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Modeling inhibitory activity of a novel antimicrobial peptide AMPNT-6 from *Bacillus subtilis* against *Vibrio parahaemolyticus* in shrimp under various environmental conditions

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ABSTRACT

Vibrio parahaemolyticus is recognized as the leading cause of human gastroenteritis associated with the consumption of seafood. NT-6 antimicrobial peptide (AMPNT-6) that is a novel antimicrobial peptide secreted by *Bacillus subtilis* NT-6 isolated from Natto which is a Chinese traditional fermented food can be an effective method to reduce *V. parahaemolyticus*. This study aimed at investigating the factors that affected the application of AMPNT-6, such as temperature, salinity, pH value and sodium pyrosulfite concentration by response surface method and modeled the effect of AMPNT-6 on inhibition to *V. parahaemolyticus* in shrimp under various environmental conditions. Design-Expert software was chosen to perform regression fit and to establish the antibiotic mathematical model. The results indicated that all the above environmental conditions had no significant interactive effects on the antibacterial activity of AMPNT-6. The quadratic polynomial mathematical model was established and obtained the quadratic regression equation of the predictive value Y (bacterial colony number) and variables x_1 (temperature), x_2 (sodium chloride concentration), x_3 (pH), x_4 (sodium pyrosulfite concentration) and x_5 (AMPNT-6 concentration) in a certain range: $Y = 6.60 + 0.34x_1 - 0.23x_3 - 0.19x_5 - 0.29x_1^2 + 0.19x_2^2 + 0.3x_4^2 - 0.35x_1x_2$ ($R^2 = 0.8963$). The experimental values were shown to be in good agreement with predicted values. The established model had a high fitting degree and could predict the inhibition effect of AMPNT-6 toward *V. parahaemolyticus* growth in shrimp under various conditions.

1. Introduction

Vibrio parahaemolyticus is a halophilic, gram-negative, food-borne pathogen which multiplies rapidly at room temperature. It is recognized as the most important food-borne pathogen that causes food poisoning (Solomakos, Pexara, & Govaris, 2012; Su & Liu, 2007). In recent years the contamination rate of *V. parahaemolyticus* has increased in shrimp culture environments year after year, and the natural germ-carry rate of shrimp, which could reach 90% in the warm seasons, remains high. *V. parahaemolyticus* has become the major harmful factor of food safety in aquatic product industry (Su & Liu, 2007; Zarei, Borujeni, Jamnejad, & Khezrzadeh, 2012). Therefore, searching for a suitable method to control *V. parahaemolyticus* growth and survival in shrimp is of great significance for ensuring people's health and the industrial development.

Using low toxicity biological antimicrobial preservative is an important approach and a common trend to control food-borne pathogenic microorganisms (Luciana, Angela, Priscila, & Thereza, 2009). Aquatic product matrix is mostly neutral or alkaline, while a number of food preservatives are only active under acid condition, such as sorbic acid and its potassium salt, benzoic acid and its sodium salt, nisin and so on (Luciana et al., 2009; Zhang & Yang, 2008). In addition, the main food-borne pathogens and spoilage bacteria in aquatic products are gram-negative bacilli (Su & Liu, 2007; Zhang & Yang, 2008), while the commonly used biological preservative (nisin) which presents a narrow antibacterial spectrum is limited to inhibit gram-positive bacteria (Luciana et al., 2009). Therefore, to develop a new aquatic biological antimicrobial preservative with broad spectrum appears to be particularly

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urgent. Antimicrobial peptides (AMPs) are gaining attention as antimicrobial alternatives to chemical food preservatives and commonly used antibiotics. *Bacillus* natto, which is recognized as the safety strain, can produce AMPs that has antimicrobial activity against bacteria, yeasts and molds according to the reports (Cao, Liao, Wang, Yang, & Lu, 2009; Sun, Wang, & Chen, 2010; Yeo, Lee, Cha, & Hahm, 2011; Zhang & Yang, 2008). The AMPs are a potential source of biological antimicrobial agent (Cao et al., 2009). In our previous researches, *Bacillus* natto NT-6 was isolated from natto, a traditional fermented food in China and Japan (Zhang & Yang, 2008), and its fermentation products were AMPs named AMPNT-6, which mainly contained three compounds of antibacterial lipopeptide homologs: fengycin, surfactin and iturin (Sun et al., 2010, 2012). Through the oral acute toxicity experiment in mouse, it was found that LD50 of the AMPNT-6 was larger than 5000 mg/kg of mouse body weight, which meant that AMPNT-6 was safe to eat (Sun et al., 2012). What's more, the research indicated that the AMPNT-6 presented a broad antimicrobial spectrum and had a strong inhibitory effect against the main food-borne pathogens and spoilage bacteria in aquatic products, such as *V. parahaemolyticus* (Xu, Wang, Sun, Liu, & Li, 2013), in a wide range of pH value from 4 to 11. It remained activity after heating to 100 °C for 30 min, and it was readily dissolved in water or organic solvents (Sun, Lu, Bie, Lu, & Yang, 2006; Sun et al., 2010). Therefore, AMPNT-6 has a good prospect of application in the control of harmful microorganisms in aquatic products as a novel biological antimicrobial agent (Banat et al., 2010; Sun et al., 2010).

The antibacterial activity of AMPNT-6 might be subjected to many factors which would reduce the effectiveness of its use in shrimp meat (Januário & Dykes, 2005). The objectives of this study were to ascertain the bactericidal efficiency of the antimicrobial peptides under the effects of different temperature, salinity, pH value, sodium pyrosulfite and other factors by response surface experimental design, and to develop mathematical model to describe the influence law of those factors on the antibacterial activity of AMPNT-6. It would provide scientific bases for the further application to control the hazards of *V. parahaemolyticus* more effectively.

2. Materials and methods

2.1. Preparation of bacterial strains

Bacillus natto NT-6, which was isolated from natto, a traditional Chinese soy paste, was obtained from The Food-borne Pathogenic Microorganisms and Toxins of the Aquatic products Green Control Laboratory, Food Science and Technology College, Guangdong Ocean University. It was grown in Luria–Bertani (LB) liquid medium at 37 °C for 24 h. *V. parahaemolyticus* ATCC17802 was purchased from Microorganisms Research Institute of Guangdong. *V. parahaemolyticus* was grown in 3% NaCl nutrient broth at 37 °C for 24 h.

2.2. Preparation of AMPNT-6

Bacillus natto NT-6, which was slant preserved, was inoculated in LB liquid medium and cultured on the shaking table at 37 °C, 150 rpm for 24 h to make the seed solution. Then the seed solution was added to the modified Landy medium at concentration of 5% (vol/vol) and subcultivated at 33 °C, 180 rpm for 36 h. After incubation, the *Bacillus* natto NT-6 culture broth was centrifuged at 5000 rpm for 15 min at 4 °C (J2-MC, Beckman, Brea, CA, USA). Cell-free supernatant was precipitated by adjusting pH to 2.0 with 6 N HCl and then overnighted at low temperature. The pellet was then collected by centrifugation at 5000 rpm for 15 min at 4 °C, after which it was dissolved in 15 mL of methanol. The pH value of the solution was adjusted to 7.0 with 1 N NaOH, and then centrifuged at 10,000 rpm for 20 min 4 °C. The supernatant was filtered through a 0.22 μm pore hydrophobic filter (Millipore, USA). Subsequently the filtrate was concentrated in a rotary vacuum evaporator (RE-52AA, Yarong Scientific Corp., Ltd., Shanghai, China) and dried in a freeze drier and preserved. It can be used after dissolving in sterile water and quantified by HPLC method.

2.3. Inhibitory effects of AMPNT-6 on V. parahaemolyticus in shrimp

Fresh shrimps 25 g were pounded and mixed with 225 mL normal saline to make homogenate, and then dispensed (10 mL) into test tubes. The amount of sodium chloride and sodium pyrosulfite diluents were added to each tube to achieve final concentrations and the pH values were adjusted respectively, as shown in Table 2. The tubes were sterilized at 121 °C for 15 min. Each tube was then inoculated aseptically with the final concentrations of 0.4, 0.8 MIC (minimum inhibitory concentration) of AMPNT-6 and 1% of *V. parahaemolyticus* to reach an initial population that the bacteria concentration was about 10^5 CFU/mL. The tubes were immediately incubated at the following temperature showing in Table 2 for 24 h, respectively. The number of colony-forming units (CFU) was used to represent the number of cultivable bacterial cells in the shrimp cultures. All experiments were replicated three times.

2.4. Methodology and design of experiments

Response surface methodology (RSM) is an empirical modeling technique used to estimate the relationship between a set of controllable experimental factors and observed results. Based on the results of preliminary experiments, response surface methodology was employed in the present work. The experimental design of the investigation was the Box–Behnken design of experiments with five independent variables to obtain the combination of values that optimizes the response within the region of the three-dimensional observation space to allow the design of a minimal number of experimental runs. The different parameters such as temperature, sodium chloride concentration, pH value, sodium pyrosulfite concentration, AMPNT-6 concentration were chosen as key variables and designated as x_1, x_2, x_3, x_4, x_5, respectively. The low, middle and high levels of each variable were designated as −1, 0 and +1, respectively, and are given in Table 1. Table 2 showed the actual design of the experiments by Box–Behnken. The behavior of the system was explained by the following second-degree polynomial equation:

$$Y = B_0 + \sum_{i=1}^{n} B_i x_i + \sum_{i<j}^{n} B_{ij} x_i x_j + \sum_{j=1}^{n} B_{jj} x_j^2 \quad (1)$$

where Y = predicted response, it can be observed that in the present study, B_0 is a constant, and B_i, B_{ij}, B_{jj} are coefficients estimated

Table 1
Code and level of variables chosen for the trials.

Symbol	Independent variables	Coded levels		
		−1	0	1
x_1	Temperature (°C)	25	30	35
x_2	Sodium chloride concentration (%)	3.0	3.3	3.6
x_3	pH	7.4	7.9	8.4
x_4	Sodium pyrosulfite concentration (%)	0.2	0.3	0.4
x_5	APNT-6 concentration (MIC)	0	0.4	0.8