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Optimization of Fermentation Parameters for the Antifungal Substances Production of Biocontrol Strains to Poplar Cancer by Response Surface Methodology

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Fungal strains LX6F2 and YGF9 were respectively isolated from poplar soil and poplar tissues, both of which have strongly antifungal activity to pathogenic *Botryosphaeria dothidia* of poplar canker. In order to improve the antifungal activity of the strains, the fermentation technology was optimized by response surface method. The optimum carbon source, nitrogen source and inorganic salts were determined by single factor experiments. The suitable concentration of carbon, nitrogen source and the basic fermentation conditions were found by orthogonal experiments. The results showed that the effects of the 8 factors on the antifungal activity of LX6F2 strain were not significant by Plackett-Burman design, so the optimized fermentation medium and conditions for strain LX6F2 were as follows: potato juice 200 g / 1000 mL, fructose 1%, beef powder 1.4%, KCl 0.1 %, initial pH value 7.0, the temperature 32°C, shaker speed 180 r/min, inoculation amount 4%, fluid volume 100 mL / 250 mL. The fermentation conditions for strain YGF9 was optimized by central composite design and response surface analysis. Through response surface plot and contour plot analysis, the optimized fermentation medium and conditions for strain YGF9 were as follows: potato juice 200 g / 1000 mL, fructose 1%, beef powder 1.4%, KCl0.1 %, initial pH value 7.0, the temperature 34.14°C, shaker speed is 188.80 r/min, inoculation amount 3.58%, fluid volume 100 mL / 250 mL. The inhibition rate of the strains could reach 75.88% and 84.98% respectively. These results improved the antifungal activity of the two biocontrol strains, and also provided guarantee for exploitation and utilization of biological agents to bio-control of poplar canker.

Key words: Poplar canker, Bio-control strains, Culture medium, Fermentation conditions, Response surface methodology.

Populus clones are widely used as a source of renewable energy and raw material for afforestation and short-rotation forestry (SRF) plantations in northern, northeastern and northwestern parts of China[1]. Species and hybrids of Populus (Salicaceae) are worldwide important in the production of fibre and energy[2]. Poplar canker is one of the main cadres disease to poplar. The infection rate of this disease can reach 80%~100% in the susceptible populus clones species, and the fatality rate is 30%~50%.

The method of control poplar canker is mainly by adjusting the space structure of poplar ecological system and other physical or chemical methods[3]. But ecological control is a relatively long process and chemical control has become a problem to environmental protection, which

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couldn't be ignored. However, people are more and more concerned about the environment safety, the prevention and control of poplar canker also have been put forward with higher safety requirements. With the development of social economy and the enhancement of people's environmental protection consciousness, andmore mature biological control technology, the biological control methods of poplar canker is likely to become the most effective method, which may replace the existing chemical fungicide. New microbial metabolites are permanently needed due to the increase of resistant pathogens, evolution of novel diseases and toxicity that of currently used are compounds[4]. Antagonistic Trichoderma species are considered as promising biological control agents against Numerous phytopathogenic fungi[5].

Given that the main aim of optimization is to maximize the production, this process can be initiated only once when a laboratory-scale purification process and a minimum set of quality control tools are available to quantify and assess the quality of the product[6]. It is important to maintain the optimal fermentation conditions throughout the production process, such as those for temperature, aeration rate, pH, moisture, contents for carbon nitrogen sources, as well as the C/N ratio of the media[7]. The optimization of fermentation medium is of primary importance in the development of any fermentation processes[8]. Optimization of fermentation conditions has been used to substantially enhance yield and productivity of many bio-processes[9]. Optimization of the fermentation process can be conducted either by changing one factor at a time or by varying several factors at the same time and looking for interactions using statistical analysis. The traditional 'one-factor at a time' technique used for optimizing a multivariable system is not only time consuming but also often easily misses the alternative effects between components[10]. In contrast, the observed behavior of fermentation results from the interactive influences of the various variables. Recently many statistical experimental design methods have been employed in bioprocess optimization. The use of statistical experimental design in the optimization of fermentation processes or media is well documented[11,12]. Among them, response surface methodology (RSM) is the one suitable for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently. RSM seeks to identify and optimize significant factors with the purpose of determining what levels of the factors maximize the response (product yield or productivity). It uses statistical experimental designs to develop empirical models that relate a response (dependent variable) to some factors (independent variables)[13]. M. V. Leal-Sanchez researched the optimization of bacteriocin production of *Lactobacillus plantarum* LPCO10, they researched the best nutrition and culture parameters, under these optimal conditions, about 3.2×10^4 times more bacteriocin per liter of culture medium was obtained than that used to initially purify plantaricin S from L. plantarum LPCO10 to homogeneity[14]. Yin Lia optimized fermentation condition (initial pH and temperature) for increase xylanase production from *Penicillium oxalicum* ZH-30 by statistical analysis using response surface methodology[15].

The objective of the present work was to apply statistical methods and Design Expert (7.1.6) to optimize fermentation parameters for enhancing the antifungi substance production by bio-control strains YGF9 and LX6F2. The early research showed that the strains YGF9 and LX6F2 had effectively inhibited to the growth of the Poplar cancer pathogeny *Botryosphaeria dothidia*. Through identified, strain YGF9 was *Trichoderma aureoviride*, and strain LX6F2 was *Fusarium equiseti*. The optimization fermentation parameters could ensure the biocontrol of Poplar canker to develop in an efficient and high quality way.

Materials and methods

MATERIALS

Strains

StrainYGF9 and strain LX6F2 were separated and conserved by the Chinese Academy of Forest Ecology, Environment and Protection of Forest protection key laboratory; poplar canker pathogens *Botryosphaeria dothidia* was supplied by the Chinese Academy of Forest Ecological Environment and Protection Institute.

Medium

Seed medium: PDA medium; initial fermentation medium: fresh potato 200g, distilled water 1000mL.

Methods

Effect of carbon sources on the antifungi activity

As supplementary carbon source, added the concentration of 2% glucose, sucrose, fructose, lactose, soluble starch, corn flour to the basal medium. After cultured the two strains for 5 days, formulated as a spore suspension of 1×10^8 Pore/mL with aquae sterilisata containing 0.01% (w / v) Tween-80. Added 100mL culture medium and 200 µL of spore suspension to each 250 mL conical flask, shaking cultured in 120 r/min, 28°C. After fermentated for 5 days, filtered to remove the mycelium and medium residue in clean benches, centrifugated the filtrate with 6000r/min at 4°Cfor 15 min, and the supernatant was filtrate with 0.22 micron filter membrane filter. Then the inhibition rate of fermentation filtrate to pathogen was measured. Choose the fermentation filtrate of a basal medium without supplementary carbon source as the control. Each experimental design group set three replicates.

Effect of nitrogen sources on the antifungi activity

As supplementary nitrogen sources, added the concentration of 1% beef extract powder, peptone, yeast extract, malt powder, ammonium sulfate, ammonium nitrate to the basal medium. The methods of culture conditions, the fermentation filtrate processing and antifungal activity testing were the same as the previous method. Each experimental design group set three replicates.

The suitable concentration ratio of carbon and nitrogen source

On the basis of carbon and nitrogen sources screening, the suitable concentration ratio of supplemental carbon and nitrogen sources was determined by orthogonal design software which were designed 2 factors 5 level orthogonal test. The concentration of the carbon source were set to 1%, 1.5%, 2%, 2.5%, 3%, the nitrogen concentration were set to 0.6%, 0.8%, 1%, 1.2%, 1.4%.

Effect of inorganic salts on the antifungi activity

To supplement $CaCl_2$, $FeSO_4$, $MgSO_4$, KCl, NaCl to the medium, and set the concentration gradient of 0.1%, 0.2%, 0.3% respectively. The methods of culture conditions, the fermentation filtrate processing and antifungal activity testing were the same as the previous method. Each experimental design group set three replicates.

Effect of fermentation conditions on the antifungal activity

The effects of the initial pH value, temperature, shaker speed, inoculation amount and liquid loading quantity on antifungal activity of bio-control strains was comprehensively analysed. 5 factors and 5 levels of orthogonal test were designed. pH levels were 5, 6, 7, 8, 9, Temperature levels were 20°C, 24°C, 28°C, 32°Cand 36°C, shaker speed levels were 100 r/min, 120 r/min, 140 r/min, 160 r/min and 180 r/min, Inoculation amount levels were as follows: 3%, 4%, 5%, 6%, 7%, fluid volume levels were: 40 mL, 60 mL, 80 mL, 100 mL and 120 mL.

Plackett-burman experiment

On the basis of above experiment the suitable conditions and levels of different factors had been determined. By Plackett-burman experiment, the significant influencing factors for producing antifungi substance of the biocontrol strains had been found, and it would be used for further optimize the fermentation culture medium and conditions.

Steepest ascent experiment design

Non-critical factors were removed by PB experiment, steepest ascent experiment was preliminary obtained the neighboring regions of the most advantage parameters. The Design-expert7.1.6 was used to design and analyze experiment.

Response surface experiments design

Based on the steepest ascent experiment, Box-Behnken design and response surface optimization analysis were chosen to obtain the optimal level of significant factor which would affect the fermentation production of antifungal substances. Using Design-expert 7.1.6 software to achieve experimental design, response surface mapping, modeling and statistical analysis. The experiment was repeated three times, the corresponding response values were the average values of the three experiments results.

RESULTS

Analysis

Effect of different carbon sources on the antifungal activity of the strains

Experimental results (Figure1) indicated that the specific requirements of the strain YGF9 on the

carbon source was not very high, in 6 kinds of selected carbon sources, almost all of the inhibition rate reached 70%, the highest inhibition rate was 73.14% when used fructose as carbon source. There was a greatly influence by different carbon sources on the antifungal activity of the strain LX6F2, if choose fructose as carbon source, the antifungal activity reached to the biggest and the highest inhibition rate was 76.27%.

Effect of different nitrogen sources on the antifungal activity of the strains

From figure 2 it indicated that there was some influence of different organic nitrogen sources and mineral nitrogen sources on the antifungal activity of strains YGF9 and LX6F2. When use beef extract powder as the nitrogen source, both of the antifungal activities of two strains were biggest and the inhibition rate 74.32% and 78.24% respectively.

The suitable concentration ratio of carbon and nitrogen source

The concentration of carbon and nitrogen sources orthogonal experiment results showed (Tab.1)that when the concentration ratio was in the fifth group, the nitrogen concentration was 1%, the nitrogen concentration was 1.4%, the strains LX6F2 and YGF9 inhibitory rate reached the maximum and were 74.91% and 78.44% respectively. Therefore the suitable concentration ratio of carbon and nitrogen source was 1% fructose and 1.4% beef powder.

The suitable inorganic salt and its concentration

As shown in figure 3, when we added 0.1% KCl, the antifungal activity of strains YGF9 and LX6F2 reached to the maximum.

The determination of basic fermentation conditions

Through 5 factors and 5 levels orthogonal experiment, the basic fermentation conditions of the strains YGF9 and LX6F2 were determined: initiation pH 7.0, temperature 28°C, shaker speed 180 r/min, inoculation amount 4%, fluid volume

Table 1. Orthogonal experimental results of carbon-nitrogen concentration ratio

No.	Fructose	Beef extract	LX6F2					YGF9				
		powder	T1	T2	T3	AV	Inhibition rate(%)	T1	T2	T3	AV	Inhibition rate(%)
1	1%	0.60%	38	37	47	40.67	52.15	47	48	55	50.00	41.18
2	1%	0.80%	40	32	39	37.00	56.47	41	47	44	44.00	48.24
3	1%	1%	42	30	35	35.67	58.04	45	39	42	42.00	50.59
4	1%	1.20%	32	29	32	31.00	63.53	32	34	34	33.33	60.79
5	1%	1.40%	27	17	20	21.33	74.91	17	18	20	18.33	78.44
6	1.50%	0.60%	54	46	38	46.00	45.88	49	50	34	44.33	47.85
7	1.50%	0.80%	47	39	41	42.33	50.20	43	38	39	40.00	52.94
8	1.50%	1%	45	46	46	45.67	46.27	42	42	42	42.00	50.59
9	1.50%	1.20%	39	42	40	40.33	52.55	39	37	39	38.33	60.79
10	1.50%	1.40%	43	45	40	42.67	49.80	25	30	32	29.00	65.88
11	2%	0.60%	30	24	30.5	28.17	66.86	38	43	42	41.00	51.76
12	2%	0.80%	42	36	35	37.67	55.68	39	33	37	36.33	57.26
13	2%	1%	37	40	46	41.00	51.76	33	40	42	38.33	54.91
14	2%	1.20%	43	40	39	40.67	52.15	38	37	42	39.00	54.12
15	2%	1.40%	39.5	35	26	33.50	60.59	45	47	40	44.00	48.24
16	2.50%	0.60%	38.5	32	29	33.17	60.98	41	42	28	37.00	56.47
17	2.50%	0.80%	40.5	42	39	40.50	52.35	40	38	40	39.33	53.73
18	2.50%	1%	43.5	37	34.5	38.33	54.91	45	45	48	46.00	45.88
19	2.50%	1.20%	34	37	35	35.33	58.44	35	33	39	35.67	58.04
20	2.50%	1.40%	31	43	34	36.00	57.65	20	24	21	21.67	74.51
21	3%	0.60%	43	46	45	44.67	47.45	48	42	44	44.67	47.45
22	3%	0.80%	46	49	53	49.33	41.96	44	45	43	44.00	48.24
23	3%	1%	50	49	48	49.00	42.35	48	53	49	50.00	41.18
24	3%	1.20%	43	43	42	42.67	49.80	46	46	45	45.67	46.27
25	3%	1.40%	45	35	42	40.67	52.15	49	42	43	44.67	47.45

Table 2. The results of the basic fermentation conditions through orthogonal experiment

No.	pH	Tem. (°C)	Rotate Speed (r/min)	inoculation amount (%)	fluid volume (mL)	YGF9 colony diameter(mm)	LX6F2 inhibition rate(%)	colony diameter (mm)	inhibition rate(%)
1	5	20	100	3	40	41.50	51.18	37	56.47
2	5	24	120	4	60	63.00	25.88	41.67	51.56
3	5	28	140	5	80	64.00	24.71	53	37.65
4	5	32	160	6	100	35.33	58.44	49.33	41.96
5	5	36	180	7	120	34.33	59.61	33	61.18
6	6	20	120	5	100	26.00	69.41	48	43.53
7	6	24	140	6	120	27.33	67.85	41.67	50.98
8	6	28	160	7	40	33.00	61.18	15.67	81.56
9	6	32	180	3	60	37.67	55.68	32	62.35
10	6	36	100	4	80	37.00	56.47	49.33	41.96
11	7	20	140	7	60	53.00	37.65	55.67	34.51
12	7	24	160	3	80	44.33	47.85	49.67	41.56
13	7	28	180	4	100	21.00	75.29	21	75.29
14	7	32	100	5	120	28.33	66.67	20.5	75.88
15	7	36	120	6	40	57.00	32.94	38	55.29
16	8	20	160	4	120	31.33	63.14	30.33	64.32
17	8	24	180	5	40	40.67	52.15	31.33	63.14
18	8	28	100	6	60	42.67	49.80	31.67	95.68
19	8	32	120	7	80	31.67	62.74	27.67	67.45
20	8	36	140	3	100	37.00	56.47	27.67	67.45
21	9	20	180	6	80	47.67	43.92	42.67	49.80
22	9	24	100	7	100	53.33	37.25	57.67	32.15
23	9	28	120	3	120	29.33	65.49	48.33	43.14
24	9	32	140	4	40	26.67	68.62	67.33	20.79
25	9	36	160	5	60	45.33	46.67	49.33	41.96

Table 3. Plackett-Burman experiment design and the results

No.	Experiment factors								Inhibition rate (%)	
	Fructose	Beef extract	KCl X_3 X_1	pH X_4 powderX_2	T X_5	Rotate speed	Inoculation Amount	fluid volume X_6	YGF9 X_7	LX6F2 X_8
1	1	1	1	-1	-1	-1	1	-1	42.91	34.41
2	-1	-1	1	-1	1	1	-1	1	64.59	61.25
3	1	1	-1	-1	-1	1	-1	1	59.16	64.59
4	1	1	-1	1	1	1	-1	-1	78.75	63.34
5	-1	1	-1	1	1	-1	1	1	51.25	62.09
6	-1	1	1	-1	1	1	1	-1	62.5	73.34
7	-1	-1	-1	1	-1	1	1	-1	52.41	58.75
8	1	-1	1	1	1	-1	-1	-1	55.41	68.75
9	1	-1	1	1	-1	1	1	1	47.59	45.83
10	-1	1	1	1	-1	-1	-1	1	38.33	41.75
11	-1	-1	-1	-1	-1	-1	-1	-1	41.66	53.34
12	1	-1	-1	-1	1	-1	1	1	46.67	66.25

100 mL / 250 ml.

Determination of significant factors by Plackett - burman test

As the aim to observe and study the 8 factors: fructose, beef powder, KCl, pH, temperature, speed, inoculation amount, fluid volume, we used the method PB two levels test to design an experiment(N = 12). Each factor was set two levels of high (+ 1) and low (1) separately, the response value was inhibition rate (Y), inhibition rate of strain YGF9 expressed as Y1, inhibition rate of strain LX6F2 expressed as Y2. Through the PB test, we screened out the significant factors of influencing antifungal activity. Design of experiments and the results were shown in table 3, the level and effect of each factor analysis were shown in table 4. Through regression fitting, two multivariate regression equations were obtained:

$$Y1 = 0.823*X_1 + 1.024*X_2 - 7.738*X_3 + 1.042*X_4 + 3.213*X_5 + 0.740*X_6 - 2.881*X_7 - 0.109*X_8 - 169.419 \quad ...(1)$$

$$Y2 = -0.306*X_1 - 0.610*X_2 - 17.929*X_3 - 2.112*X_4 + 4.015*X_5 + 0.338*X_6 - 1.029*X_7 - 0.042*X_8 - 62.693 \quad ...(2)$$

Decision coefficient of the equation 1 was $R^2 = 0.9873$, which means that the equation of the regression was very good. Decision coefficient of the equation 2 was $R^2 = 0.7435$, which means that the equation of the regression was preferably.

The table 4 showed that the impact response value of eight factors, for the strain YGF9, there were three significant factors (P - value < 0.05), the level of impact was in the following order: shaker speed > temperature > inoculum amount.

Table 4. Evaluation for the effect of PB experiment design

No.	Factor	Levels		YGF9			LX6F2		
		-1	1	Estimate	t-Value	p-Value	Estimate	t-Value	p-Value
X_1	Fructose(%(	0.8	1.2	1.65	1.7	0.1392	-0.61	-0.19	0.8637
X_2	Beef extract powder (%)	1.2	1.6	2.05	1.84	0.0885	-1.22	-0.54	0.7344
X_3	KCl (%)	0.08	0.12	-1.55	-2.04	0.1565	-3.59	-1.55	0.3540
X_4	pH	6.5	7.5	0.52	0.63	0.5716	-1.06	-0.44	0.7685
X_5	Temperature(°C)	26	30	6.43	6.97	0.0044	8.03	4.01	0.0917
X_6	Rotate speed(r/min)	170	190	7.4	8.12	0.0029	3.38	1.57	0.3789
X_7	Inoculation amount (%)	3	5	-2.88	-3.19	0.0394	-1.03	-0.40	0.7741
X_8	fluid volume (mL)	80	120	-2.17	-1.69	0.0777	-0.85	-0.30	0.8127

Table 5. Design and data from the steepest ascent experiment

No.	Experiment factors			
	Temperature (°C) X_5	Rotate speed (r/min) X_6	Inoculation amount (%)X_7	Inhibition rate(%)
1	30	190	5	46.14
2	31	195	4.5	53.69
3	32	200	4	76.28
4	33	205	3.5	64.63
5	34	210	3	49.21

Table 6. Factors and levels for central composite experiment

Factors	Level		
	-1	0	1
Tempreture(X_5)°C	29	32	35
Rotate speed(X_6)r/min	185	200	215
Inoculation amount(X_7)%	3	4	5

According to the results, the three factors were chosen to carry out the steepest climbing test. For strain LX6F2, eight factors were not significant (P - value > 0.05), therefore the fermentation conditions of the strain wasn't optimized further more. Through a series of experiments, we confirmed the optimized fermentation medium and conditions for strain LX6F2 were as follows: potato juice 200 g / 1000 mL, fructose 1%, beef powder

1.4%, KCl0.1 %, initial pH value is 7.0, the temperature 32°C, shaker speed 180r/min, inoculation amount 4%, fluid volume 100 mL / 250 mL.

Determination the scope of the center value by the steepest ascent experimental

By evaluation of the effect of the PB test and its fitting equation, there were two factors out

Table 7. Design and results from central composite experiment

Std	Run	Tempreture (°C)	rotate speed(r/min)	inoculation amount(%)	inhibition rate(%)	Predicted Value(%)
8	1	35	200	5	72.24	71.12
12	2	32	215	5	65.46	66.90
17	3	32	200	4	79.95	79.49
15	4	32	200	4	79.17	79.49
10	5	32	215	3	74.01	72.95
11	6	32	185	5	67.14	68.20
13	7	32	200	4	78.42	79.49
5	8	29	200	3	53.49	54.61
4	9	35	215	4	70.92	70.60
14	10	32	200	4	79.42	79.49
2	11	35	185	4	84.81	84.87
6	12	35	200	3	79.95	81.33
9	13	32	185	3	75.42	73.98
1	14	29	185	4	49.02	49.34
7	15	29	200	5	54.36	52.98
3	16	29	215	4	61.35	61.29
16	17	32	200	4	80.49	79.49

Table 8. Coefficient estimates for regression equation

Parameters	Estimate	Std.error	t-Value	F-Value	p-Value
X_5-Tempreture	11.2125	0.523748	101.3753	458.3107	< 0.0001
X_6-rotate speed	-0.58125	0.523748	11.27836	1.231632	0.3038
X_7-inoculation amount	-2.95875	0.523748	62.81125	31.9133	0.0008
X_5* X_6	-6.555	0.740692	-0.14567	78.31961	< 0.0001
X_5* X_7	-2.145	0.740692	-0.715	8.38648	0.0231
X_6* X_7	-0.0675	0.740692	-0.0045	0.008305	0.9299
X_5^2	-9.23125	0.721937	-1.02569	163.5018	< 0.0001
X_6^2	-3.73375	0.721937	-0.01659	26.74804	0.0013
X_7^2	-5.24875	0.721937	-5.24875	52.85831	0.0002

Table 9. ANOVA analysis of coefficient of the regression equation

Variance Source	Degree of freedom	Sum of squares	Mean squares	F-Value	p-Value
Model	9	1853.6737	205.9637	93.85467	< 0.0001
Linear	3	1078.49768	359.4992	5.911788	0.0090
Quadratic	3	584.881601	194.9605	88.84067	< 0.0001
Cros Product	3	190.294425	63.43148	6.979828	0.0456
Lack of fit	3	12.897675	4.299225	1.056763	0.4100
Pure Ermr	4	2.4638	0.61595		
Total Erm	17	87370.1872	5139.423		

R^2=0.9918ÿ Adj R^2=0.9812ÿPre R^2=0.8875ÿ Adeq Precision=31.2762ÿCV=2.0888ÿ PRESS=210.2125

of the three factors which had significant effect on the antifungal activity, temperature and rotation speed showed positive effect, namely the Estimate > 0. 05. Its value should be improved appropriately so as to improve the response value. And the other factor inoculation amount showed negative effect, namely Estimate < 0; its value should be reduced appropriately so as to improve the response value. According to the proportion of the three factors effects, their changing direction and stepping length had been setting for steepest climbs experiments of strains YGF9. The experimental design and the results were shown in table 5.

The table 5 showed that the inhibition rate was the highest in experiment 3, which suggested that the optimum point probably appears nearby. Therefore, we select 32°C, 200 r/min, inoculum concentration 4% as the center to carry out the response surface experiment.

The response surface analysis and the optimal fermentation technology

The factors and levels were showed in table 6. The regression fitting of the test results of table 7 was implemented by software SAS9.1, the quadratic response surface regression model was established. The following regression equation for antifungal activity:

$$Y=101.3753\,X_5+11.27834\,X_6+62.8112\,X_7-0.1457\,X_5{*}X_6-0.715\,X_5{*}X_7-4.5\,X_6{*}X_7-1.0257\,X_5^2-0.0166\,X_6^2-5.2488\,X_7^2-2845.9806$$

Y is the response that is inhibition rate (%) and X_5, X_6, X_7 are coded values of the test variables, Tempreture (°C), rotate speed(r/min), inoculation amount (%), respectively.

The results of coefficient estimates for regression equation were showed in table 8. The parameters which had small value of "Prob>F" less than 0.05 indicated that model terms were significant. The table showed that the regression equation of the liner term X5 and X7, quadratic term of $X5^2$, $X6^2$ and $X7^2$, interaction term X5* X6 affect model highly significant ($P < 0.01$), the interaction term X5* X7 impact model significantly ($P < 0.05$), liner term X6 and interaction term X6* X7 impact model were not significant ($P > 0.05$). It showed that the response of the model value change was relatively complex, it wasn't a simple linear relation, surface effect was remarkable.

ANOVA analysis of coefficient of the regression equation was showed in table 9. Through F-Value check out, this model had highly significant and reliability (P<0.000 1), and both of the liner term and quadratic term were significant, it means that equation of quadratic fitting

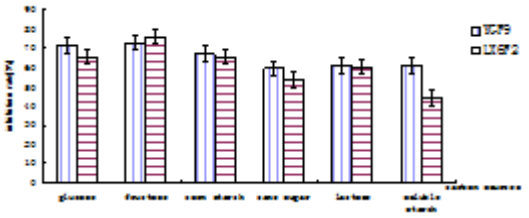

Fig. 1. Effect on different carbon sources to antimicrobial activity

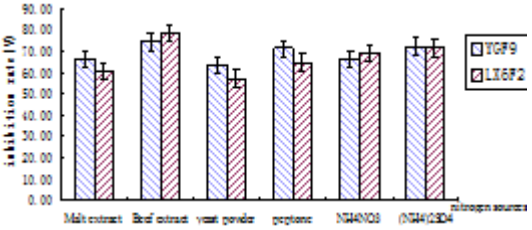

Fig. 2. Effect on different nitrogen sources to antimicrobial activity

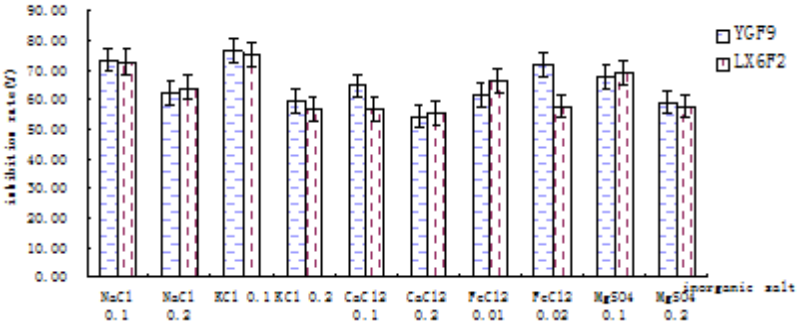

Fig. 3. Effect on different kinds and concentration inorganic salt to antimicrobial activity

regression was suitable. The $P_{Lack\ of\ fit}$ = 0.4100, which was not significant. If $P_{Lack\ of\ fit}$<0.05, which means that the model may have not included all appropriate functions of independent variables or the experimental region may be too large for the quadratic model used[16]. The determination coefficient R^2 (99.18 %) was sufficient. Adjusted R^2 is a modification of R^2 that adjusts for the number of explanatory terms in a model. Unlike R^2, the adjusted R^2 increases only if the new term improves the model more than what would be expected by chance [17]. The adjusted R^2 is 0.9812. The coefficient of variation (CV) indicating the degree of precision with which the treatments were compared, is 2.0888. Relatively lower value of CV indicates a better precision and reliability of the experiments carried out.

With the aim to better understand the relationship between the independent variables, we used the design expert 7.1.6 software to make the three dimensional response surface plot and contour plot. Known from the analysis of software, when X_5=34.14, X_6= 188.80, X_7=3.58, the response value Y reached the maximal values. As the X_5, X_6, X_7 far away from the extreme value, Y values decreased gradually. It was appeared as concentric contour of the declining response value in the contour plot.

The response surface plot can reflect when they were made sure in a value range one factor affect the change trendency of response value after the other factor is determined. Contour plot shape of the two factors can reflect the strong and weak of interaction effect. Circular shows the interaction effect isn't significant, and ellipse means that the interaction effect is significant[18].

Figure 4 showed the response surface plot and contour plot of temperature and rotate speed on inhibitive rate of strain YGF9 when the inoculation amount was zero level. Through the

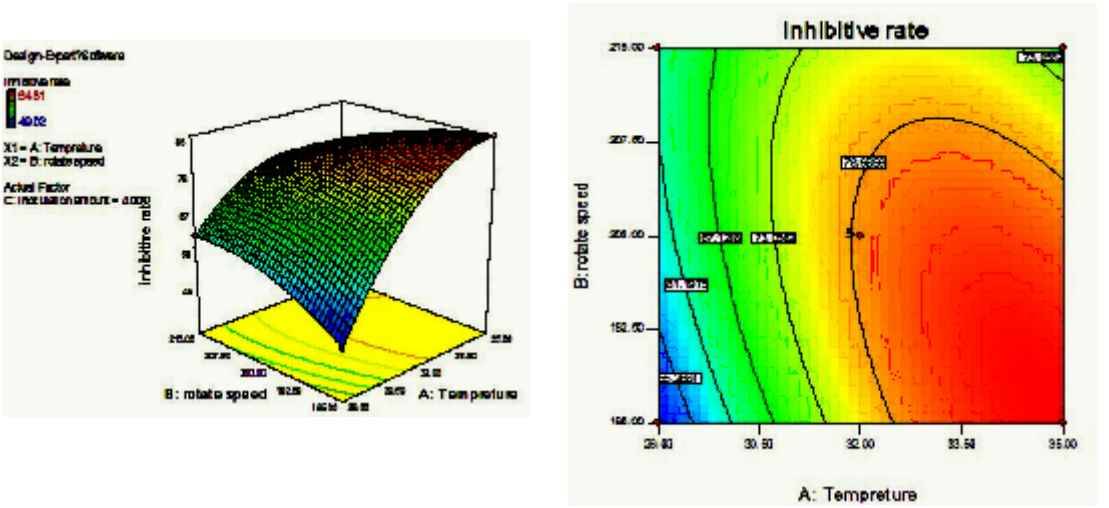

Fig. 4. Response surface plot and contour plot of temperature and rotate speed on inhibitive rate of YGF9

contour plot we can see that the reciprocal action of the two factors was significant. In a range of value, along with the increasing of temperature and rotational speed, the inhibition rate increased. When the rotate speed was 208.93r/min, inhibition rate increased along with the increasing of the fermentation temperature. When the temperature was 31.85°C^ÿ35°C and rotational speed was 185r/min^ÿ208.93r/min respectively, the inhibition rate could maintain above 78.9856%.

Figure 5 showed the response surface plot and contour plot of temperature and inoculation amount on inhibitive rate of strain YGF9 when the rotate speed was zero level. Through the contour plot we can see that the reciprocal action of the two factors was significant. In a range of value, along with the increase of temperature, the inhibition rate increased. Along with the increase of inoculation amount, the inhibition rate appeared the trend of firstly increased then decreased. When the temperature was 31.62°C, inhibition rate decreased when inoculation amount increased. When the inoculation amount was 4.56%, inhibition rate increased when increasing the fermentation

temperature. When the temperature was 31.62°C^ÿ35°C and inoculation amount was 3%^ÿ4.56% respectively, the inhibition rate could maintain above 78.6467%.

Figure 6 showed that when the temperature was zero level, the response surface plot and contour plot of rotate speed and inoculation amount on inhibitive rate of strain YGF9. We can see that the shape of the contour was almost circular, which indicated that the interaction effect of the two independence variables wasn't significant.

Verification on the results of RSM

According to the RSM results, the optimization fermentation technology of strain YGF9 was that: potato juice 200 g / 1000 mL, fructose 1%, beef powder 1.4%, KCl0.1 %, initial pH value 7.0, the temperature 34.14°C, rotate speed 188.80 r/min, inoculation amount 3.58%, fluid volume 100 mL / 250 mL. In order to facilitate operation and control in the actual production, we adjusted the temperature to34°C, rotate speed to190r/min and inoculation amount to 3.5%.

In order for verification accuracy and availability of the results of response surface

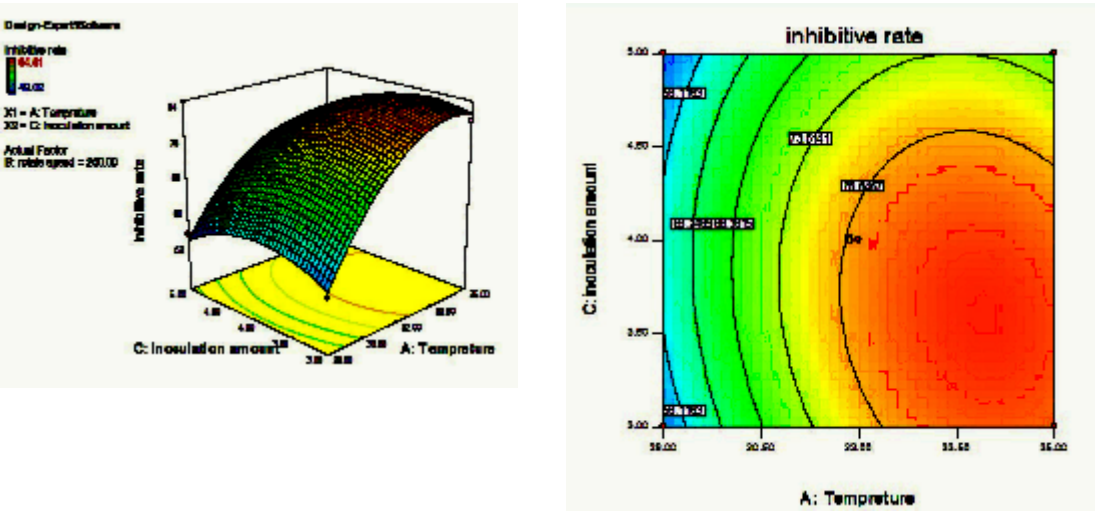

Fig. 5. Response surface plot and contour plot of temperature and inoculation amount on inhibitive rate of YGF9

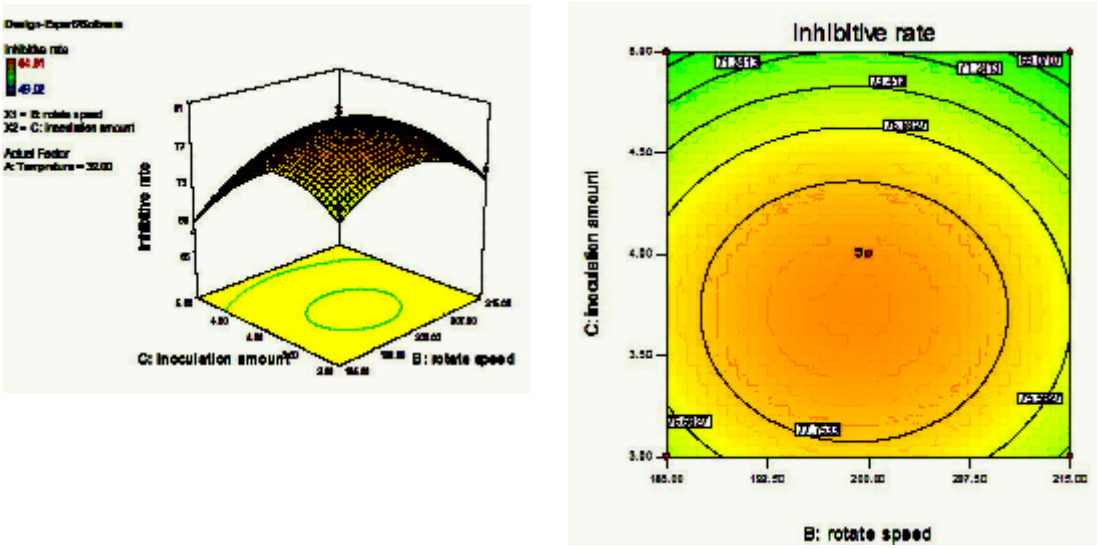

Fig. 6. Response surface plot and contour plot of rotate speed and inoculation amount on inhibitive rate of YGF9

analysis, fermentation verification test had been done three times. The results showed that the average inhibition rate of strain YGF9 was 84.98% under the optimization condition. It's very close to the predicted value Y =85.5667%. This confirms that using the RSM to optimization the fermentation was feasible and effective.

DISCUSSION

We optimized the fermentation of two biocontrol strains in this study. By single factor, orthogonal experiments and response surface analysis, the optimization fermentation technology of strain LX6F2 was: potato juice 200 g / 1000 mL, fructose 1%, beef powder 1.4%, KCl0.1 %, initial pH value is 7.0, the temperature 32°C, shaker speed 180 r/min, inoculation amount 4%, fluid volume 100 mL/250 mL; the optimization fermentation technology of strain YGF9 was that: potato juice 200 g/1000 mL, fructose 1%, beef powder 1.4%, KCl0.1 %, initial pH value 7.0, the temperature 34°C, rotate speed 190r/min and inoculation amount 3.5%, fluid volume 100 mL/250 mL. The inhibition rate of the strains could reach to 75.88% and 84.98% respectively.

Optimization of fermentation technology is fundamental for using excellent microbial resources. Only in a reasonable nutrition conditions and the suitable cultivation environment, it is possible to maximum utilize the useful metabolites of microorganisms. Culture medium ingredients, concentration and fermentation conditions of the microorganism, have important influence on product quality and yield[19]. The optimization fermentation technologies of strains we studied in this research ensure the quality of the two biocontrol strains to produce antifungal active substances. It provides the premise conditions for exploitation and utilization of biological agents, and lays a foundation for bio-control of poplar canker.

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REFERENCES

1. Jun Liang, Zhiqiang Sun, Zhiwei Qua, et al. Long-term effect of an ectomycorrhizal inoculum and other treatments on survival and growth of Populus hopeiensis Hu et Chow. *Forest Ecology and Management*, 2010; **259** : 2223-2232.
2. Nixon DJ, Stephens W, Tyrrel SF, Brierley EDR. The potential for short rotation energy forestry on restored landfill. *Bioresource Techonol,* 2001; **77**: 237-245.
3. HUANG Feng-long, JIAO Yi-jie, LIANG Jun, et al. Relationship between crown structure of poplar and poplar canker in different site conditions. *Journal of Beijing Forestry University,* 2011; **33**(2):72-76.
4. Demain, A.L. Microbial natural products: alive and well in 1998. *Nat. Biotechnol*, 1998; **16**: 3-4.
5. Sarhan, M. M., Ezzat, et al. Application of Trichoderma hamatum as a Biocontroller against Tomato Wilts Disease Caused by Fusarium oxysporum f. lycopersici. *Egyptian J. Microbiol,* 1999; **34**: 347-376.
6. Michel Thiry, Doriano Cingolani. Optimizing scale-up fermentation processes. *Trends in Biotechnology*, 2002; **20**(3): 103-105.
7. Kim, K. A., Noh, et al . Kinetics of cellulose saccharification by Trichoderma reesei cellulases. *Journal of Microbiology and Biotechnology*, 2000; **10**: 69-74.
8. Isar, J. Isar, L. Agarwal, et al. A statistical method for enhancing the production of succinic acid from Escherichia coli under anaerobic conditions. *Bioresoure Technology*, 2006; **97**: 1443-1448.
9. Chisti Y, Moo-Young M. Bioprocess intensification through bioreactor engineering. *Trans Inst Chem Eng,* 1996; **74A**: 575-583.
10. B.V.V. Ratnam, M. Narasimha Rao, M. Damodar Rao, et al. Optimization of fermentation conditions for the production of ethanol from sago starch using response surface methodology. *World Journal of Microbiology & Biotechnology*, 2003; **19**: 523-526.
11. Altaf, Md. Altaf, B.J. Naveena, et al . Use of inexpensive nitrogen sources and starch for L (+) lactic acid production in anaerobic submerged fermentation. *Bioresoure Technology*, 2007; **98**: 498-503.

12. Frances, G.L. Allan, B.F. Nowak. The effects of nitrite on the shortterm growth of silver perch (Bidyanus bidyanus). *Aquaculture*, 1998; **163**: 63-72.
13. Sarat Babu IMANDI, Sita Kumari KARANAM, Hanumantha Rao GARAPATI. Optimization of fermentation medium for the production of Lipopeptide using artificial neural networks and genetic algorithms. *International Journal of Natural and Engineering Sciences*, 2008; **2**(2): 105-109.
14. M. V. Leal-Sanchez, R. Jimenez-Díaz, J. L. Ruiz-Barba, et al . Optimization of bacteriocin Production by batch fermentation of Lactobacillus plantarum LPCO10. *Applied and enviromental microbiology*, 2002; **68**(9): 4465-4471.
15. Yin Lia, Zhiqiang Liub, Hui Zhaoa, et al. Statistical optimization of xylanase production from new isolated Penicillium oxalicum ZH-30 in submerged fermentation. *Biochemical Engineering Journal*, 2007; **34**: 82-86.
16. Martínez KD, Pilosof AMR. Relative viscoelasticity of soy protein hydrolysate and olysaccharides mixtures at cooling conditions analyzed by response surface methodology. *Food Hydrocolloids*, 2012; **26**: 318-322.
17. Muhammet Saban Tanyildiziule Bulut, Veyis Selen and Dursun Özer. Optimization of lactic acid production with immobilized Rhizopus oryzae. *African Journal of Biotechnology*, 2012; **11**(34): 8546-8552.
18. Yong-Bin Wang, Yun-Xiang Wang. Optimization of cultivation conditions for extracellular polysaccharide fermented by Ustilago maydis using response surface methodology. *China Brewing*, 2006; **5**: 56-60.
19. Qian Mingyong. (ed): Optimization control of fermentation engineering. In: jiangsu science and technology press, Nanjing, 1998; pp 46-58.