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# Isolation and Optimization of Growth Condition of *Bacillus* sp. from Fermented Shrimp Paste for High Fibrinolytic Enzyme Production

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**Abstract** The fibrinolytic enzyme has been found in the traditional fermented foods including the traditional shrimp paste product. In this study, six Vietnamese traditional shrimp paste products collected in three regions (north, south and middle of Vietnam) were screened for fibrinolytic enzyme and the related bacteria were isolated and identified. The fibrinolytic enzyme was found in all Vietnamese shrimp paste products, in which the products in the middle of Vietnam had higher fibrinolytic enzyme activity (2.43–2.95 FU/ml) than those in other regions. The isolated bacterium which produced the highest fibrinolytic enzyme was identified as a strain of *Bacillus* sp., closely related to the species of *Bacillus weihenstephanensis* with 99 % identities. The optimal fermentation conditions were also investigated using response surface methodology based on Box–Behnken design for high fibrinolytic enzyme production by the isolated strain. As a result, the fibrinolytic enzyme activity reached to 6.85 FU/ml at the optimal fermentation condition of 1.50 % of shrimp shell powder, 1.44 % of NaCl, at 33 °C and 32-h fermentation. Thus, the fibrinolytic enzyme produced by the strain of *Bacillus* sp. in this study might be used as a thrombolytic agent in pharmaceutical industries.

**Keywords** *Bacillus* sp. · Box–Behnken design · Fibrinolytic enzyme · Response surface methodology

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## 1 Introduction

Although the advanced innovations of thrombolytic therapies have been applied in heart disease treatments, there is still a high mortality rate in the world caused by vascular diseases such as stroke and coronary artery disease. As reported by the World Health Organization (WHO) in 2000, heart diseases occupied 29 % of total fatality rate in the world [1]. Cardiovascular diseases, especially stroke and heart diseases, account for 17 million people death annually and predicted that the number will be increased to 23.3 million in 2030 [2]. The accumulation of fibrin clots in blood vessels is a main cause of stroke and many serious cardiovascular diseases. Thrombolytic agents dissolve fibrin clots by making fibrinolytic pathways in human body or imitating natural thrombolytic molecules. The common clinical thrombolytic agents, which have been applied in treatments, are derived bacterial or recombinant DNA technology products [3]. Many thrombolytic agents such as urokinase, streptokinase, genetically tissue-type plasminogen activators (t-PA) have also been widely applied in thrombolytic treatments, but they seem not to be effective because of many undesirable side effects. To be specific, the patients may be vulnerable to resistance to reperfusion, occurrence of acute coronary reocclusion, allergic reactions and bleeding complications [3,4]. In addition, some thrombolytic agents such as urokinase and t-PA which are so expensive may become obstacles for long treatment-abiding patients [5]. Therefore, the finding for new safe and inexpensive fibrinolytic enzyme is very essential.

Fibrinolytic enzyme was first successfully discovered in natto—a Japanese traditional fermented soybean [6]. Afterward, many researches showed that fibrinolytic enzymes can be widely found not only in nature but also in a variety of foods such as earthworm secretions [7], marine creatures [8], fermented red bean [9], fruiting bodies of Korean *Cordyceps*

*militaris* [10], chive [11]. A total of thirteen Asian traditional fermented food samples, including fermented black beans, instant soybean paste, fermented shrimp paste, pickled gourami fish, tempeh, light soy sauce, sweet bean paste sauce, yellow bean sauce, fermented bean curd (red), fermented bean curd (white), fermented cow milk, glutinous rice in wine and stinky bean curd powder, were screened for fibrinolytic enzyme activity, and the fermented shrimp paste, one of popular Asian seasonings, was demonstrated to have the strongest fibrinolytic activity among these materials [1,12]. Many kinds of bacteria presented in fermented food products are able to secrete fibrinolytic enzymes, and members of *Bacillus* sp. are considered as a potential source for fibrinolytic enzyme production [13]. The fibrinolytic enzymes could be found in many fermented foods with different strains of *Bacillus* such as Japanese Natto with *Bacillus natto* [6], Chinese Douchi with *Bacillus amyloliquefaciens DC-4* [14] and *B. subtilis LD-8547* [15], Korean Doen-jang with *Bacillus* sp. *DJ-4* [16], Korean Chungkook-Jang with *Bacillus* sp. *CK* [17], and Chinese fermented shrimp paste with *Bacillus subtilis DC-33* [12].

In Vietnam, the fermented shrimp paste is a traditional food produced by natural fermentation of whole fresh shrimp mixed with salt (10–15 %, w/w) exposed to sunlight for 1–3 months before utilization. The Vietnamese fermented shrimp paste has various colors from dark-brown to reddish-brown, and different dried matters and flavors due to the different production processes, such as the amount of salt and fermentation conditions, and the presence of different naturally occurring bacteria in the product. Until now, there is no report on the presence of fibrinolytic enzymes as well as the fibrinolytic enzyme-producing bacteria in the Vietnamese traditional fermented shrimp pastes. Therefore, the objectives of this study are to screen for bacteria which produce high amount of fibrinolytic enzymes in Vietnamese traditional fermented shrimp pastes and then optimize their growing conditions for maximizing fibrinolytic enzyme production using response surface methodology based on Box–Behnken design.

## 2 Materials and Methods

### 2.1 Sample Collection

Six fermented shrimp pastes collected from three regions in Vietnam were screened for fibrinolytic enzyme activity. Two shrimp paste products were obtained in the north of Vietnam coded as SP1, a commercial product of Tam Duc Fish Co. and SP2, a commercial product of Trung Thanh Co.; three shrimp paste products were collected from middle of Vietnam including SP3, a traditional handmade product in Thanh Hoa province, SP4, a traditional handmade product in Nha Trang city and SP5, a commercial product of PT FISACO Co.; one traditional handmade sample in the south of Vietnam was collected from coastal village in Tien Giang province (SP6). The dry matter contents of these products were in a range of 26–58 %.

### 2.2 Substrate and Chemicals

In this study, shrimp shell powder (SSP), used as the sole carbon/nitrogen sources, was prepared as follows: Shrimp shell was collected from market, washed with tap water, then dried in an oven at 120 °C. After 30 min, it was ground into powder form and stored in desiccators with knobbed lid to minimize the moisture. Other chemicals consisted of bovine fibrinogen, thrombin, sodium chloride, yeast extract, peptone, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$ and D-glucose were purchased from Sigma Chemical Company (Singapore).

### 2.3 Screening of Crude Fibrinolytic Enzyme in the Shrimp Pastes

The shrimp paste products were screened for fibrinolytic enzymes, which exist naturally in these samples. The samples were diluted by phosphate buffer to ensure that all samples had the same dry matter content before extraction and determination. Samples were shaken for 10 min, filtered by Whatman paper, and then fibrinolytic activity was evaluated using Fibrin degradation assay provided by Japan Bio Science Laboratory Co., Ltd. (JBSL) with slight modifications [18].

### 2.4 Fibrinolytic Activity Evaluation

Fibrinolytic enzyme activity was evaluated according to the fibrin degradation assay provided by Japan Bio Science Laboratory Co., Ltd. (JBSL) with slight modifications [18]. First, 0.4 ml of fibrinogen and 0.1 ml of 245 mM phosphate buffer (pH 7) were loaded to a test tube and incubated for 5 min at 37 °C. Then, 0.1 ml of thrombin solution was added and incubated at 37 °C for 10 min to form fibrin clot. After adding 0.1 ml enzyme solution , the solution was incubated at 37 °C with stirring for 20 min, 40 min, 60 min; 2.0 ml of 0.2 M trichloroacetic acid (TCA) was added and mixed well to stop the enzyme reaction. The reaction mixture was then incubated at 37 °C for 20 min, centrifuged at 6,500*g* for 5 min. Then, 1 ml of supernatant containing fibrinolytic enzyme was measured using a spectrophotometer at 275 nm. For control, all steps were done following the above process except the enzyme addition step, in which the enzyme solution was added after terminating the reaction by TCA. In this assay, 1 fibrin degradation unit (FU) of enzyme activity is defined as a 0.01-per-min increase in absorbance at 275 nm of the reaction mixture.

Enzyme activity was calculated using the following formula:

$$\text{Fibrinolytic enzyme activity (FU/ml)} = (\mathrm{OD_s} - \mathrm{OD_c})/(0.01 \times 60 \times 0.1)$$

where $OD_s$ is the optical density value of sample, $OD_c$ is the optical density value of control.

### 2.5 Isolation of Bacteria

The shrimp paste product which showed the highest activity was used to isolate the fibrinolytic enzyme-producing bacteria. The sample was diluted at $10^{-3}$, $10^{-4}$ and $10^{-5}$ concentration, and then 100 µl diluted solutions were spread onto LB agar plates and incubated at 37 °C for 24 h. Growth on LB agar plates was observed, and separate colonies were purified by sub-culturing on LB agar plates.

### 2.6 Fibrinolytic Enzyme Production by Isolated Bacteria

To determine the bacteria isolate with highest fibrinolytic enzyme activity, each isolate was cultured in seed culture containing (w/w) 0.1 % $K_2HPO_4$ and 0.05 % $MgSO_4 \cdot 7H_2O$ at 37 °C for 24 h according to the method of Wang and Yeh [19]. Then, 1 ml of seed culture was inoculated into culture medium consisted of (w/w) 1 % SSP, 0.1 % $K_2HPO_4$ and 0.05 % $MgSO_4 \cdot 7H_2O$ at 37 °C for 24 h. After 24-h shaking, the culture medium was centrifuged at 10,000*g* for 15 min and the supernatant was collected as the raw fibrinolytic enzyme.

### 2.7 Box–Behnken Design

Four factors affecting the growth of bacteria, temperature ($X_1$), shrimp shell powder ($X_2$), sodium chloride ($X_3$), fermentation time ($X_4$), were chosen as independent variables, and fibrinolytic activity ($Y$) was used as dependent response. Table 1 shows three levels of each factor and corresponding values. A model involved 25 experiments was set up by Box–Behnken design according to Design Expert software 7.0.0 trial version (Stat Ease, USA).

**Table 1** The coded level of factors for Box–Behnken design

| Variables | Code | Range and level | | |
|---|---|---|---|---|
| | | −1 | 0 | 1 |
| Temperature (°C) | $X_1$ | 30 | 35 | 40 |
| Shrimp shell powder (%) | $X_2$ | 0.5 | 1 | 1.5 |
| Sodium chloride (%) | $X_3$ | 0.5 | 1 | 1.5 |
| Fermentation time (h) | $X_4$ | 24 | 48 | 72 |

Box–Behnken design is a three-level second-order design used in response surface methodology [20]. It contributes particular subset of the factorial combinations from the $3^k$ factorial design. In Box–Behnken design, each variable was varied at 3 levels which were coded as −1, 0, 1 [21]. The Box–Behnken design used in this study was established for four independent factors which varied at three levels, and the relationship among independent variables and dependent response was fitted by second-order model as shown in equation (1).

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{n} \beta_{ij} X_i X_j \quad (1)$$

where $Y$ is the measure response, $\beta_o$, $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are the intercept, linear coefficient of $X_i$, quadratic coefficient of $X_i$, and two-factor interaction between $X_i$ and $X_j$, respectively. $X_i$ and $X_j$ are the coded value of the $i$th and $j$th independent variables. The significance of the model was evaluated based on the estimated coefficient and $p$ value obtained from $F$ test. The coefficient of determination, denoted as $R^2$, indicated how well data points fit the line or curve. The relationships among variables and response in response surface methodology were expressed in three-dimensional surface.

### 2.8 Optimization of Growing Condition Using Response Surface Methodology

According to method of Prafulla et al. [22], the bacteria were grown in an Erlenmeyer flask containing 25 ml of liquid medium consisted of (g/l) D-glucose, 10; yeast extract, 10; $K_2HPO_4 \cdot 3H_2O$, 1; $MgSO_4 \cdot 7H_2O$, 0.5. The medium was adjusted to a pH of 7.0–7.5 and incubated at 37 °C in orbital shaker at 180 rpm for 24 h. For the optimization process, 5 % (v/v) of inoculum culture was transferred into the Erlenmeyer flask containing 25 ml medium composed of (g/l) D-glucose, 20; soy peptone, 10; yeast extract, 10; $K_2HPO_4 \cdot 3H_2O$, 1; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.5. Different sets of temperature, SSP, NaCl concentrations and fermentation time were investigated: 30–40 °C, 0.5–1.5 %, 0.5–1.5 % and 24–72 h, respectively. The pH of medium was adjusted to 7.0–7.5. After appropriate incubation temperature and fermentation time at 180 rpm, samples were centrifuged at 10,000 rpm for 30 min to get the supernatant. Centrifuge step was performed at 4 °C. All experiments were conducted in triplicates.

### 2.9 Statistical Analysis

Analysis of variance was used to analyze data. F test was carried out to compare treatment means at $p < 0.05$, and $p$ value from $F$ test was compared with 0.05. The higher the $F$ value and the lower the $p$ value ($p$ value <0.05), the higher is

the significance. Based on *p* value and estimated coefficient, the significance of variables to response was evaluated.

Design Expert 7.0.0 trial version (Stat Ease, USA) was used to design matrix of relationship among variables and response. The optimal treatment was contributed by solving regress equation and analyzing response surface contour plots. The predicted maximize/minimize response was supplied together with the optimal condition based on the input response from empirical experiments.

## 3 Results and Discussion

### 3.1 Fibrinolytic Enzymes in Traditional Fermented Shrimp Pastes

Figure 1 shows the activities of fibrinolytic enzymes extracted from six traditional shrimp paste products in Vietnam. The results indicated that all Vietnamese traditional shrimp paste products contained the fibrinolytic enzyme, in which the products produced in the middle region of Vietnam contained higher fibrinolytic enzyme than those in the north or south of Vietnam. Among them, SP3 had the highest fibrinolytic activity (2.95 FU/ml), followed by SP4 (2.80 FU/ml), SP5 (2.43 FU/ml) and SP1 (1.98 FU/ml), whereas SP2 and SP6 showed the lowest fibrinolytic activity (<1.00 FU/ml). The fibrinolytic enzymes in the shrimp paste products were naturally produced by bacteria from air, utensils or ingredients which were present in nature [1]. Therefore, the difference in fibrinolytic enzyme activities in the shrimp paste products might be due to the source of bacteria and their growth condition such as fermentation condition and ingredients. Each bacterium had its own optimal condition for the growth and for the secondary metabolite secretion resulting in the difference in the fibrinolytic enzyme production. Wang et al. [23] reported that the nattokinase activity in the culture supernatant of *Bacillus subtilis* TKU007 with shrimp shell powder (SSP) as the sole carbon/nitrogen source reached a maximum (6.7 FU/ml) at optimal condition of 1 % SSP, pH 7 and on 3rd day of fermentation, whereas the *Pseudomonas sp.* TKU015 secreted a maximum nattokinase activity (2.3 FU/ml) at optimal condition of 1 % SSP, pH 7 and on 2nd day of fermentation [18].

The bacteria in the SP3, which produced the highest fibrinolytic enzyme, were isolated and identified.

### 3.2 Isolation and Identification of Bacteria and Enzyme Production

Three strains of bacteria isolated from the fermented shrimp paste SP3 were cultured for evaluating their capacity of producing fibrinolytic enzyme. All bacteria were of Gram-positive, rod-shaped, aerobic and endospore-forming bacteria.

Table 2 shows the fibrinolytic enzyme activities in the cultures inoculated with bacterial isolates. The fibrinolytic enzymes were significantly different, ranging between 1.22 and 2.85 FU/ml though the fermentation conditions for fibrinolytic enzyme production were similar. Among the three isolated strains, M2 strain produced the highest fibrinolytic activity with 2.85 FU/ml. The M2 was identified as a strain of

**Table 2** Fibrinolytic enzyme activities in the cultures inoculated with isolated bacteria

| Sample | Enzyme activities (FU/ml) |
|---|---|
| Culture 1 | 1.22 ± 0.24a |
| Culture 2 | 2.85 ± 0.14c |
| Culture 3 | 1.51 ± 0.02b |

Culture 1, Culture 2 and Culture 3 are cultures inoculated with isolated bacterium strains M1, M2 and M3, respectively, for producing fibrinolytic enzymes
All data are the means of triplicate experiments
Data followed by the different letter in the same column are significantly different ±SDs

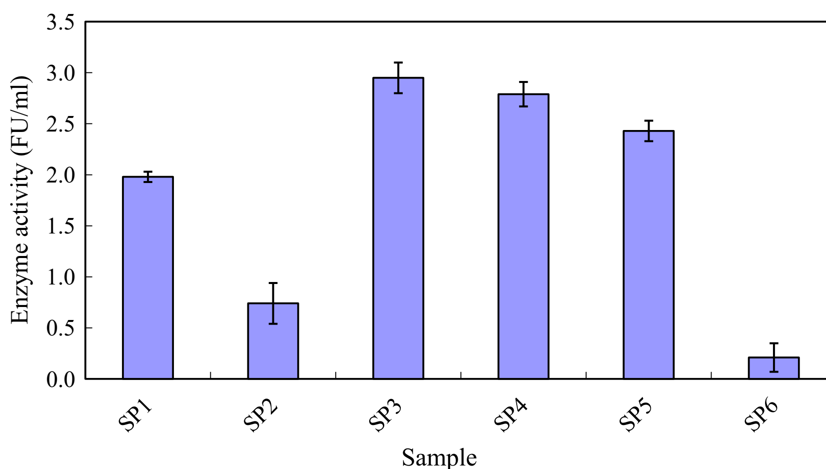


**Fig. 1** Fibrinolytic enzyme activities of Vietnamese traditional shrimp paste products. SP1, a commercial product of Tam Duc Fish Co.; SP2, a commercial product of Trung Thanh Co.; SP3, a traditional handmade product in Thanh Hoa province; SP4, a traditional handmade product in Nha Trang city; SP5, a commercial product of PT FISACO Co.; SP6, a traditional handmade product in Tien Giang province (SP6). All data are the means of triplicate experiments ±SDs

*Bacillus* sp., which was most closely related to the species of *Bacillus weihenstephanensis* with 99 % identities according to 16S rRNA sequencing (data not shown). Previous studies also reported that *Bacillus* sp. isolated from shrimp paste product was considered as one of the most potential microorganisms for fibrinolytic enzyme production [1,12].

### 3.3 Optimization of Growth Conditions for High Fibrinolytic Enzyme Production Using Response Surface Methodology

Growth conditions including temperature, shrimp shell powder (SSP), sodium chloride and fermentation time were chosen as four variables to optimize enzyme production using response surface methodology; these factors were considered to significantly affect the fibrinolytic enzyme secretion by *Bacillus* sp. M2 in the submerged fermentation. The polynomial proposed models for fibrinolytic activity regressed by considering the significant terms. The data were analyzed by multiple regression analysis and the regression coefficients for equation were determined as following equation:

$$Y = +5.26 - 0.25X_1 + 1.54X_2 + 0.27X_3 - 0.45X_4 \\ -0.14X_1X_2 - 0.82X_1X_3 + 1.22X_1X_4 \\ +1.42X_2X_3 - 1.01X_2X_4 + 0.12X_3X_4 - 2.06X_1^2 \\ -0.90X_2^2 - 1.14X_3^2 - 1.47X_4^2$$

where $X_1$, $X_2$, $X_3$ and $X_4$ were coded independent variables of temperature, SSP, NaCl and fermentation time, respectively, and Y was predicted response of fibrinolytic activity.

The results which was tested by the $F$ test for analysis of variance (ANOVA) using Design Expert software are summarized in Table 3. The suitability and adequacy of model was expressed by determination of coefficient ($R^2$). The closer the $R^2$ value to 1 was, the better the correlation between empirical and predicted values was. In this study, the $R^2$ value was found to be 0.91, indicating that the designed model was positive and confident model (Table 3). The ANOVA of the quadratic regression model showed that $F$ value and $p$ value were 7.02 and 0.0019, respectively. These numbers indicated a good relation between the experimental and predicted values of the response. Hence, the model used in this study was statistically significant at the 95 % confidence level ($p < 0.05$). The estimated coefficient together with corresponding $p$ value indicated how significant each factors and interaction between factors were. In this case, $X_2$, $X_1^2$, $X_3^2$, $X_4^2$ were found to be significant model terms. Moreover, the interactions between $X_1$ and $X_4$, $X_2$ and $X_3$, $X_2$ and $X_4$ were also significant and fitted to the model. The lack of fit measured the failure of the model to represent data in the experimental domain at points which are not included in the regression.

**Table 3** Analysis of variance (ANOVA) for the experimental results of response surface quadratic model

| | *df* | Coefficient estimate | $F$ value | Prob > $F$ |
|---|---|---|---|---|
| Model | 14 | 5.26 | 7.02 | 0.0019* |
| $X_1$ | 1 | −0.25 | 1.10 | 0.3190 |
| $X_2$ | 1 | 1.54 | 41.73 | <0.0001* |
| $X_3$ | 1 | 0.27 | 1.27 | 0.2852 |
| $X_4$ | 1 | −0.45 | 3.58 | 0.0879 |
| $X1_1X_2$ | 1 | −0.14 | 0.12 | 0.7372 |
| $X_1X_3$ | 1 | −0.82 | 3.99 | 0.0736 |
| $X_1X_4$ | 1 | 1.22 | 8.69 | 0.0146* |
| $X_2X_3$ | 1 | 1.42 | 11.87 | 0.0063* |
| $X_2X_4$ | 1 | −1.01 | 6.04 | 0.0338* |
| $X_3X_4$ | 1 | 0.12 | 0.092 | 0.7683 |
| $X_1^2$ | 1 | −2.06 | 17.55 | 0.0019* |
| $X_2^2$ | 1 | −0.90 | 3.38 | 0.0958 |
| $X_3^2$ | 1 | −1.14 | 5.38 | 0.0428* |
| $X_4^2$ | 1 | −1.47 | 8.95 | 0.0135* |
| Residual | 10 | | | |
| Lack of fit | 10 | | | |
| $R^2$ | 0.91 | | | |

* $p < 0.05$

**Table 4** The optimal condition for fibrinolytic enzyme production using response surface methodology

| | Optimal values (in a range) | Optimal values (targeted) |
|---|---|---|
| *Variables* | | |
| Temperature (°C) | 32.68 | 33 |
| Shrimp shell powder (%) | 1.50 | 1.50 |
| Sodium chloride (%) | 1.44 | 1.44 |
| Fermentation time (h) | 32.35 | 32 |
| *Response* | | |
| Fibrinolytic activity (FU/ml) | 7.21 | 6.85 |

Table 4 shows the optimal conditions supplied by software and the targeted conditions carried out in this study. The optimal conditions were contributed based on Eq. (1), and the response was also predicted by the Design Expert software. The condition consisted of 1.50 % of SSP ($X_2$), 1.44 % of NaCl ($X_3$), 32.68 °C ($X_1$) and 32.35 h ($X_4$) was corresponded with 7.21 FU/ml of the predicted response. In practical experiment, the optimal condition was maintained with slight modification for suitable setting. The optimal conditions were adjusted to fermentation temperature of 33 °C and fermentation time of 32 h, while SSP and NaCl concentrations were maintained at 1.50 and 1.44 %, respectively. Under targeted conditions, the obtained fibrinolytic activity after doing practical experiment was 6.85 FU/ml, which was not significantly different from the predicted value (7.21 FU/ml). Therefore, the targeted conditions can be applied in producing fibrinolytic enzyme in large scale.

The fibrinolytic enzyme activity produced by *Bacillus* sp. M2 in this study under optimal fermentation conditions was significantly higher than that previously reported [18,23].

Wang et al. [18] reported that 1 % SSP was more suitable as an inducer for nattokinase production by *Pseudomonas* sp. TKU015 and the fibrinolytic enzyme activity reached a maximum level at only 2.3 FU/ml. The production of fibrinolytic enzyme by *Bacillus subtilis* TKU007 was also reported, and the optimal pH, optimal temperature, pH stability and thermal stability were 8, 40 °C, pH 4–11 and <50 °C, respectively, with enzyme activity of 6.7 FU/ml [23]. These results indicated that the production of fibrinolytic enzyme was depend on fermentation conditions and microbial strains.

## 4 Conclusion

The Vietnamese traditional shrimp pastes are the source of fibrinolytic enzyme which was produced by the bacteria naturally existing in those products. The isolated bacterium from the shrimp paste was *Bacillus* sp. M2 strain, which was considered as the most potential microbial source for fibrinolytic enzyme production. In this study, the fibrinolytic enzyme was achieved to 6.85 FU/ml by *Bacillus* sp. M2 strain under the optimal growth conditions using response surface methodology. As a result, the new bacterial strain from the Vietnamese traditional shrimp pastes can be used to produce high amount of thrombolytic agents in industrial and pharmaceutical fields.

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**Conflict of interest** The authors have declared no conflict of interest.

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