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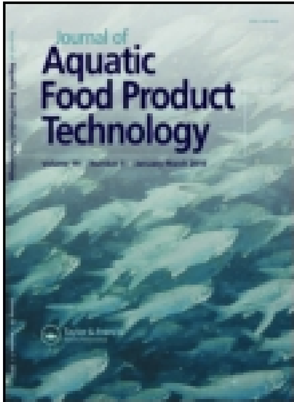

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Optimization of Enzymatic Hydrolysis of Channel Catfish Bones for Preparing Antimicrobial Agents

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*The bones of channel catfish (*Ictalurus punctatus*), a kind of fish processing waste, were hydrolyzed with one of five proteases (alcalase, neutrase, papain, pepsin, and trypsin) in order to generate antibacterial agents. The antibacterial activity of hydrolysates recovered through enzyme hydrolysis was tested by radial diffusion assay (RDA). Pepsin hydrolysate was found to have the greatest antibacterial activity. Thus, the conditions of hydrolysis with pepsin were optimized by response surface methodology (RSM). After screening and optimization, a quadratic model was proposed. The model predicted that the maximum diameter of clear zone, which suggests the optimum antibacterial activity, was around 20.2 mm with a hydrolysis condition of pH 3.5, reaction temperature of 40°C, enzyme-substrate ratio of 1.97/100 (g/g), substrate concentration of 0.15 g/mL, and reaction time of 4 h. Verification experiments under this condition showed a clear zone of 19.8 mm, which agreed well with the model's predicted value. This result indicates that RSM is an effective way to optimize the enzymatic hydrolysis of channel catfish bones to increase antibacterial activity, and the bones of channel catfish are promising resources for generating antibacterial components.*

Keywords antibacterial activity, catfish bone, enzymatic hydrolysis, response surface methodology (RSM), protease

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Introduction

Channel catfish (*Ictalurus punctatus*) was originally found in the Gulf states and Mississippi Valley north to the prairie provinces of Canada and south to Mexico. Since then, it has been widely raised in the United States as well as many other countries. In the 1980s, it was introduced to China. After that, the yield of farm-raised catfish in China rapidly increased, especially in recent years.

With the increased yield of farm-raised channel catfish, catfish has been processed into a variety of products. The process produces a lot of by-products including bones, skins, viscera, and heads, which account for more than half of the total fish weight (Yang et al., 2007). Fish bones are one of the major by-products with a wet weight accounting for 16.3% of the total wet weight of live fish and 25.0% of the dry weight (Cameron, 1985). Moreover, fish bones are perishable due to rich protein and fat contents (Ferraro et al., 2010), thus they are an important source of environmental contamination (Bhaskar et al., 2008). Therefore, technology development for utilizing these bones would not only provide economic benefits but also reduce environmental stress.

Attempts to develop methods for utilizing this by-product include transformation into fish meal for livestock and aquaculture industries (Nguyen et al., 2007), extraction of oil and gelatin (Sathivel et al., 2009; Yang et al., 2008), and obtaining bioactive components through enzymatic hydrolysis (Theodore et al., 2008).

Enzymatic hydrolysis of proteins to obtain bioactive components has attracted public interest recently (Kadam and Prabhasankar, 2010). Bioactive components were shown to have a wide range of properties and functions including antibacterial, antithrombotic, antioxidant, and opioid activities, lowering blood pressure and cholesterol levels, increasing mineral absorption/bioavailability, and modulating immunological response (Hartmann and Meisel, 2007). Recently, one bioactive component from enzymatic hydrolysis of proteins, an antibacterial peptide, has especially captured the attention of researchers because of its efficacy against pathogens without known damage to the host. This peptide can be used as biopreservatives, potential pharmaceuticals, and protective agents for postharvest preservation (Bolscher et al., 2006; Keymanesh et al., 2009; Marshall and Arenas, 2003). To date, antibacterial peptides generated *in vitro* from lactoferrin, casein, whey, and hen ovotransferrin proteins by enzyme hydrolysis have been reported (Haque and Chand, 2008). In addition, they have been found to be functional against different Gram-positive and Gram-negative bacteria, yeasts, and filamentous fungi (Keymanesh et al., 2009). It appears promising to use them as new substitutes for chemical preservatives, antibiotics, and pesticides.

Recently, enzymatic hydrolysates from fish bones have been reported to have antioxidant effects and calcium binding capacity (Je et al., 2007; Jung et al., 2006). However, to our knowledge, there has been no known report about their antibacterial property.

Response surface methodology (RSM) is an important experimental design and a critical technique in process optimization. It can optimize and analyze the effects of each parameter as well as interactions between parameters, thus reducing the number of experimental trials and saving time and labor. RSM is also a useful method to study enzymatic hydrolysis and has been successfully applied in chicken breast meats (Kurozawa et al., 2008), whey protein (Guo et al., 2009), and shrimp processing discards (Guerard et al., 2007).

The objective of this study was to investigate the antibacterial property of enzymatic hydrolysates from channel catfish bones. A number of conditions including enzyme reaction time, temperature, pH, enzyme-substrate ratio, and substrate concentration were

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optimized using response surface methodology in order to obtain a maximum antibacterial activity.

Materials and Methods

Preparation of Channel Catfish Bones

Channel catfish, 7-months old, was kindly provided by Naruhito Aquaculture Co., Ltd. (Tianjin, China). In the laboratory, the fresh fish was washed twice with tap water and then killed. The bones were separated manually by knife and rinsed with cold distilled water to get rid of meat residues that adhered to them. After that, the bones were heated at 85°C to help inactivate endogenous enzymes and to facilitate the removal of fat (Bhaskar et al., 2008). Then, the heat-treated bones were allowed to cool and dry. The dried bones were ground to 1- to 3-mm particles in diameter and stored at −20°C in polyethylene bags until use.

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Enzymatic Hydrolysis of Channel Catfish Bones Using Various Proteases

To produce enzymatic hydrolysates from the bones of channel catfish, enzymatic hydrolysis was performed using alcalase, neutrase, papain, pepsin, and trypsin (Nuoao Co., Tianjin, China) with their respective optimal conditions (Table 1). Briefly, 50 mL of 0.1 M buffer solution was added to 10 g of bone sample in a 250 mL flask. Appropriate pH and temperature for each enzyme were applied. Then, 0.2 g pre-incubated enzyme (pre-incubated for 30 min) was added. As a control experiment, the bones were subjected to the same process as described above except that heat-inactivated enzyme was applied. The flask was hermetically covered with Parafilm and aluminum foil, and the enzymatic hydrolysis was carried out for 5 h with shaking at 170 rpm. At the end of the reaction, the mixture was heated in a boiling water bath for 10 min in order to inactivate the enzyme. After that, the mixture was rapidly cooled in ice and centrifuged at 3,000 *g* for 20 min. The supernatants were collected, lyophilized, and stored at −20°C until use.

Table 1
Conditions for the hydrolysis of channel catfish bones

Enzyme	Activity (U/g)	Temperature (°C)	pH	Buffer	Diameter of the clear zone (mm)***
Control	Same to each enzyme	Same to each enzyme	Same to each enzyme	Same to each enzyme	0.00 ± 0.00^c
Trypsin	2.0×10^4	37	8.0	0.1 M PB*	9.50 ± 0.71^b
Papain	6.5×10^5	50	7.0	0.1 M PB	0.00 ± 0.00^c
Neutrase	2.0×10^5	50	7.0	0.1 M PB	0.00 ± 0.00^c
Alcalase	2.0×10^5	60	8.5	0.1 M PB	0.00 ± 0.00^c
Pepsin	1.25×10^4	37	3.0	0.1 M GHB**	14.75 ± 1.06^a

*Phosphate buffer. **Glycine-HCl buffer. ***The results are the means ± standard deviation of duplicates. Different superscript letters (a, b, and c) in the last column indicate significant ($p < 0.05$) differences among different treatments with Duncan's multiple range tests.

Antibacterial Assays

The antibacterial activity of hydrolysates was determined using the radial diffusion assay (RDA) with some modifications (Li et al., 2007). Briefly, *Escherichia coli* strain TAU8739, isolated from a fish processing plant by Tianjin Agricultural University, China, was grown overnight in Luria-Bertani (LB) broth after three continuous transfers per 24 h growth. The bacteria were centrifuged at 3,000 *g* for 15 min at 4°C, washed twice using cold 20 mM phosphate buffered saline (PBS, pH 7.4), and resuspended in cold PBS. The bacterial concentration was adjusted to 1×10^6 colony forming units (CFU)·mL^{-1} with a spectrophotometric method and confirmed with tryptic soy agar plating. Bacterial suspension (100 μL) was added to the previously autoclaved 15 mL LB broth with 2.0% agar, which was cooled to 45°C and poured into 90 mm sterile Petri dishes. After the agar solidified, sample wells were made using a sterile puncher, 6 mm in diameter. A 20 μL test sample filtered through a Millipore filter (0.22 μm) was added to each well and incubated at 37°C for 18 h. Antibacterial activities were assessed by recording the diameter of the clear zone.

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Experimental Design

Among the five enzymatic hydrolysates, the one with the highest antibacterial activity was chosen for further optimization of working conditions by RSM. Design Expert 7.1.3 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for experimental design, and analysis of variance (ANOVA) was used for statistical data analysis. The RSM can be divided into screening and optimization steps.

A fractional factorial design (FFD; 2^{5-1}) was used for screening factors that influenced the enzymatic hydrolysis process significantly. Insignificant factors were eliminated in order to obtain a smaller, more manageable set of factors (Ahmad et al., 2008). The five factors selected were pH, temperature (T), enzyme-substrate ratio (E/S), substrate concentration (S), and time of hydrolysis (t). The response measured was the antibacterial activity. The levels designed for the factors are shown in Table 2. A total of 16 sets of experiments were employed. The factors that were identified as important or significant were then investigated more thoroughly in subsequent experiments.

The significant factors identified from FFD were further optimized using a central composite design (CCD). The CCD can be used for the prediction and verification of a model equation as well as optimization of the response. CCD experimental design is preferred and widely used for fitting a second-degree polynomial model represented by Equation 1,

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j, \quad (1)$$

where y was the response variable; β_0, β_i, β_{ii}, and β_{ij} were the intercept, linear, quadratic, and interaction regression coefficients of the model, respectively; and x_i and x_j were the coded independent variables. In developing the regression equation, the test variables were coded according to Equation 2,

$$x_i = (X_i - X_i^*)/\Delta X_i; \quad i = 1, 2, \ldots, k; \quad (2)$$

where x_i was the independent variable coded value; X_i was the independent variable actual value; X_i^* was the independent variable actual value on the center point; and ΔX_i was the step change value (Ahmad et al., 2008). The statistical significance of the second-order

Table 2
Design matrix and results of two-level fractional factorial design on the hydrolysis of channel catfish bones

		Experimental factors and code levels					Diameter of the clear zone (mm)	
Std.	Run	pH A	T (°C) B	E/S (g/100 g) C	S (g/100 mL) D	t (h) E	Actual value	Predicted value
1	12	2.5 (−1)	30 (−1)	1 (−1)	10 (−1)	5 (1)	9.00	9.69
2	2	3.5 (1)	30	1	10	3 (−1)	12.00	12.44
3	13	2.5	40 (1)	1	10	3	12.00	11.69
4	4	3.5	40	1	10	5	17.00	16.19
5	1	2.5	30	3 (1)	10	3	9.00	8.81
6	14	3.5	30	3	10	5	12.00	11.56
7	11	2.5	40	3	10	5	12.00	10.81
8	7	3.5	40	3	10	3	15.00	15.31
9	10	2.5	30	1	20 (1)	3	11.00	9.69
10	5	3.5	30	1	20	5	12.00	12.44
11	3	2.5	40	1	20	5	11.00	11.69
12	9	3.5	40	1	20	3	16.00	16.19
13	15	2.5	30	3	20	5	8.00	8.81
14	16	3.5	30	3	20	3	12.00	11.56
15	8	2.5	40	3	20	3	10.00	10.81
16	6	3.5	40	3	20	5	15.00	15.31

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model equation was determined by the *F* value. The proportion of variance explained by the model was given by the multiple coefficients of determination, R^2.

The significant factors identified from FFD, pH, T, and E/S were considered with eight factorial points (2^k, $k = 3$) and six axial or star points (2 k, $k = 3$). Three replicates at the center points of them were also considered. Design Expert 7.1.3 was used for regression and graphical analysis of the data obtained. Optimal values of design variables were obtained by the regression analysis. All experimental designs were randomized. Experiments were performed in duplicate, and mean values were applied.

Results and Discussion

Preparation of Hydrolysates from Channel Catfish Bones Using Various Proteases

The biological activities of protein hydrolysates depend on the protein substrate, the enzyme specificity, and the hydrolysis conditions (Balti et al., 2010). Since each enzyme has its specific cleavage positions on polypeptide chains, alcalase, neutrase, papain, pepsin, and trypsin were used, respectively, to generate bone hydrolysates. Hydrolysates obtained were evaluated for their antibacterial activities. As shown in Table 1, the order of antibacterial activity was pepsin hydrolysate > trypsin hydrolysate > alcalase, neutrase, and papain hydrolysates. Therefore, among the five hydrolysates, pepsin hydrolysates exerted the highest antibacterial activity. In addition, the difference of antimicrobial activities between pepsin and trypsin hydrolysates was significant ($p < 0.05$). Thus, pepsin was

chosen for further optimization studies under the hydrolysis conditions used to prepare hydrolysates.

Pepsin hydrolysates produced at lower pH had better antibacterial effects than those produced by other enzymes. The results indicate that potent antibacterial peptides from catfish bones were generated by enzymes having a cleavage site specificity similar to pepsin, which preferentially cleaves at the carboxyl terminus of phenylalanine and leucine residues (Tomita et al., 1991).

Screening Factors for Peptic Hydrolysis of Channel Catfish Bones

FFD is an experimental design consisting of a carefully chosen fraction of the experimental runs of a full factorial design. The FFD was carried out according to Table 2. The coded factor and response values (diameter of clear zone indicating antibacterial activity) are also shown in Table 2. The estimated main effects of the factors together with their interactions were plotted on a half normal probability graph. As shown in Figure 1, while the effects neglected were normally distributed, mean zero and variance tended to fall along a straight line. In contrast, significant effects had non-zero means and did not lie along the straight line. The larger the effect, the further away it was from the straight line (Design-Ease, 2006). Figure 1 shows that the interaction of AB, and factors A (pH), B (T), and C (E/S)—especially factors A and B—significantly affected the hydrolysis reaction.

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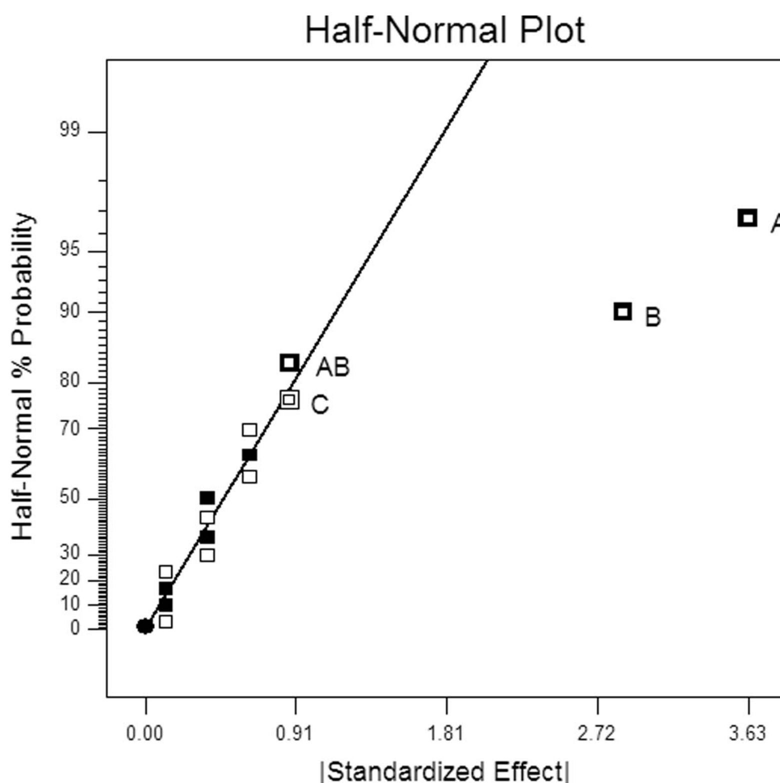

Figure 1. Half normal probability plot of the diameter of the clear zone.

Table 3
Analysis of variance (ANOVA) for 2^{5-1} fractional factorial design on the hydrolysis of channel catfish bones

Source	Sum of squares	*df*	Mean square	*F* value	Prob > *F*
Model	91.75	4	22.94	35.10	<0.0001
A	52.56	1	52.56	80.44	<0.0001
B	33.06	1	33.06	50.60	<0.0001
C	3.06	1	3.06	4.69	0.0532
AB	3.06	1	3.06	4.69	0.0532
Residual	7.19	11	0.65	–	–
Cor. total	98.94	15	–	–	–

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Based on the results in Table 2, ANOVA was performed to obtain the quantitative information of each effect, as shown in Table 3. Factors with values of "Prob > *F*" less than 0.05 indicated significant; i.e., significantly affected the measured response. In this test, "Prob > *F*" values for factors of A and B were highly significant ($p < 0.01$), while factor C and the interaction of AB were close to significant ($p \approx 0.05$). Other factors and interactions did not affect the hydrolytic efficiency significantly. Since this step is a coarse optimization, those critical values should be carefully considered for further application. The interaction of AB included factor A and factor B. Therefore, factor C was selected for further optimization. These results suggest that the important independent variables were pH, T, and E/S, which were further optimized for peptic hydrolysis of channel catfish bones. This screening result was consistent with the result of Guerard et al. (2007), who reported that pH and temperature played a dominant role in the hydrolysis of shrimp processing discards.

Optimization of the Hydrolysis Parameters

Based on the results from the initial FFD, the significant factors of pH, T, and E/S were further studied, modeled, and optimized using CCD. The parameters of S and t that were shown to not significantly influence the measured response (antibacterial activities) were set at their corresponding central levels (S: 15 g/100 mL; t: 4 h) in the screening design. Seventeen groups were used in this procedure, as shown in Table 4. All 17 groups were tested, and the results were analyzed via multiple regressions (Table 4). The ANOVA for the response surface quadratic model is shown in Table 5. The model *F* value of 23.57 implied that the model was significant. The *p* value was 0.0002; i.e., there was a 0.02% chance that an error was caused by noise, which also implied that the regression model was significant. The fitness of the model was examined by the determination coefficient (R^2). In this case, the R^2 was 0.9681, which indicated that the model did not explain only 3.20% of the total variations. The Pred R^2 was 0.8419, which somewhat agreed with the Adj R^2 of 0.9270. The Lack of Fit *F* value (0.40) implied that it was not significantly related to the pure error. At the same time, a relatively low coefficient of variation (C.V. = 9.45%) indicated a better precision and reliability of experiments carried out (Design-Ease, 2006).

Table 4
Design matrix and results of CCD on the hydrolysis of channel catfish bones

		Experimental factors and code levels			Diameter of the clear zone (mm)	
Std.	Run	pH A	T (°C) B	E/S (g/100 g) C	Actual value	Predicted value
1	13	2.50 (−1)	30.00 (−1)	1.00 (−1)	7.6	7.59
2	17	3.50 (1)	30.00	1.00	8.7	8.55
3	16	2.50	40.00 (1)	1.00	8.5	9.27
4	11	3.50	40.00	1.00	17.6	17.53
5	3	2.50	30.00	3.00 (1)	8.5	9.16
6	7	3.50	30.00	3.00	9.6	9.42
7	9	2.50	40.00	3.00	8.9	9.64
8	14	3.50	40.00	3.00	16.6	17.20
9	2	2.16 (−1.68)	35.00	2.00	12.3	11.30
10	8	3.84 (1.68)	35.00	2.00	18.3	18.46
11	1	3.00 (0)	26.59 (−1.68)	2.00	6.7	6.80
12	5	3.00	43.41 (1.68)	2.00	15.7	14.76
13	12	3.00	35.00	0.32 (−1.68)	7.9	7.86
14	10	3.00	35.00	3.68 (1.68)	9.7	8.90
15	15	3.00	35.00 (0)	2.00 (0)	16.8	16.35
16	4	3.00	35.00	2.00	17.5	16.35
17	6	3.00	35.00	2.00	14.6	16.35

Table 5
Analysis of variance (ANOVA) for the quadratic model of the hydrolysis of channel catfish bones

Source	Sum of squares	*df*	Mean square	*F* value	Prob > *F*
Model	277.07	9	30.79	23.57	0.0002
A-pH	61.97	1	61.97	47.44	0.0002
B-T	76.56	1	76.56	58.61	0.0001
C-E/S	1.31	1	1.31	1.00	0.3502
AB	26.65	1	26.65	20.40	0.0027
AC	0.25	1	0.25	0.19	0.6780
BC	0.72	1	0.72	0.55	0.4820
A^2	3.03	1	3.03	2.32	0.1717
B^2	43.65	1	43.65	33.42	0.0007
C^2	89.42	1	89.42	68.45	<0.0001
Residual	9.14	7	1.31	–	–
Lack of fit	4.56	5	0.91	0.40	0.8240
Pure error	4.58	2	2.29	–	–
Cor. total	286.22	16	–	–	–

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A second-order polynomial model for the antibacterial activity was obtained from regression analysis of CCD results using Design Expert 7.1.3 as seen in Equation 3,

$$y = 16.35 + 2.13x_1 + 2.37x_2 + 0.31x_3 + 1.83x_1x_2 - 0.18x_1x_3 - 0.30x_2x_3 - 0.52x_1^2 - 1.97x_2^2 - 2.82x_3^2, \quad (3)$$

where y was the response factor, the diameter of the clear zone; and x_1, x_2, and x_3 were the values of the independent factors, pH, T, and E/S, respectively. The model coefficients and probability values could be obtained by the software (data not shown). The coefficients of the quadratic model included three linear coefficients (x_1, x_2, x_3), three quadratic coefficients (x_1^2, x_2^2, x_3^2), and three interact coefficients (x_1x_2, x_1x_3, x_2x_3) of the response surface model. The significance of each coefficient was determined by the *p* values listed in Table 5. The smaller the *p* value, the more significant the corresponding coefficient.

From Table 5, the partial coefficients of the regression model showed that T was the most important factor because it had a significant effect at the linear ($p = 0.0001$), the quadratic ($p = 0.0007$), and the interactive (with pH) level ($p = 0.0027$). Significance of "T" meant that a small change of it could cause a large variation in response. Actually, changes of T affected the rate of two independent processes, enzymatic hydrolysis and thermal inactivation of pepsin itself. When T was lower than 35°C, the rate of hydrolysis decreased, resulting in a small diameter of the clear zone. When T was higher than 40°C, the diameter of the clear zone was also small due to thermal inactivation of pepsin. Similar results were observed in some food protein sources such as chicken meat (Kurozawa et al., 2008) and catla (Bhaskar et al., 2008) under hydrolytic reactions.

The linear main effect of pH ($p = 0.0002$) was much more significant than its quadratic main effect ($p = 0.1717$). Further pH increases in the pH range (2.5–3.5) were used, which increased the diameter of the clear zone. Changes in pH affect ionization of free substrates and free pepsins. Pepsin activity is affected by pH due to its effect on ionization of enzyme-substrate complex. The result was consistent with the report by Dubois et al. (2005).

In the case of E/S, its level had a highly significant effect on the diameter of the clear zone at the quadratic level ($p < 0.0001$). The negative symbol of the quadratic coefficient meant that by either increasing or decreasing the level of E/S the diameter of the clear zone decreased. The phenomenon and its related reasons have been discussed by Carreira et al. (2004).

The diameters of the clear zones for different levels of variables could also be predicted from respective response surface plots generated by the proposed models, as shown in Figures 2a–2c. These figures depicted the interaction between two independent variables when the third variable was maintained at the central point. Figure 2a showed the effect of T, pH, and their interaction on antibacterial activity with a constant E/S of 2/100 (g/g) during enzymatic hydrolysis. A higher pH and T enhanced the diameter. At a low temperature (30°C, for instance), the diameter was slightly affected by pH; whereas, at a higher temperature (40°C, for instance), pH was extremely relevant to the augmentation of antibacterial activity. Thus, the appropriate maximal antibacterial activity was determined at a relatively high pH and T. Figure 2b showed the effect of E/S, pH, and their interaction on antibacterial activity with temperature at 35°C. At a constant pH, E/S demonstrated quadratic effects on the response. Generally, higher enzyme concentration resulted in a hydrolysate with a greater degree of hydrolysis (DH). Therefore, the antibacterial activity

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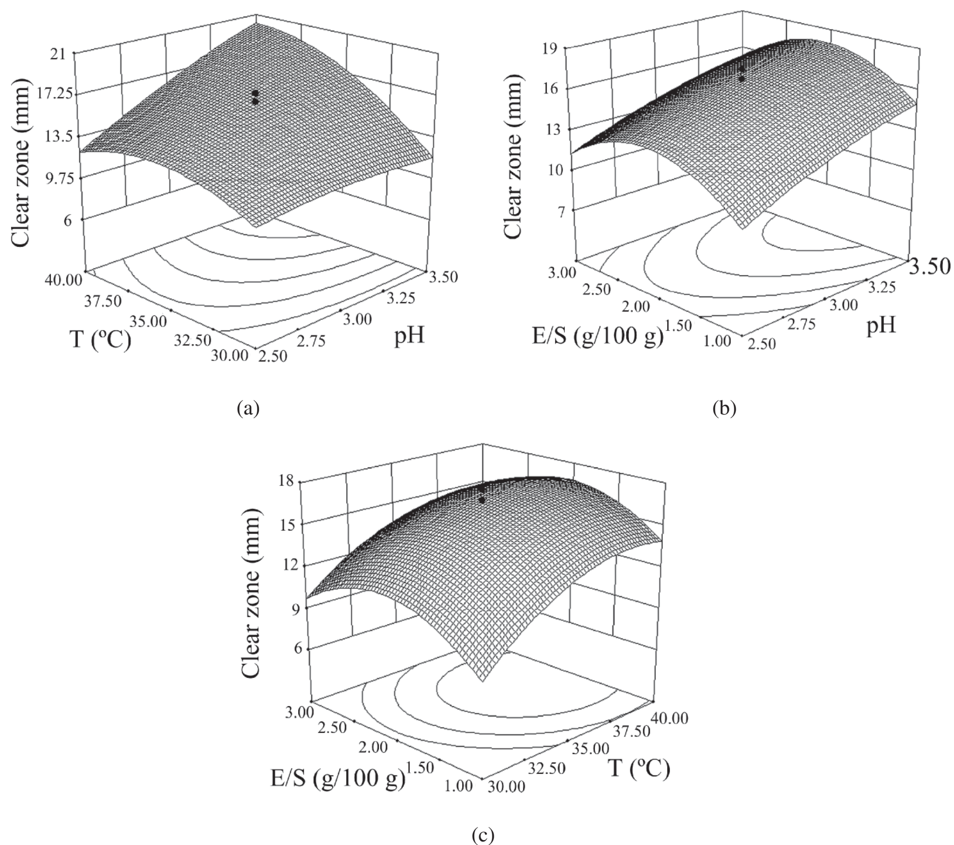

Figure 2. Response surface plot of the diameter of the clear zone during enzymatic hydrolysis: (a) effect of temperature (T), pH, and their interaction on the antibacterial activity with E/S of 2 g/100 g; (b) effect of E/S, pH, and their interaction on the antibacterial activity with 35°C; (c) effect of temperature (T), E/S, and their interaction on the antibacterial activity with pH 3.

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was not positively correlated with DH. The result here was consistent with the finding that greater DH can not lead to higher inhibition of angiotensin-I converting enzymes (Guo et al., 2009). The effect of T, E/S, and their interaction on antibacterial activity with a pH of 3 is shown in Figure 2c. The maximum clear zone was obtained with T of 38°C and E/S of 2/100 (g/g).

From the RSM result, an optimal antimicrobial activity could be obtained at pH of 3.5, temperature of 40°C, and E/S of 1.97/100 (g/g). Under the above conditions, the maximum diameter of the clear zone predicted by the model was 20.2 mm. In order to verify whether the prediction was correct, triplicate experiments were performed under the above conditions, which generated a result showing that the average diameter was 19.8 mm. Thus, the experimental result agreed well with the predicted value from the model equation. Since the antibacterial activity of catfish bone hydrolysates was higher than other protein hydrolysates, such as *Tenebrio molitor* protein (Liu et al., 2009), the antibacterial agents obtained using the optimum conditions could be used for further studies such as investigating the mechanism of their antimicrobial activity and applications in food systems.

Conclusions

Five proteases were used for hydrolyzing channel catfish bones in order to obtain antimicrobial agents. Pepsin hydrolysate was shown to have the most effective antimicrobial activity among all the hydrolysates. A further RSM was applied to optimize pepsin hydrolysis conditions for obtaining maximum antimicrobial activity. The predicted optimum conditions were pH of 3.5, reaction time of 4 h, temperature of 40°C, and E/S of 1.97 g/100 g. Under these conditions, the predicted diameter of clear zone was 20.2 mm and the experimental value was 19.8 mm, suggesting that actual experimental value agreed well with the predicted value from the model.

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