Modeling the Antimicrobial Activity of Lipopeptides against *Pseudomonas spp* in Shrimp Meat Using a Response Surface Method

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Abstract: The antibacterial activity model of antibacterial lipopeptide against *Pseudomonas* in shrimp was researched in the article. The minimal inhibitory concentration of antibacterial lipopeptides against *Pseudomonas* was 0.6 g/mL with double dilution method. Response surface methodology was used to establish the antibacterial activity model of antibacterial lipopeptide against *Pseudomonas* in shrimp meat .The correlation coefficient (R) between the experimental and predicted values was 0.998 which suggested that the model equations was accurate. A reduction over two log-cycles of *Pseudomonas* cells could be realized under the conditions of temperature below 4.3 , time over 6 h and lipopeptides concentration over 0.3 mg/g by analyzing the response surface plot and contour plot of the model equations.

Key words: *Pseudomonas spp*; lipopeptide; *Bacillus amyloliquefaciens*

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响应面法建立脂肽对虾肉中假单胞菌抗菌活性的模型

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摘要：本文研究了脂肽对虾肉中假单胞菌的抗菌活性模型。通过二倍稀释法测定抗菌脂肽对假单胞菌的最小抑菌浓度为 0.6 mg/mL,并采用响应面实验建立了抗菌脂肽对虾肉中假单胞菌的抗菌活性模型。验证实验结果表明实验值与预测值之间的相关系数（R）为 0.998，这说明该模型的方程是准确的。通过分析该模型方程的响应面曲线和等高线可以得到，当温度低于 5.4 ℃、时间高于 6 h、抗菌脂肽浓度超过 0.3 mg/g 时，虾肉中的假单胞的杀灭对数值可超过 2。

关键词：假单胞菌；脂肽；淀粉液化芽孢杆菌

1 Introduction

The increasing trend of limiting the use of chemical food preservatives has generated considerable interests in the use of natural alternatives. With several desirable properties, such as heat-tolerant, relatively broad antimicrobial spectrum, low toxicity, antimicrobial peptides may serve as a potentially significant group of natural food preservatives. Antibiotic peptides could be produced by animals, plants and microorganisms. However, compared to that produced by animals and plants, antibiotic peptides produced by microorganisms can be easily produced in large scale because of their unlimited sources of raw material, which leads to a better applying prospect.

Nisin produced by *Lactococcus lactis* is currently the most researched, and commercially the most important member of a large class of antimicrobial peptides produced by bacteria. It exhibits antimicrobial activity toward a wide range of Gram-positive bacteria, in particular against foodborne pathogens in food, such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* [1~3]. However, it has a narrow antibacterial spectrum and is inactive against Gram-negative bacteria like *Pseudomonas spp* and *Shewanella putrefaciens*, which are the most common specific spoilage organisms (SSOs) on chilled aquatic products under aerobic conditions [4~6]. Therefore the application of nisin in aquatic products is limited.

Nevertheless, some antimicrobial lipopeptides

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(AMLPs) including surfactin, fengycin and iturin, produced by *Bacillus subtilis* [7-9], and *Bacillus amyloliquefaciens*[10], have a broad spectrum of antimicrobial activity, including Gram-positive and Gram-negative bacteria, fungi, protozoa, and viruses . AMLPs have a better prospect in aquatic products preservation compared to nisin for their antimicrobial activity against Gram-negative bacteria，and may serve as a new potentially significant member of natural food preservatives in aquatic products.

Litopenaeus vannamei has become the primary species of penaeid shrimp currently being reared in China. *Pseudomonas spp* is one of the specific spoilage organisms on chilled Litopenaeus vannamei under aerobic conditions [11]. *Bacillus amyloliquefaciens* strain ES-2, screened from a medicinal plant in our laboratory, was discovered to produce AMLPs including surfactin and fengycin [10].In order to promote the application of AMLPs in Litopenaeus vannamei preservation, the sensitivity of *Pseudomonas spp* to AMLPs was assayed, and the model of antimicrobial activity against *Pseudomonas spp* in shrimp meat was also researched. That would provide technological support and a theoretical basis for commercial application of AMLPs in Litopenaeus vannamei preservation.

2 Marerials and methods

2.1 AMLPs preparation

The strain ES-2 was inoculated into a 250-mL shake flask containing 70 mL of Luria-Bertani (LB) (5 g/L beef extract; 10 g/L peptone; 5 g/L NaCl; pH 7.2) medium and cultivated at 30 ℃ and 150 rpm for 24 h. Preculture (5%, v/v) was inoculated into a 500-mL shake flask containing 100 mL of Landy medium [12] and cultivated for 38 h at 33 ℃ and 180 rpm for the production of antimicrobial lipopeptides. At the end of cultivation, the culture was centrifuged at 11,000×g for 15 min to collect fermentation broth. The fermentation broth was fed into a column with X-5 resin at 30 g/L resin concentration. The separation conditions of lipopeptides from the crude lipopeptide were as follow: feed flow rate of 0.5 BV/h, 80% aqueous ethanol as eluant agent, elute flow rate of 1 BV/h. The eluate solution was monitored by absorbance at 210 nm and 230 nm, and then it was pooled. The pooled fractions were freeze-dried under vacuum to obtain AMLPs powder [13].

2.2 Preparation of inocula

The bacterium used was *Pseudomonas spp*, isolated from the spoiled Litopenaeus vannamei by our laboratory. The bacterial strains were grown in Luria Broth (LB) medium at 25 ℃ and 150 rpm. The strain *Pseudomonas spp* was inoculated into a 250-mL shake flask containing 70 ml of LB medium and cultivated at 25 ℃ and 150 rpm for 20 h. The cells were harvest by centrifugation at 7000×g for 10 min, and then they were resuspended to the desired concentration described in the follow experiments with sterile water as the working inoculums.

2.3 Preparation of Steriled Shrimp meat

Live Litopenaeus vannamei was purchased from a local supermarket. Shelled shrimps were obtained after the heads, shells and intestines of Litopenaeus vannamei being removed. The shelled shrimps were washed in sterile distilled water for three times, and then they were cut into pieces using sterile scalpel blades.

2.4 Determination of Minimal Inhibitory Concentration (MIC)

A modified microdilution technique described by Vorland was used to determine MIC[14]. MIC was tested by adding 200 µl two-fold serial LB medium dilutions of AMLPs from 2.4 mg/mL to 0.0187 mg/mL or LB medium control, and 5µL 4×10^6 CFU/mL *Shewanella putrefacien* inocula in each well. Microtiter plates were incubated at 25 ℃ for 1 day. The MIC was determined as the lowest concentration at which growth was inhibited.

2.5 Methodology and Design of Experiments

25 g shrimp meat was inoculated with 2.5 mL 10^8 CFU/mL *Pseudomonas spp* inocula to give a final 10^7 CFU/g concentration. Then modeling the inhibition of *Pseudomonas spp* in Shrimp meat experiments were performed as following design.

RSM was employed in the present work and was used to establish the model of inhibition the *Pseudomonas spp* cells in shrimp meat under different parameters, such as temperature, time, and lipopeptides concentration. The experimental design of the investigation was a Box-Behnken design of experiments. Temperature, time, and lipopeptides concentration were chosen as key variables and designated as X1, X2, and X3, respectively. The low, middle, and high levels of each variable were designated as -1, 0, and +1, respectively, as

shown in Table 1. The measurable response (dependent variable) was a function of the number of decimal reduction of the *Pseudomonas spp*, as follows:

$$Y = \log_{10} N_0/N_t \quad (1)$$

Where N_t = the final number of survivals (CFU/g) and N_0 = the initial number of cells (CFU/ g).

Table 1 Code and level of factors chosen for the trials

Factor	Symbol		Levels[a]		
	Coded	Uncoded	-1	0	+1
Temperature/℃	χ_1	X_1	4	16	28
Time of treatment/h	χ_2	X_2	6	12	18
Concentration (mg/g)	χ_3	X_3	0.3	0.6	0.9

[a] $\chi_1 = (X_1 - 16)/12$; $\chi_2 = (X_2 - 12)/6$; $\chi_3 = (X_3 - 0.6)/0.3$.

2.6 Counting of Surviving Cells

Survivors in the treated and untreated sample were estimated by the viable count method using nutrient agar media. The plates were incubated at 25 ℃ for 48 h, and then the colonies were enumerated.

2.7 Statistical Analysis

All experiments were carried out in at least three different experiments, and the standard deviation values were calculated from the triplicate experiments.

3 Results and Discussion

3.1 Determination of the MIC

Inhibited activity of antimicrobial lipopeptides was determined by microdilution technique on *Pseudomonas spp*. Results demonstrated that bacterial growth could not be detected when antimicrobial lipopeptides were diluted to 0.6mg/ml, but it could when concentrations were 0.3 mg/mL. Thus, the MIC of antimicrobial lipopeptides to *Pseudomonas spp* was 0.6 mg/mL.

3.2 Regression Model of Response

The mean values of the response (log-cycle reduction for cells of *Pseudomonas spp*) obtained under the different experimental conditions are summarized in Table 2. The variability associated with test samples, was small again as indicated by the standard deviation values given in parentheses. The experimental data (Table 2) were analyzed using Design Expert (Version 6.0.5). Multiple regression analysis of the experimental data gave the following second-order polynomial equation 2:

$$Y=1.46-1.23\chi_1+0.28\chi_2+0.32\chi_3-0.092\chi_1\chi_2-0.26\chi_1\chi_3+0.090\chi_2\chi_3+0.15\chi_1^2+0.15\chi_2^2+0.057\chi_3^2 \quad (2)$$

Table 2 Box-Behnken design matrix and the experimental results

Trial No.	χ_1	χ_2	χ_3	Ya	
				Actual	Predicted
1	-1	-1	0	2.59(0.03)	2.62
2	1	-1	0	0.39(0.05)	0.35
3	-1	1	0	3.33(0.02)	3.37
4	1	1	0	0.76(0.06)	0.73
5	-1	0	-1	2.40(0.04)	2.32
6	1	0	-1	0.39(0.05)	0.38
7	-1	0	1	3.46(0.02)	3.47
8	1	0	1	0.43(0.05)	0.51
9	0	-1	-1	1.11(0.02)	1.16
10	0	1	-1	1.49(0.03)	1.54
11	0	-1	1	1.67(0.04)	1.62
12	0	1	1	2.41(0.02)	2.36
13	0	0	0	1.42(0.03)	1.46
14	0	0	0	1.51(0.05)	1.46
15	0	0	0	1.47(0.01)	1.46
16	0	0	0	1.40(0.04)	1.46
17	0	0	0	1.46(0.02)	1.46

[a] Mean, in parentheses, standard deviation values.

A summary of the analysis of variance (ANOVA) for the selected quadratic model was shown in Table 3. The correlation measure for testing the goodness of fit of the regression equation was the adjusted determination coefficient (R^2_{adj}). The value of R^2_{adj} (0.9942) for Eq. 5 being close to 1 indicates a high degree of correlation between the observed and predicted values. The value of the R^2_{adj} (0.9942) suggests that only about 0.58% of the total variation was not explained by the model. Statistical testing of the model was done in the form of ANOVA, which is required to test the significance and adequacy of the model. Here the ANOVA of the regression model demonstrated that the model was highly significant, as was evident from the calculated F value (315.48) and a very low probability value ($P<0.0001$). Moreover, the computed F value was much greater than the tabulated F value ($F_{0.01(9,4)}=14.66$), indicating that the treatment differences were highly significant. The model also showed statistically insignificant lack of fit, as was

evident from the lower calculated F value (4.84) than the tabulated F value ($F_{0.05(9,3)}$=8.81) even at 0.05 level. The model was found to be adequate for prediction within the range of variables employed.

Table 3 Analysis variance (ANOVA) for regression equation

Source	Sum of Squares	DF	Mean Square	F Value	Prob＞F
Model	14.05	9	1.56	315.48	<0.0001
Lack of Fit	0.027	3	0.009058	4.84	0.0808
Pure Error	0.00748	4	0.00187		
Cor Total	14.08	16			

R=0.9975 R^2=0.9950 R^2_{adj} =0.9942.

The coefficient values of Eq. 2 were calculated and tested for their significance using Design Expert and are listed in Table 4. The P values are used as a tool to check the significance of each of the coefficients, which in turn may indicate the pattern of the interactions between the variables. The smaller the value of P values, the more significant is the corresponding coefficient. It can be seen from this table that all the linear coefficients and quadratic term coefficients were significant, the P values being very small (P＜0.05). All of the interactions were significant (P＜0.05).

Table 4 Regression coefficients and their significance of the quadratic model

Model term	Parameter estimate	Df	Std. error	95% CI Low	95% CI High	Prob > F
Intercept	1.46	1	0.031	1.38	1.53	<0.0001
χ_1	-1.23	1	0.025	-1.29	-1.17	<0.0001
χ_2	0.28	1	0.025	0.22	0.34	<0.0001
χ_3	0.32	1	0.025	0.26	0.38	<0.0001
χ_1^2	0.15	1	0.034	0.077	0.24	0.0025
χ_2^2	0.15	1	0.034	0.077	0.24	0.0025
χ_3^2	0.057	1	0.034	-0.021	0.14	0.1223
$\chi_1\chi_2$	-0.092	1	0.035	-0.18	-0.009	0.0339
$\chi_1\chi_3$	-0.26	1	0.035	-0.034	- 0.17	0.0002
$\chi_2\chi_3$	0.090	1	0.035	-0.007	0.17	0.0376

3.3 Verification of Model

To validate the adequacy of the model equation (Eq. 2), a total of six verification experiments were carried out under different lipopeptides combinations of process parameter (within the tested area) and the results are shown in Table 5. The validation data were analyzed using the SPSS software (version 10.0, SPSS). The correlation coefficient (R) between the experimental and predicted values was 0.998. The results of the analysis indicated that the experimental values were found to be significantly in agreement with the predicted ones, and also suggested that the model of Eq. 2 was satisfactory and accurate.

Table 5 Verified results of the model equation

Trial No.	Variable			Response	
	X_1	X_2	X_3	Observed	Predicted
1	4	9	0.45	2.31	2.26
2	4	15	0.75	2.89	2.93
3	16	9	0.45	1.20	1.17
4	16	15	0.75	1.63	1.66
5	28	9	0.75	0.54	0.50
6	28	15	0.45	0.45	0.40

3.4 Determination of inactivation conditions

The graphical representations of the regression Eq. 2, called the response surfaces and the contour plots, were obtained using the Design Expert and presented in Fig. 1~3. The initial concentration of *Pseudomonas* spp *in* shrimp meat in the experiment was 10^7 cfug/g, which is considered as the maximum level for acceptability for aquatic products. The maximum level of the total Viable Count for aquatic products sold for human consumption is 10^5 CFU/g. Thus a reduction over two log-cycles for *Pseudomonas* spp was the criterion of AMLPs inactivation in this investigation.

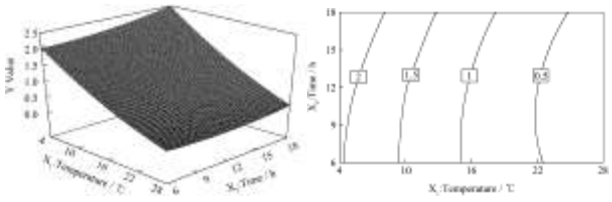

Fig.1 Response surface plot and its corresponding contour plot of the effect of time and temperature mutual interactions on *Pseudomonas* spp of inactivation

Fig.1 showed the effect of temperature and time with Lipopeptides concentration at the minimal value (0.3 mg/g, –1level). From Fig.1, it could be seen that the

reduction of *Pseudomonas* spp increased with temperature decreasing and time increasing, reaching a reduction over two log-cycles when temperature was below 4.3 ℃, and time was over 6 h.

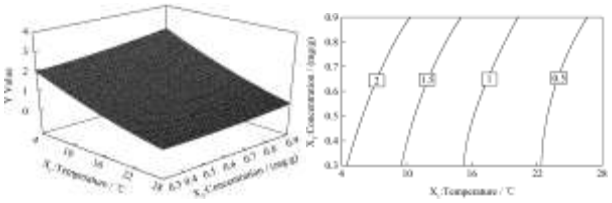

Fig.2 Response surface plot and its corresponding contour plot of the effect of temperature and concentration mutual interactions on *Pseudomonas* spp of inactivation

Fig.2 showed how the reduction of *Pseudomonas spp* varied with temperature and AMLPs concentration at a fixed temperature (6 h, –1level). From Fig. 2, it could be seen that the reduction of *Pseudomonas* spp increased with temperature decreasing and AMLPs concentration increasing, reaching a reduction over two log-cycles when temperature was below 4.3 ℃, and AMLPs concentration was over 0.3 mg/g.

The contour plot in Fig.3, which gave the reduction of *Pseudomonas* spp as a function of time and AMLPs concentration at a fixed temperature (4.3 ℃, –0.97 level), showed that the reduction of *Pseudomonas* spp increased with time prolonging and AMLPs concentration increasing, reaching a reduction over two log-cycles when AMLPs concentration was over 0.3 mg/g, and time was over 6 h.

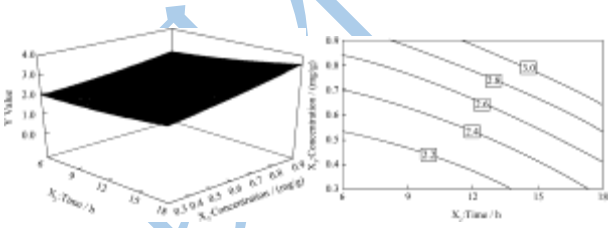

Fig.3 Response surface plot and its corresponding contour plot of the effect of time and concentration mutual interactions on *Pseudomonas* spp of inactivation

Analyzing the temperature-time-concentration plots, a significant synergistic effect was observed among the three parameters. Decreasing the temperature of a certain treatment gave the opportunity to decrease the treatment time and concentration. As a consequence, a reduction over two log-cycles of *Pseudomonas* spp cells could be realized when the temperature was below 4.3 ℃, time was over 6 h, and AMLPs concentration was over 0.3 mg/g.

Conclusion

Here, we mainly examined the effect of several external factors (the most commonly controlled factors in food processing) on AMLPs sterilizing *Pseudomonas spp* cells and other external factors (such as stress), and internal factors (such as pH and ion strength) were not studied. However, it is important that these works will more completely explain AMLPs sterilizing effects and lipopeptides applications.

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