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Optimization of Medium Components for Improving the Antifungal Activity from *Pseudomonas protegens* XL03

Feifei Wang, Qizhong Zhang*

Institute of Hydrobiology, Jinan University, Engineering Research Center of Tropical and Subtropical Aquatic Ecological Engineering Ministry of Education, Eutrophication and Red Tide Prevention of Guangdong Higher Education Institutes, Guangzhou, People's Republic of China

Email address

zhangqzdr@126.com (Qizhong Zhang)

*Corresponding author

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Abstract

Pseudomonas protegens XL03 showed the antifungal activity against *Saprolegnia* sp., and its cell-free disruption supernatant (CFDS) inhibited the hyphal growth with an antifungal zone diameter of 19.34±1.20 mm. In order to improve the production of the antifungal compounds which resided in intracellular, Plackett-Burman design (PBD) and Response surface methodology (RSM) were applied to optimize the medium of the strain XL03. Firstly, the process parameters that significantly affected the antifungal compounds production (tryptone, glucose and NaCl) were identified by PBD; Subsequently, the optimum levels of these significant parameters were determined using RSM, which showed as follows: tryptone (7.50 g/L), glucose (8.79 g/L) and NaCl (1.27 g/L); Finally, the experimental validation was conducted under the optimal conditions， and the antifungal activity (antifungal zone diameter) increased about 4 mm in comparison with that on the un-optimized medium.

1. Introduction

Saprolegniasis is a widespread disease in freshwater aquaculture, and causes a huge economic losses in aquaculture industry. The pathogens, *Saprolegnia* spp., have a ubiquitous distribution [1]. *Saprolegnia* spp. produced a large number of infectious spores at low temperatures [2]. Fish and fish eggs were commonly parasitized by the spores. Fungal pathogens infected the surface of the fish's body, thus caused epidermal damage and cellular necrosis, and finally resulted in diseased fish death.

In the aquaculture industry, chemical drugs were mainly used to prevent and cure the fungal infection with their effectiveness and convenience. Malachite green was extremely effective for control of saprolegniasis, and was widely used as a therapeutic agent in aquaculture in past decades [3]. However, it has been banned for usage in fish food around the world because of its potential carcinogenicity, mutagenicity and teratogenicity [4]. Meanwhile, other chemicals, such as bronopol, sodium chloride, potassium permanganate, hydrogen and peroxide had been applied to control of saprolegniasis [1, 5], but these chemicals have no good efficacy for treatment of saprolegniasis. Besides all, a large amount of chemical drugs resulted in the environmental pollution and residues in edible aquatic animals, and were further harmful to human health. Therefore, there is an urgent

need to find a safe and effective agent for controlling saprolegniasis.

Recently, microbiological control, which exploited the interaction of microorganisms to inhibit the growth of pathogens, had received more and more attention [6]. Some anti-*Saprolegnia* bacteria strains were discovered，such as *Aeromonas* sp., *Serratia marcescens*, *Bacillus subtilis*, *B. amyloliquefaciens* and *Streptomyces* sp. [6, 7]. Moreover, the saprolegniasis of *Bidyanus bidyanus* was successfully treated with the strain A199 [8]. This provided the feasibility of microbiological agents to prevent and cure the saprolegniasis.

In this study, the strain XL03 exhibited the efficient antifungal activity against *Saprolegnia* sp., and the antifungal compounds resided in the cytoplasm. In order to improve the production of the antifungal compounds, statistical methods have been applied for optimization of fermentation medium for the maximum antifungal activity.

2. Materials and Methods

2.1. Tested Strains and Media

Saprolegnia sp. was isolated from the diseased fish tissue, and cultured on the potato dextrose agar (PDA) medium at 25°C. To obtain the fresh spore suspension, the *Saprolegnia* hyphae were placed into the Erlenmeyer flask with sterile water and were incubated at 25°C for 48 h, and quantified by Hu's method [9]. All the fungal materials stored at 4°C for usage.

Pseudomonas protegens XL03 was isolated with the gradient dilution method from sediment of Baimang River in Xili, Guangzhou, China. It was cultured in Luria Bertan (LB) broth medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 28°C in an incubator shaker for 48 h, and its antifungal activity was evaluated with the following methods.

2.2. Antifungal Activity Assay of CFDS *in vitro*

The fermentation broth was centrifuged at 5,974 g for 15 min at 4°C, and the cells were collected. The pellet was rinsed with phosphate buffer saline (PBS, 49.5% Na_2HPO_4, 50.5% NaH_2PO_4, 0.02 M, pH 6.8) for two times, and subjected to sonication (Ultrasonic processor, UH-950B, China) for 4 s burst at 100 W and with 6 s cool period in a total of 20 min. The sonicate solution was centrifuged at 15,777 g for 30 min at 4°C, the supernatant was filtered through 0.45 μm filters to yield the CFDS.

Antifungal activity of CFDS was determined against *Saprolegnia* hyphal growth using agar well diffusion method [10]. Four hyphal PDA blocks (8-mm diameter) were put around the well at about three centimeters on the PDA plate, and the well was added with CFDS (200 μL), then the PDA plates were incubated at 25°C for 72 h. Finally, the antifungal zone diameters were measured and recorded, and the sterile PBS used as a blank control.

2.3. Selection of Basal Medium

The antifungal activity of CFDS depended on growth of the strain XL03 on eight different media (Table 1). Eight kinds of culture media (200 mL) were inoculated with 2 mL of the seed culture, and incubated at 28°C, 160 rpm for 48 h. The cells were collected from the culture broth by centrifugation at 5,974 g post 48 h, and the antifungal activity of CFDS from different media was evaluated under the same conditions. The best basal medium was used for next optimization.

Table 1. *Components of eight media used for selecting basal media for the strain XL03.*

BMP1		**BMP2**		**BMP3**		**BMP4**	
Tryptone	**10.0g**	**Tryptone**	**20.0g**	**Tryptone**	**5.0 g**	**Tryptone**	**10 g**
Yeast extract	5.0g	$MgSO_4.7H_2O$	1.5 g	Yeast extract	1.0 g	Na_2HPO_4	2.5 g
NaCl	10.0g	K_2HPO_4	1.5 g	Beef extract	3.0 g	Glucose	2.0 g
Distilled water	1.0L	Glycero	10 mL	NaCl	1.0 g	Beef heart infusion	5 mL
		Distilled water	1.0 L	Glucose	10g	NaCl	5.0g
				$MgSO_4.7H_2O$	1.5 g	Distilled water	1.0L
				K_2HPO_4	1.5 g		
				Glycero	10 mL		
				Distilled water	1.0 L		
BMP5		BMP6		BMP7		BMP8	
Tryptone	10 g	Potato	200g	Tryptone	17 g	$(NH4)_2SO_4$	20 g
Beef extract	3.0 g	Glucose	20g	Soya peptone	3.0 g	Glucose	23 g
NaCl	5.0 g	Distilled water	1.0 L	Glucose	2.5 g	NaCl	5.0 g
Distilled water	1.0 L			K_2HPO_4	2.5 g	KNO_3	5.5 g
				NaCl	5.0 g	Distilled water	1.0 L
				Distilled water	1.0 L		

2.4. Screening for Medium Components Using Plackett-Burman Design (PBD)

The PBD is a statistical technique used for screening the significant variables that had an important impact on the response in the study [11]. The medium components, codes and levels of the different variables of the experimental design were listed in Table 2. The eight medium components at two levels, high (1) and low (-1) related to the 12 tests to determine their effects on the antifungal activity (Table 3). Every test was conducted in triplicates and the diameter of antifungal zone was noted as response. The variables were considered to be significant on antifungal compound

production with confidence levels above 95%, and all the data analysis was conducted by Minitab 16.0 software [12].

Table 2. *Variables, their codes and levels under PBD.*

Medium components	Codes	Low level (-1)	High level (1)
Tryptone (g/L)	A	0.75	7.5
Yeast extract (g/L)	B	0.15	1.5
NaCl (g/L)	C	0.15	1.5
Beef extract (g/L)	D	0.45	4.5
Glucose (g/L)	E	1.50	15
$MgSO_4.7H_2O$ (g/L)	F	0.225	2.25
K_2HPO_4(g/L)	G	0.225	2.25
Glycero (ml/L)	H	1.5	15

Table 3. *PBD and the experimental response obtained for strain XL03. The variables of A, B, C, D, E, F, G, H represents tryptone, yeast extract, NaCl, beef extract, glucose, $MgSO_4.7H_2O$, K_2HPO_4, glycerol in the BMP3, respectively.-1 and 1 are code values. All experiments were done at the same time. Values are expressed as mean ± SD from three replicates.*

Trials	Variables								Diameter of antifungal zone in mm
	A	B	C	D	E	F	G	H	
1	-1	-1	-1	1	1	1	-1	1	10.86±1.56
2	-1	1	1	1	-1	1	1	-1	12.78±0.86
3	1	-1	1	1	-1	1	-1	-1	13.64±1.00
4	1	1	-1	1	-1	-1	-1	1	12.10±1.38
5	-1	1	1	-1	1	-1	-1	-1	18.68±1.28
6	-1	1	-1	-1	-1	1	1	1	8.00±0.00
7	1	1	-1	1	1	-1	1	-1	19.70±1.14
8	-1	-1	-1	-1	-1	-1	-1	-1	8.00±0.00
9	1	-1	1	-1	-1	-1	1	1	19.74±1.26
10	1	1	1	-1	1	1	-1	1	21.70±1.58
11	1	-1	-1	-1	1	1	1	-1	18.90±1.08
12	-1	-1	1	1	1	-1	1	1	12.20±1.18

2.5. Optimization of Significant Medium Components Using Response Surface Methodology (RSM)

RSM is one of the methods to evaluate the relationship between a set of controlled experimental factors and observed results [13]. RSM was applied to determine the optimum levels of the three most significant medium components (tryptone, glucose, NaCl) through Design Expert 8.0 software in this study. The significant variables like tryptone, glucose and NaCl were designated as A, B, and C, respectively. The low, middle, and high levels of each factor were designated as −1, 0, and 1, respectively, and shown in Table 4. The experimental plan consisted of 17 trials, and the experimental design was shown in Table 5. All the experiments were done in triplicate and the diameter of antifungal zone was taken as response. The regression analysis involving three significant independent variables (tryptone, glucose, NaCl) was performed to estimate the response function as a second order polynomial equation:

$$Y = b_0 + \sum b_i x_i + \sum b_{ij} x_i x_j + \sum b_{ii} x_i^2 \quad (1)$$

Where Y is the estimate response, b_0 is constant, b_i is the linear coefficient, b_{ij} is the quadratic coefficient and b_{ii} is the interaction coefficient.

The Design Expert software was applied to the regression analysis. The statistical adequacy of the model was determined through analysis of variance (ANOVA) with a confidence level of 95% ($p < 0.05$). The quality of the polynomial model equation was also judged by the coefficient of determination (R^2) and adjusted R^2. Three-dimensional surface response plots were drawn by changing two variables within the experimental range, and the other variables were held at the moderate point. An optimum level of the variables for maximum antifungal activity was obtained by regression analysis of the software.

Table 4. *Variables, their codes and levels for RSM.*

Variables	Code	Level		
		1.00	0.00	-1.00
Tryptone (g/L)	A	7.5	4.125	0.75
Glucose (g/L)	B	15	8.25g	1.5
NaCl (g/L）	C	1.5	0.825	0.15

Table 5. *Response surface design for different medium components with three independent variables and antifungal activity in every trial. Values are expressed as mean ± SD from three replicates.*

Trials	Tryptone	Glucose	NaCl	Diameter of antifungal zone in mm
1	0	1	1	22.78±1.56
2	-1	0	-1	22.13±0.94
3	0	0	0	23.07±1.52
4	1	0	-1	21.98±1.58
5	0	1	-1	22.40±1.05
6	0	0	0	22.98±1.34
7	-1	-1	0	21.70±0.65
8	0	-1	-1	22.00±1.14
9	0	0	0	22.95±0.86
10	-1	1	0	22.55±1.21
11	1	0	1	22.86±1.24
12	1	1	0	22.49±1.23
13	1	-1	0	22.48±1.82
14	0	-1	1	22.23±1.87
15	-1	0	1	22.00±0.96
16	0	0	0	22.91±2.10
17	0	0	0	22.76±1.98

2.6. Experimental Validation of Optimization

The statistical model and the optimization were experimentally validated by culturing the strain XL03 on the un-optimized and optimized media at 28°C for 48 h. In order to compare the antifungal activity of strain XL03 on the un-optimized and optimized media, CFDS was extracted with equal volume, and evaluated its antifungal activity under the same conditions.

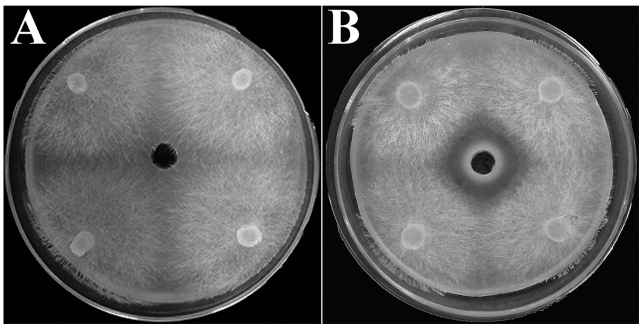
2.7. Statistical Analysis

All the experiments were carried out in three replicates. Student *t*-test was carried out in statistical analysis system software package (SPSS 19.0), and statistically significant difference was present at $p< 0.05$. All experimental data were expressed as the mean ± SD (standard deviation).

3. Result

3.1. Inhibition of *Saprolegnia* Hyphal Growth by CFDS

CFDS displayed antifungal activity against *Saprolegnia* hyphal growth in plate tests. The antifungal zone diameter of 19.34±1.20mm revealed CDFS could inhibit the hyphal growth in comparison with the blank control (Figure 1).



Note: CFDS, cell-free disruption supernatant; A, blank control; B, experiment group.

Figure 1. *Inhibition efficacy of CFDS against Saprolegnia hyphae.*

3.2. Appropriate Basal Medium

The eight kinds of media had a different effect on the antifungal activity (Figure 2). Based on the size of antifungal zone diameter, BMP3 had the highest antifungal activity, and was selected for further study.

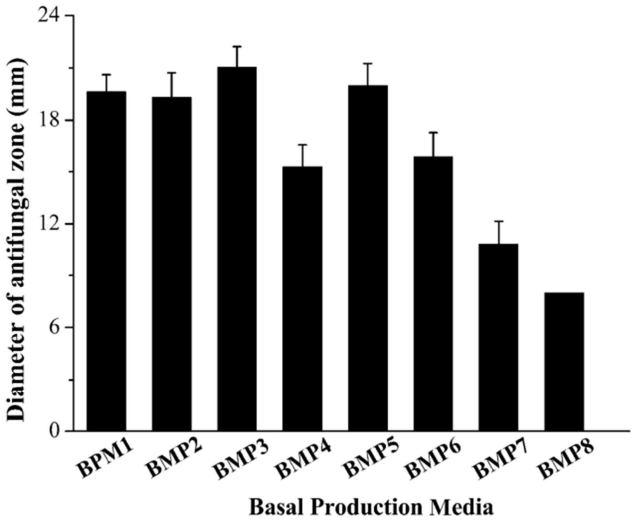


Figure 2. *Effects of eight kinds of media on the antifungal zone diameter of the strain XL03.*

3.3. The Most Important Medium Components for the Best Antifungal Activity

The effects of eight variables on the antifungal activity were presented in Table 6. The low p value (<0.05) indicated that tryptone and glucose were the significant impact factors. Although the p value of NaCl (0.084) is greater than 0.05, it is still an important factor in contrast to those of other factors. Therefore, tryptone, glucose and NaCl were selected as the most important components consisting of an appropriate medium for the best antifungal activity of the strain XL03.

Table 6. *Statistical analysis of antifungal activity under Plackett-Burman design.*

Medium components	Effect	Standard error	*t* value	*p*-value	sequence
Tryptone	5.877	0.6915	4.25	0.024	1
Yeast extract	1.603	0.6915	1.16	0.330	5
NaCl	3.530	0.6915	2.55	0.084	3
Beef extract	-2.290	0.6915	-1.66	0.196	4
Glucose	4.630	0.6915	3.35	0.044	2
$MgSO_4.7H_2O$	-0.757	0.6915	-0.55	0.622	8
K_2HPO_4	1.057	0.6915	0.76	0.500	7
Glycero	-1.183	0.6915	-0.86	0.455	6

3.4. Optimization of Selected Media Components

The significant factors（tryptone, glucose, NaCl）were picked for further optimization using the RSM. The results of the seventeen experiments were shown in Table 5. The experimental data were fitted to a second-order polynomial equation by means of Design Expert 8.0 software. The response of antifungal activity was calculated by the following second-order polynomial equation:

$$Y=22.93 + 0.18A + 0.23B + 0.17C - 0.21AB + 0.25AC + 0.038BC - 0.37A^2 - 0.26B^2 - 0.32C^2$$

Where A, B and C were the coded values of tryptone, glucose, NaCl, respectively. Statistical testing of the fitted model was checked by ANOVA, and the results were shown in Table 7. The model F value of 39.12 implied the model is significant and p value was smaller than 0.0001. The parameters values indicated that the model was suitable for this experiment. The insignificant lack of fit value of 0.09 suggested that the obtained experimental data were in good fit with the model. The predictedR^2 of 0.9518 was in reasonable agreement with the Adjusted R^2 value of 0.9554.

Meanwhile, in order to provide a better visualization of the statistically significant factors, the response surface 3D plots were applied to show the interactions between the important factors on the antifungal activity. The effect of tryptone and glucose on the antifungal activity was in Fig 3A. It could be seen that the antifungal activity increased with increment of tryptone when glucose was at a moderate concentration. The same trend could be seen in the effects of glucose and NaCl on the antifungal activity (Fig 3B). The antifungal activity

increased with increasing concentration of tryptone and NaCl (Fig 3C). The 3D plots clearly showed that moderate level of glucose and higher levels of both tryptone and NaCl resulted in the maximum antifungal activity.

Table 7. *Summary of ANOVA for response surface quadratic model analysis of variance using RSM.*

Source	Sum of Squares	df	Mean Square	*F* Value	*p*-value Prob>*F*	
Model	2.78	9	0.31	39.12	< 0.0001	significant
Residual	0.055	7	7.885E-003			
Lack of Fit	3.475E-003	3	1.158E-003	0.090	0.9620	insignificant
Pure Error	0.052	4	0.013	39.12	< 0.0001	
Core Total	2.83	16	0.31			
R^2	0.9805					
Adj. R^2	0.9554					
Pred. R^2	0.9518					

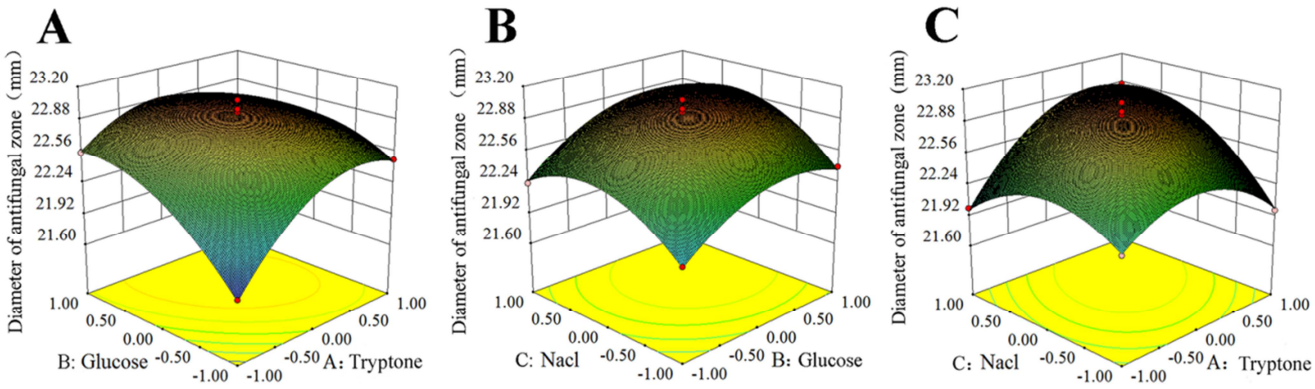


Figure 3. *Response surface 3D plotes shows the relationship between the antifungal activity and the interactive effects of variables. (A) Effects of tryptone and glucose on the antifungal activity. (B) Effects of glucose and NaCl on the antifungal activity. (C) Effects of tryptone and NaCl on the antifungal activity.*

3.5. Experimental Validation of Optimization

The optimum conditions for the significant variables and the predicted values of the responses (Antifungal activity) were shown in Table 8. According to the quadratic model, the predict maximum antifungal zone diameter was 22.88 mm when the optimal values of test factors were tryptone of 7.50 g/L (coded value= 1), glucose of 8.79 g/L (coded value =0.08), and NaCl of 1.27 g/L (coded value= 0.66), respectively. Under the optimal conditions, the experimental antifungal zone diameter was 22.96±1.28 mm, which increased about 4 mm in comparison with that on the un-optimized medium. Meanwhile, the experimental value was close to the predicted value of 22.88 mm. Therefore, this indicated that the model was adequate for the study.

Table 8. *Predicted and experimental values of the responses at optimum conditions. Values are expressed as mean ± SD from three replicates.*

Variables	Level		Antifungal activity (mm)		
	Unoptimized	Optimized	Unoptimized	Optimized (Predicted)	Optimized (Experimenal)
Tryptone	5.00g	7.50g	19.34±1.20	22.88	22.96±1.28
Glucose	10.00g	8.79g			
NaCl	1.00g	1.27g			

4. Discussion

Certain *Pseudomonas* spp. strains are plant-protecting bacteria and widespread in the rhizosphere of plant [14]. *P. protegens* CHA0was also called *P. fluorescens* CHA0 [15]. Several fluorescent *Pseudomonas* spp. strains protect plants from diseases caused by different soil-borne fungal pathogens, such as *Gaeumannomy cesgraminis* var.*tritici* [16], *S. sclerotiorum* [17] and *F. graminearum* [18]. Although some *P. protegens* strains were widely used as biological antifungal agents in the plant field, so far, they were not reported to have anti-*Saprolegnia* activity in aquaculture. However, *P. protegens* XL03 showed good antifungal activity against *Saprolegnia* sp. in our study, so it could be used for biological control of *Saprolegnia* spp. in aquaculture.

Medium components played an important role in the microbial metabolites research, and the success or failure of the whole optimization experiment was determined by the levels of carbon source and nitrogen source [19]. Therefore, in view of significance of media components and their optimum levels to the antifungal compounds of microorganisms, we have attempted to optimize medium components for improving the antifungal compounds production of *P. protegens*XL03. The significant medium components for enhancing the antifungal activity were screened by PBD. The results showed that tryptone and glucose have a significantly

impact on the antifungal compounds production, and the effects of tryptone and glucose on the metabolites production of *Pseudomonas* spp. has previously been reported [20, 21]. The optimum levels of the medium components were obtained with RSM. In present study, the antifungal zone diameter of the strain XL03 increased about 4 mm on the optimized medium in comparison with that on the un-optimized medium, so the results indicated the antifungal compounds production from the strain XL03 was enhanced. However, in the actual mass fermentation production, some factors should always be taken into consideration for the maximum production, such as incubation temperature, rotation speed, pH, oxygen demand, incubation time and inoculum level [20, 22], because these factors would also have an important impact on the antifungal compounds production.

5. Conclusions

The study discovered that *Pseudomonas protegens*XL03 has efficient antifungal activity against *Saprolegnia* sp.. Furthermore, the culture medium optimized using PBD and RSM will be useful for enhancing production of the antifungal compounds on a large scale from *P. protegens* XL03, and applied for controlling saprolegniasis in aquaculture.

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