Glucose 20g·L-1;
Media for carbon source test: Potato 200 g·L-1, Carbon source (Glucose / Sucrose / Soluble starch / Dextrin / Maltose / Corn protein / Lactose / Mannitol / Fructose) 20 g·L-1;
Media for nitrogen source test: Potato 200 g·L-1, Glucose 20 g·L-1, Nitrogen source (Peptone / Yeast extract powder / Beef extract / Tryptone / Urea / Potassium nitrate / Ammonium chloride / Peanut meal / Bran / Ammonium phosphate) 2 g·L-1;
Media for inorganic salt test: Potato 200 g·L-1, Glucose 20 g·L-1, Inorganic salt (CuSO4·5H2O / ZnSO4·7H2O / MgSO4·7H2O / FeSO4·7H2O / CaCO3 / K2HPO4·3H2O / KH2PO4 / NaCl) 1 g·L-1;
Media for pH test: The pH of the sterilized PDB medium was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 with 1.0 mol·L-1 HCl and 1.0 mol·L-1 NaOH, respectively.

Test methods

Screening of optimal components in fermentation medium: Single-factor experiment: Two 8 mm discs of strain L131 were inoculated into 35 kinds of carbon source, nitrogen source, inorganic salt and pH test medium respectively. Set up three replicates, and shake culture at 25℃ and 175 rpm for 7 days. The different fermentation broths were mixed evenly with PDA medium at 1:5, poured into plates, left to stand, and then inoculated with *A. alternata*, with three replicates, and sterile water mixed with PDA medium at 1:5 as the control group. Culture at 25℃ and measure the diameter when the control is full grown. We calculated the inhibition rate by using the following formula (Yasmin & Shamsi, 2019).

$$I = \frac{C - T}{C} \times 100\%$$

where, I = Per cent growth inhibition, C = Growth in control, T = Growth in treatment.

Orthogonal experiment: The optimal combination of components was obtained by L9 (34) orthogonal experimental design (Table 1) with 4 factors of carbon source, nitrogen source, inorganic salt, and pH, and 3 levels of the better three components of each factor in the single-factor test with intuitive analysis.

Optimization of fermentation medium components additions: Based on the optimal components obtained by orthogonal test, the addition of carbon source, nitrogen source and inorganic salt was optimized by response surface experiment. A group of optimal liquid medium suitable for fermentation of strain L131 was screened out.

Single-factor experiment: The effect of lactose addition (15 g·L-1、20 g·L-1、25 g·L-1、30 g·L-1、35 g·L-1), beef extract addition (1.5 g·L-1、2 g·L-1、2.5 g·L-1、3 g·L-1、3.5 g·L-1) and NaCl addition (1 g·L-1、1.25 g·L-1、1.5 g·L-1、1.75 g·L-1、2 g·L-1) on the inhibition rate was investigated sequentially using one-factor-at-a-time experimentation. The initial fermentation condition was 25℃, 175 rmp, 7 d, and pH 6.0.

Response surface experiment: According to the results of single factor test, the parameter range of response surface test for each component addition was determined (Song *et al*., 2018; Wei *et al*., 2012). A 3-factor, 3-level response surface test was conducted using the Box-Behnken experimental design and analysis method to determine the optimal parameters for the fermentation of strain L131 (Guowei *et al*., 2016). Analyze and process the data with Design Expert 8.0.6.

Optimization of fermentation conditions: Set the liquid volume as 50 mL, 75 mL, 100 mL, 125 mL, 150 mL (250 mL conical flask), fermentation time as 4 d, 5 d, 6 d, 7 d, 8 d, fermentation temperature as 15℃, 20℃, 25℃, 30℃, 35℃. The inhibition rate of fermentation broth was determined and the optimal fermentation conditions were selected.

Results

Screening of optimal components in fermentation medium: Strain L131 was fermented media with different carbon sources, nitrogen sources, inorganic salts and pH, and its inhibition effect varied widely (Fig. 1).

Nine carbon source and no carbon source added were screened, and the results showed that the 3 kinds with the higher inhibition rates were: Lactose (73.58%)＞Mannitol (73.22%)＞Soluble starch (72.21%). Their inhibition rates were significantly better than those of other carbon sources at the P=0.05 level, ranging from 4.05% to 5.42% higher than that of the initial carbon source (glucose).

Ten nitrogen source and no nitrogen source added were screened, and the results showed that the 3 kinds with the higher inhibition rates were: Bran (72.70%)＞Beef extract (72.30%)＞Tryptone (67.12%). Their inhibition rates were significantly better than those of other nitrogen sources at the P=0.05 level, ranging from 3.13%~8.71% higher than that of the initial nitrogen condition.

Eight Inorganic salt and no Inorganic salt added were screened, and the results showed that the 3 kinds with the higher inhibition rates were: NaCl (70.64%)＞KH2PO4 (69.93%)＞Not added (67.66%) (Fig. 2). The inhibition rate of fermentation using added NaCl or KH2PO4 medium was significantly higher than not added inorganic salts, increasing by 2.27% to 2.98%.

In the pH screening test, the best inhibition of fermentation was shown when the pH was 5, 6 and 7.

Table 1. Factors and levels of orthogonal design.

Level	Factor			
	A Carbon source	B Nitrogen source	C Inorganic salt	D pH
1	Soluble starch	Bran	KH2PO4	5
2	Lactose	Beef extract	NaCl	6
3	Mannitol	Tryptone	Not added	7

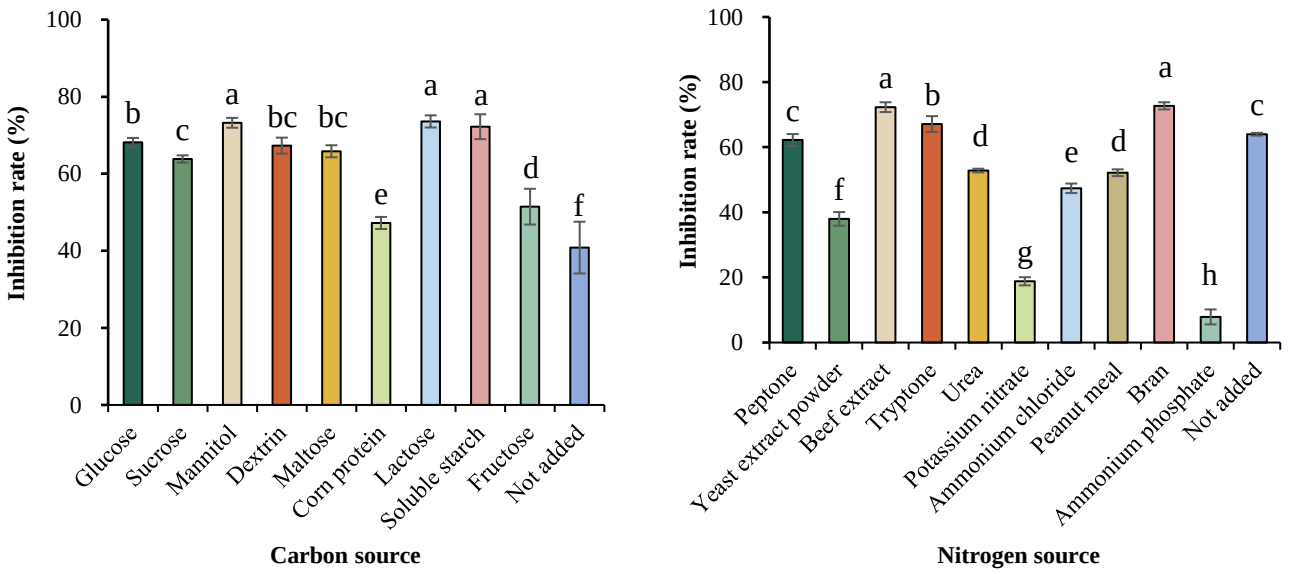

Fig. 1. Inhibition rate of fermentation with different carbon and nitrogen sources.
Different lowercase letters indicate significant differences at the p=0.05 level.

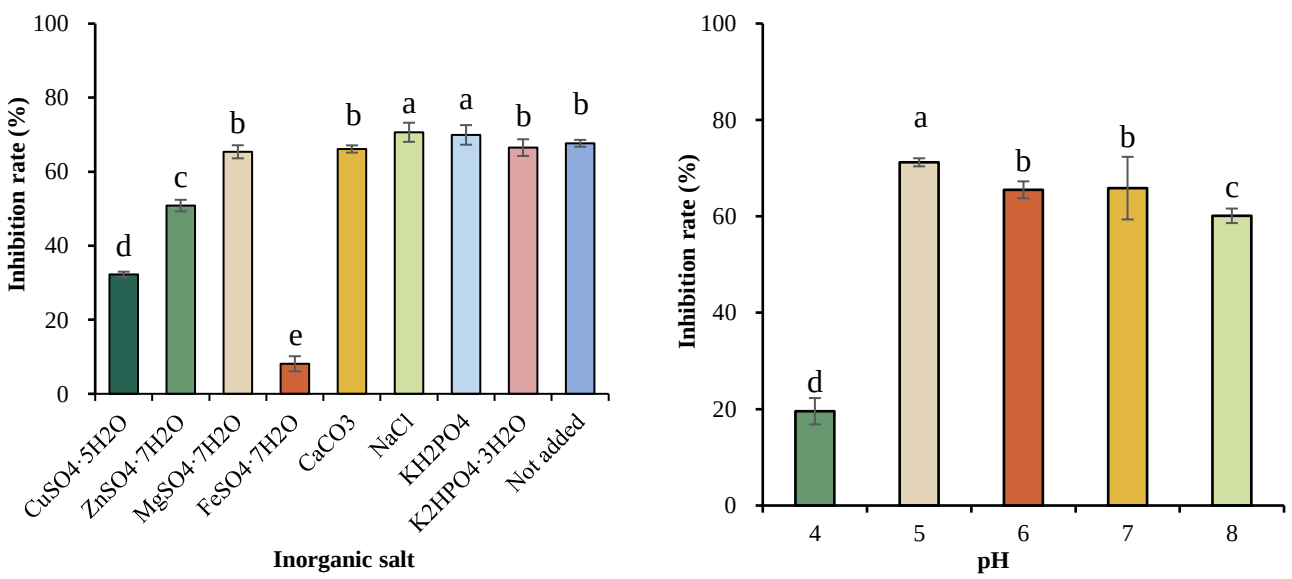

Fig. 2. Inhibition rate of fermentation with different inorganic salt and pH
Different lowercase letters indicate significant differences at the P=0.05 level.

The intuitive analysis of the orthogonal experiments can be seen (Table 3). Factor D has the largest R value of 2.69, which is the primary factor affecting the inhibition rate. The R values of factor B and factor C are 2.05 and 2.17 respectively; The R value of factor A is only 1.70. The order of importance of the influence on the inhibition rate was pH, inorganic salt, nitrogen source, and carbon source. The results showed that the optimal components of fermentation medium was A2B2C2D1, that is, lactose, beef paste, NaCl and pH 5 had the highest inhibition rate of 75.12% (Table 2).

Optimization of fermentation medium components additions: As shown in (Fig. 3), the highest inhibition rate (77.02%) was observed when lactose was added at 25 g·L-1, and the over addition was detrimental to the fermentation of strain L131. When beef extract was added at 2.5 g·L-1, the highest inhibition rate (82.60%)

was achieved against *A. alternata*. There was no significant difference in the inhibition rate of the fermentation broth when NaCl was added at 1.00~2.00 g·L-1, all of which ranged from 75.77% ~ 77.66%. Therefore, the optimal additions of lactose, beef extract and NaCl to the liquid fermentation medium were 25 g·L-1, 2.5 g·L-1 and 1.75 g·L-1, respectively.

Response surface experiment: The model building and significance examination was done by using medium 5 in the orthogonal experiment. The inhibition rate (Y) was used as the response value, and the amount of lactose (X1), beef extract (X2), and NaCl (X3) were used as the 3 factors (Dertli *et al*., 2016; Chen *et al*., 2018). And the variable levels are represented by -1, 0, and 1 (Table 4). Design Expert 8.0.6 was used to design a 3-factor, 3-level response surface optimization test to determine the optimal addition combination. The response surface test data are shown in (Table 5).

The regression equation was obtained by fitting the scores in Tab. 5 using Design expert V8.0.6.1 software: Y = 81.37 + 0.72X1 - 0.49X2 - 0.071X3 + 0.072X1X2 + 0.012X1X3 + 0.39X2X3 - 0.76X12 - 1.26X22 - 0.36X32. From the equation, it can be concluded that in this model, the inhibition rate is most affected by the amount of lactose, followed by the amount of beef extract, and least affected by the amount of NaCl.

Analysis of variance and significance of the inhibition rate scores (Table 6) shows that the F-value of the model is 31.79 and p<0.0001, implying that the model is significant. X1、X2、X12、X22 are extremely significant terms, X2X3、X32 are significant terms, and X3、X1X2、X1X3 are insignificant terms. The F-value of the lack of fit is 1.63 and p(=0.3163) > 0.05, implying that the lack of fit is not significant relative to the pure error, indicating that the model has a high reliability. R2 = 0.9761 and R2Adj = 0.9454 indicate that the model has a low error and a strong fit. The difference between R2Pred = 0.7729 and R2Adj is also within a reasonable range. Adeq Precision = 17.524 > 4 also confirms that the model is ideal.

Figure 4 represents the contour and 3D surface maps of the effect of the interaction of lactose, beef extract and NaCl addition on the inhibition rate (Wang *et al*., 2018). The top part of Fig. 4-above shows the interaction between lactose and beef extract addition at the central level of another factor, indicating that the inhibition rate increases and then decreases with the increase of lactose and beef extract addition. The middle part of Fig. 4-middle shows the interaction between lactose and NaCl addition at the central level of another factor, indicating that the inhibition rate increases and then decreases with the increase of lactose and NaCl addition. The bottom part of Fig. 4-below shows the interaction between beef extract and NaCl addition at the central level of another factor, indicating that the inhibition rate increases and then decreases with the increase of beef extract and NaCl addition.

Table 2. The intuitive analysis of the orthogonal experiments.

Test number	Factors				Inhibition rate (%)
	A	B	C	D	
1 (A1B1C1D1)	Soluble starch	Bran	KH2PO4	5	72.69 ± 0.34bc
2 (A1B2C3D2)	Soluble starch	Beef extract	0	6	69.95 ± 0.44de
3 (A1B3C2D3)	Soluble starch	Tryptone	NaCl	7	70.88 ± 0.5cde
4 (A2B1C3D3)	Lactose	Bran	0	7	72.73 ± 1.75bc
5 (A2B2C2D1)	Lactose	Beef extract	NaCl	5	75.12 ± 0.16a
6 (A2B3C1D2)	Lactose	Tryptone	KH2PO4	6	69.09 ± 2.84e
7 (A3B1C2D2)	Mannitol	Bran	NaCl	6	73.87 ± 0.80ab
8 (A3B2C1D3)	Mannitol	Beef extract	KH2PO4	7	71.58 ± 0.67bcd
9 (A3B3C3D1)	Mannitol	Tryptone	0	5	73.16 ± 0.92abc

Note: 0 means not added; different lowercase letters indicate significant differences at the P=0.05 level

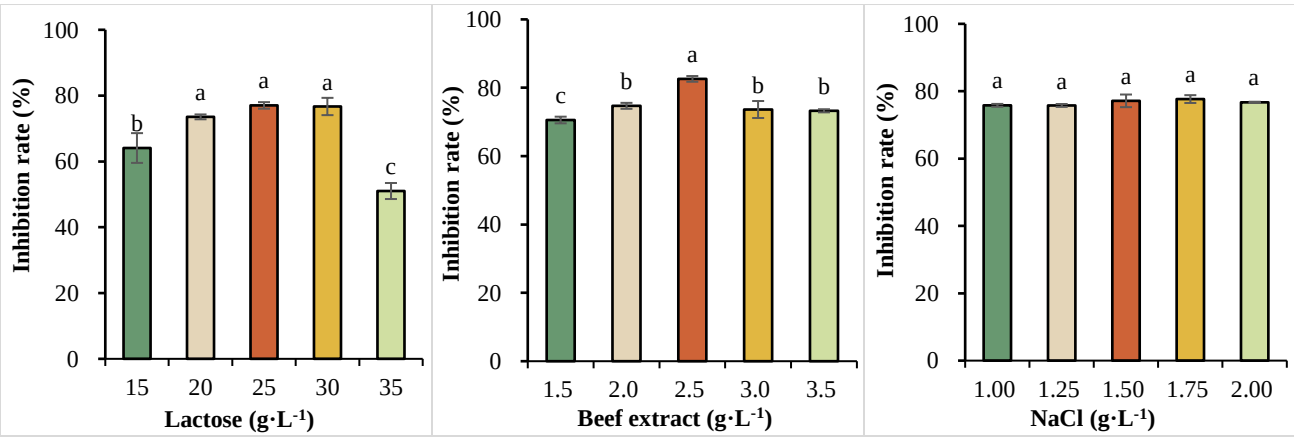

Fig. 3. Effects of lactose, beef extract and NaCl addition on the inhibition rate
Different lowercase letters indicate significant differences at the P=0.05 level.

Table 3. Range analysis results of various factors.

Index	Factor			
	A	B	C	D
K1	213.52	219.29	213.36	220.97
K2	216.94	216.65	219.87	212.91
K3	218.61	213.13	215.84	215.19
k1	71.17	73.09	71.12	73.66
k2	72.31	72.22	73.29	70.97
k3	72.87	71.04	71.95	71.73
R	1.7	2.05	2.17	2.69

Note: K1, K2 and K3 are the sum of all levels of each factor; k1, k2 and k3 are the average values of the level of each factor; R value of are the range of k1, k2 and k3

Table 4. Variables and level of Box-Behnkendesign.

Level	Factor		
	X1 Lactose addition (g·L-1)	X2 Beef extract addition (g·L-1)	X3 NaCl addition (g·L-1)
-1	20	2.0	1.50
0	25	2.5	1.75
1	30	3.0	2.00

Table 5. The results of response surface optimization test for strain L131 fermentation.

Code	Factors and levels			Y Inhibition rate (%)
	X1 (g·L-1)	X2 (g·L-1)	X3 (g·L-1)	
1	-1	-1	0	78.97
2	1	-1	0	80.54
3	-1	1	0	78.02
4	1	1	0	79.88
5	-1	0	-1	79.80
6	1	0	-1	80.95
7	-1	0	1	79.53
8	1	0	1	80.73
9	0	-1	-1	80.74
10	0	1	-1	78.79
11	0	-1	1	79.92
12	0	1	1	79.53
13	0	0	0	81.66
14	0	0	0	81.53
15	0	0	0	81.12
16	0	0	0	81.26
17	0	0	0	81.29

Table 6. Variance analysis of response surface experiments results.

Source	Sum of squares	df	Mean square	F Value	P-value Prob > F	Sig.
Model	17.33	9	1.93	31.79	＜0.0001	**
X1	4.18	1	4.18	68.93	＜0.0001	**
X2	1.95	1	1.95	32.19	0.0008	**
X3	0.041	1	0.041	0.67	0.4399	
X1X2	0.021	1	0.021	0.35	0.5743	
X1X3	0.000625	1	0.000625	0.010	0.9219	
X2X3	0.61	1	0.61	10.04	0.0157	*
X12	2.41	1	2.41	39.72	0.0004	**
X22	6.72	1	6.72	110.95	＜0.0001	**
X32	0.56	1	0.56	9.18	0.0191	*
Residual	0.42	7	0.061			
Lack of Fit	0.23	3	0.078	1.63	0.3163	
Pure Error	0.19	4	0.048			
Cor Total	17.76	16				

Note: "*" Indicated significant impact on the results ($0.01<p<0.05$); "**" Indicated that the impact on the results was extremely significant ($p<0.01$)

The maximum predicted response value (inhibition rate) was 81.60% at the combination of lactose 27.33 g·L-1, beef paste 2.39 g·L-1 and NaCl 1.70 g·L-1 for each factor of the model. Tests were conducted according to the best combination given by the model, and the actual inhibition rate obtained was 81.55%, which was similar to the predicted value, proving the validity of the model. The inhibition rate was 15.14 percentage points higher than that of fermentation with PDB medium (66.41%) before optimization (Fig. 5).

Optimization of fermentation conditions: The highest inhibition was 76.75% at 7 d of fermentation time, which was not significantly different from 6 d, but significantly better than 4, 5 and 8 d (Fig. 6). The highest inhibition rate was 78.16% at a medium volume of 100 mL (250 mL conical flask), which was not significantly different from 50 mL, 75 mL and 125 mL, but significantly better than 150 mL. At the fermentation temperature of 20℃, the highest inhibition rate was 78.07%, which was significantly better than other temperatures.

The results showed that the optimal fermentation conditions were at 100 mL medium, 20℃ and 7 d.

Discussions

Biological control, as an environmentally friendly way to prevent and control plant diseases, has attracted much attention in recent years. In the early stage of this study, a biocontrol strain L131 with good antifungal activity was screened and obtained from vegetable rhizosphere soil and was identified as *P. citrinum*. *Penicillium* fungi, as a fungus with biocontrol potential, can produce various secondary metabolites, including Polyketides, Alkaloids, Terpenoids, Macrolides, and other types of compounds to inhibit the growth of pathogenic bacteria (Zhang *et al*., 2019). In order to maximize its inhibitory effect, the composition and cultivation conditions of the fermentation medium were optimized. The components of the fermentation medium affect the supply of nutrients and microbial cell metabolism, and the productivity of the fermentation process of inhibition substances depends on the medium used. Among the main culture nutrients, carbon and nitrogen sources usually play a dominant role in fermentation productivity, as these nutrients are directly related to biomass and metabolite formation

(López *et al*., 2010). In addition, the nature and concentration of the carbon source can regulate secondary metabolism through phenomena such as catabolite repression (Hajjaj *et al*., 2001). Due to the multiple quantification and variables involved in the process, orthogonal and response surface design methods have been used in many studies to optimize fermentation medium. (Grothe *et al*., 1999). The fermentation medium components, addition of components and fermentation conditions were optimized by various methods in order to better exploit the inhibitory effect of strain L131 against *A. alternata*.

The optimal medium components were screened by orthogonal test results for lactose, beef extract, NaCl, pH 5. Modeling using Design Expert software predicted the highest inhibition rate of 81.60% at the optimal addition levels of 27.33 g·L-1、2.39 g·L-1、1.70 g·L-1 for lactose, beef extract, and NaCl, respectively. The verification test shows that the inhibition rate is 81.55%, which is close to the predicted value and 15.14% higher than that before optimization. At the same time, the optimum fermentation conditions were as follows: liquid volume 100/250 mL, 20℃, and fermentation for 7 d. This experiment provides a theoretical basis for the application of biocontrol bacteria in practical production in the future. However, this experiment is only carried out under the shaking table condition. If it is to be applied to actual production, it is necessary to further test its control effect in larger-scale fermentation and give full play to its biological control function (Zhang, 2012).

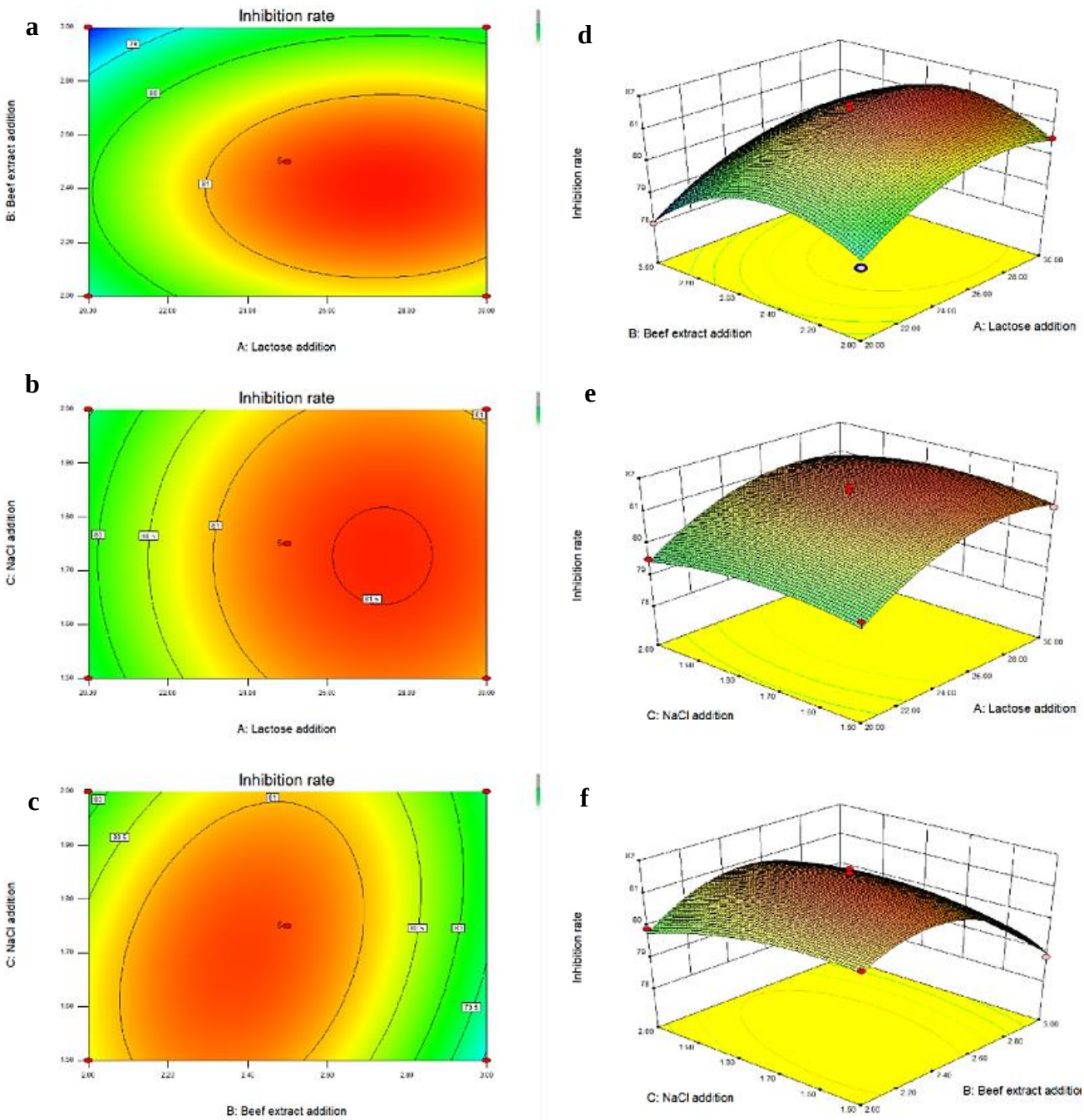

Fig. 4. Contour and 3D surface maps of interactions between factors.

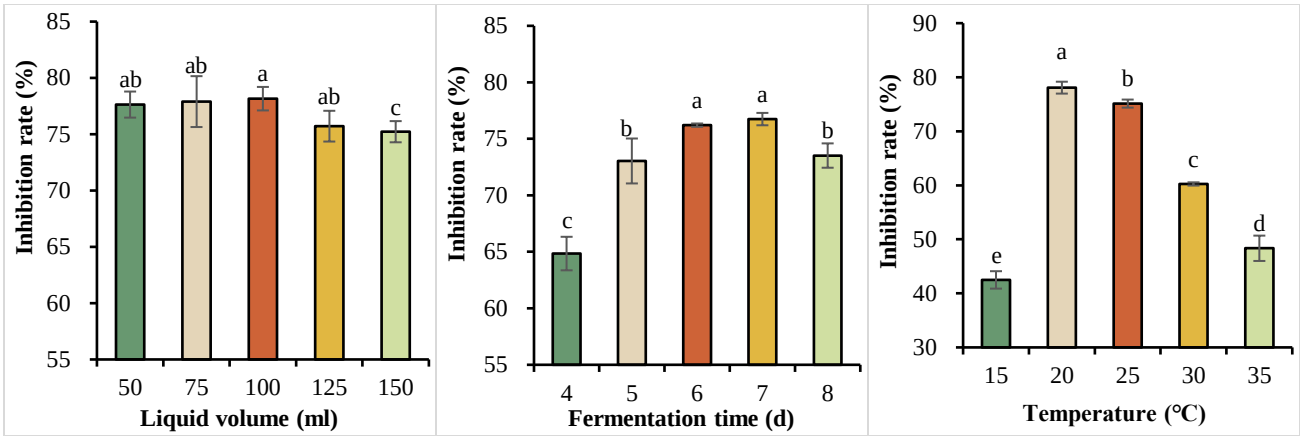

Fig. 6. Inhibition rate of fermentation broth after treatment with different fermentation time, liquid volume and fermentation temperature. Different lowercase letters indicate significant differences at the P=0.05 level.

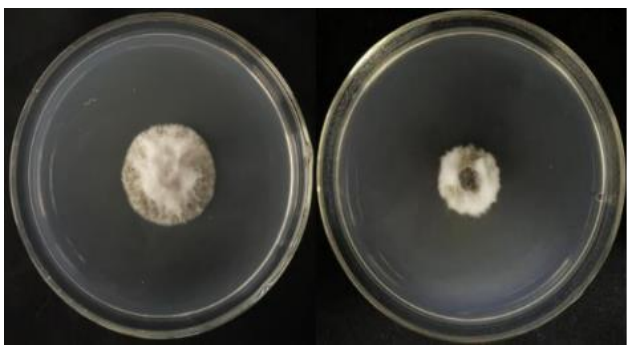

Fig. 5. Comparison of inhibition rate between initial (left) and optimized (right) fermentation.

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