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OPTIMIZATION OF ENZYMATIC HYDROLYSIS CONDITIONS FOR ANTIMICROBIAL ACTIVITY AGAINST *Pantoea* Spp. CAUSING RICE LEAF BLIGHT

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Abstract: The Central Composite Design (CCD) within the Response Surface Methodology (RSM) was applied to optimize the enzymatic hydrolysis process. This process used Alcalase® to hydrolyze *Bactronophorus thoracites* protein with the goal of maximizing its antimicrobial effects. Four distinct parameters were identified as independent variables: pH (A: 8.5–10.5), temperature (B: 45–65 °C), hydrolysis time (C: 120–360 min), and enzyme-to-substrate ratio (D: 1.45%–2.65% w/v). Meanwhile, the antimicrobial activity was chosen as the response variable, specifically against *Pantoea ananatis* (Y_1) and *Pantoea stewartii* (Y_2). According to the findings, the constructed quadratic polynomial model showed a significant correlation with the experimental data, as evidenced by the coefficient of determination (R^2) values for antimicrobial activity: Y_1 being 0.9893 ($p < 0.0001$) and Y_2 at 0.9848 ($p < 0.0001$). Optimal antimicrobial activity for *Bactronophorus thoracites* protein hydrolysates (BTPH) was recorded at 46.748% against *P. ananatis* and 40.768% against *P. stewartii*. This result was observed under the optimal conditions of pH 9.5, temperature 55ºC, hydrolysis duration of 240 minutes, and 2.05% w/v enzyme-to-substrate ratio. There was a notable alignment between the actual and predicted values from our models, with the Residual Standard Error (RSE) values falling under 5%. Furthermore, the established Minimum Inhibitory Concentration (MIC) was 250µg/mL, and the Minimum Bactericidal Concentration (MBC) was 500µg/mL for both *P. ananatis* and *P. stewartii*. In conclusion, the findings suggest that the refined BTPH has great promise as an effective bioactive component for agricultural use.

Keywords: *Alcalase®, Antimicrobial, Response Surface Methodology (RSM), Rice pathogens, Shipworms.*

1. Introduction

Leaf blight, a disease caused by *Pantoea* bacteria, is a significant issue in many rice-growing countries, causing substantial economic losses (Chukwu et al., 2019). The disease can cause up to 70% yield reduction in some regions. The disease was first linked to *Pantoea agglomerans* in Venezuela in 2002 (González et al., 2015), and later found in India in 2008 and Korea in 2009 (Lee et al., 2010; Mondal et al., 2011). Recent Malaysian outbreaks were attributed to *P. stewartii*, *P. ananatis*, and *P. dispersa* (Azizi et al., 2019a, b; Toh et al., 2019), with *P. stewartii* and *P. ananatis* suggested as the primary agents (Azizi et al., 2019a, b). The symptoms include water-soaked lesions evolving into brown leaf stripes, and in severe cases, yellowing or burning patterns along leaf borders.

Although numerous strategies for managing leaf blight in rice have been published, the precise procedure for controlling *Pantoea* spp. has not been determined. For instance, *Xanthomonas oryzae* leaf blight can be controlled by spraying agrochemical chemicals on the diseased plants (Khan et al., 2012). Even though agrochemical chemicals are beneficial from a crop production perspective, excessive use might have significant environmental implications and acute toxicity issues due to their bio-magnification and persistency, resulting in rapid pathogen resistance towards the chemical (Sharma et al., 2000; Sundin & Wang 2018; Valarmathi, 2020). Therefore, the world needs novel active biomolecules to fight the emerging and re-emerging plant pathogens (Hamed et al., 2018).

The extraction process plays a crucial role in the identification of bioactive components when producing bioactive compounds from natural sources. Protein hydrolysis is an extraction process

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that uses an enzyme in an acidic or alkaline setting to dismantle protein peptide bonds. It's a well-established technique for altering the nutritional and functional characteristics of marine protein and creating antimicrobial peptides through enzyme-driven protein hydrolysis. This technique involves the use of protease under conditions that can be precisely managed, such as the duration of hydrolysis, temperature, enzyme-to-substrate ratio and pH (Amin & Cheng, 2019; Rosa et al., 2011; Jamal et al., 2022; Jayaprakash & Perera, 2020). Over the past few decades, much of the scientific research in this area has concentrated on mollusks, specifically in the quest for antimicrobial peptides (AMPs) (Avila, 2006; Benkendorff, 2010, 2014).

Many previous studies have effectively utilized enzymatic hydrolysis for the extraction of bioactive compounds from diverse sources (Amin & Cheng, 2019; Wang et al., 2013; Jayaprakash & Perera, 2020). The capability to attain elevated extraction efficiency under favorable conditions has established enzymatic hydrolysis as a commonly employed extraction technique. Moreover, enzymatic hydrolysis is recognized for its environmentally friendly attributes, as it generally produces fewer detrimental byproducts and waste when compared to the conventional chemical methods. Consequently, enzymatic hydrolysis is highly regarded as a promising and sustainable extraction approach.

At present, there is a significant demand to establish a reliable model that optimizes variables like pH, temperature, hydrolysis duration, and enzyme-to-substrate ratio to determine the best condition for mollusks (Amin & Cheng, 2019). Both observational and statistical strategies can shed light on this optimization (Adebo et al., 2018; Hamid et al., 2015; Wang et al., 2017). In this realm, the Response Surface Methodology (RSM) stands out as an effective tool. RSM combines mathematical and statistical insights to fit empirical models with real-world data, often using linear or quadratic equations to describe the system being studied. This makes RSM an asset for research projects aiming to optimize experimental settings (Adebo et al., 2018). One of RSM's most notable advantages is its efficiency in minimizing experimental runs, reducing costs, and saving time. Such benefits have made it a popular choice for enhancing the extraction of antioxidants and antimicrobial compounds from natural origins (Hamid et al., 2015; Seema & Rajesh, 2015; Wang et al., 2017).

B. thoracites offers potential as a source for synthesizing bioactive peptides and other beneficial protein derivatives. Using proteins from this organism, enzymatic hydrolysis can create these peptides. The antibacterial properties of peptides from mollusks have been discussed in several studies (Avila, 2006; Rosa et al., 2011; Mitta et al., 1999; Jamal et al., 2022a,b; Charlet et al., 1996; Hubert, 1966). These peptides have shown effectiveness against rice diseases like the one caused by *Xanthomonas oryzae* (Sharma et al., 2000) and may serve agricultural roles, from combating pathogens to medicinal applications (Kaspar et al., 2019; Robertsen & Musiol-Kroll, 2019; Sarika et al., 2012). These peptides also exhibit a wide array of biological benefits, including antiviral and antidiabetic to anticancer activities (Agrawal et al., 2017; Gogineni & Hamann, 2018). Additionally, their potential as antimicrobial agents have been underscored by previous studies like Sathoff et al. (2019), Datta et al. (2015), and Seo et al. (2014).

However, previous studies on *B. thoracites* protein hydrolysates have not explored the optimization of Alcalase® enzyme hydrolysis for antimicrobial potential. Therefore, this study seeks to optimize the enzymatic hydrolysis conditions with Alcalase® to maximize the antimicrobial activity of *B. thoracites* against rice pathogens.

2. Materials and Methods

Sample Collection

B. thoracites was collected from a mangrove forest at Kelanang Beach, Banting, Selangor, Malaysia, (2° 48'44.5" N, 101° 22'08.5" E). *B. thoracites* were safely packed in a plastic bag and chilled for transportation to the Plant Molecular Biology Lab (UPM).

Preparation of Crude Extract

Shipworms were cleaned and homogenised at 4°C. This mixture was dispersed with cold deionised water and packed in small plastic bags, then frozen overnight at -80°C. They were then freeze-dried until a stable weight was reached. Post-drying, the samples were ground into powder, sieved to ensure fineness, and stored in capped bottles at -20°C for future use.

Protein Hydrolysate Preparation

The Response Surface Methodology (RSM) and Central Composite Design (CCD) in Design-Expert® software were applied for optimization of conditions. 6g of *B. thoracites* was dissolved into 100mL phosphate buffers. According to the RSM design, protein and enzyme solutions were mixed to get the desired enzyme concentration. This mixture was shaken at 100 rpm and heated to 95°C for 15 minutes to deactivate the enzyme. Post-heating, the samples were centrifuged at 2600g for 15 minutes at 4°C to remove impurities. The resulting supernatant, the protein hydrolysate, was collected. After assessing the degree of hydrolysis, it was freeze-dried. The final product, *Bactronophorus thoracites* protein hydrolysates (BTPH), was then stored at -20°C for further research.

Experimental Design, Modelling and Statistical Analysis

The Design-Expert® software (version 13.0) was used to design experiments and analyze data. Using the Central Composite Design (CCD) method, four factors were assessed over five levels to study the antimicrobial activity against *P. ananatis* and *P. stewartii*. Table 1 shows the selected variables and their levels, while Table 2 presents the experiment layout. A total of 30 tests were conducted, including three central point repeats. These were randomized to account for unforeseen factors. Based on the

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Malaysian Journal of Science 43(3): 1-12 (September 2024)

data, a second-order polynomial model or a simpler one was used, as shown in Equation (1).

$$Y = z_0 + \sum z_1\ A + \sum z_{12}\ AB + \sum z_{123}\ AA \qquad (1)$$

Equation (1) represents formula which " Y " symbolizes the dependent variable, which pertains to the antibacterial action against *P. ananatis* and *P. stewartii*. The term "z_0" indicates the model's average value, "z_1" is the coefficient linked to parameter A, while "z_{12}" and "z_{123}" are coefficients corresponding to the interaction terms AB and AA, respectively. The equation considers the quadratic, linear, and interactive effects of process variables on the outcomes. The importance of each process variable's coefficient was gauged using the Student's t-test. Additionally, the relevance, accuracy, and influence of process variables on the results were scrutinized using Analysis of Variance (ANOVA).

Table 1. The four variables and their associated coded levels for optimizing enzymatic hydrolysis conditions.

Symbol	Independent Variable	Coded level				
		-2	-1	0	+1	+2
A	pH	8.5	9.0	9.5	10	10.5
B	Temperature (°C)	45	50	55	60	65
C	Hydrolysis time (min)	120	180	240	300	360
D	Enzyme-to-substrate ratio (E/S) % (w/v)	1.45	1.75	2.05	2.35	2.65

Table 2. Central Composite Design (CCD) design for optimizing enzymatic hydrolysis conditions under different levels of process variables.

Run	Independent Variables			
	A	B	C	D
1	8.5	55	240	2.05
2	9	50	180	1.75
3	9	50	180	2.35
4	9	50	300	1.75
5	9	50	300	2.35
6	9	60	180	1.75
7	9	60	180	2.35
8	9	60	300	1.75
9	9	60	300	2.35
10	9.5	45	240	2.05
11	9.5	55	120	2.05
12	9.5	55	240	1.45
13	9.5	55	240	2.05
14	9.5	55	240	2.05
15	9.5	55	240	2.05
16	9.5	55	240	2.05
17	9.5	55	240	2.05
18	9.5	55	240	2.05
19	9.5	55	240	2.65
20	9.5	55	360	2.05
21	9.5	65	240	2.05
22	10	50	180	1.75
23	10	50	180	2.35
24	10	50	300	1.75
25	10	50	300	2.35
26	10	60	180	1.75
27	10	60	180	2.35
28	10	60	300	1.75
29	10	60	300	2.35
30	10.5	55	240	2.05

Independent variables: A, pH; B, Temperature (°C); C, Hydrolysis time (min); D, Enzyme to substrate ratio (% w/v).

Determination of Antimicrobial Activity

Protein hydrolysates were tested for antimicrobial effects against *P. ananatis* and *P. stewartii*, with an unhydrolyzed sample as control. They were mixed with Luria Broth and incubated at 30°C for 24 hours. Antimicrobial activity was assessed by measuring absorbance at 600 nm, following guidelines from Mohamad Asri et al. (2020). The bacterial inhibition percentage was calculated using a formula (Equation 2). The most effective samples were then freeze-dried for further analysis.

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Malaysian Journal of Science 43(3): 1-12 (September 2024)

$$Inhibition\% = \frac{(24\ h\ negative\ control - 0\ h\ negative\ control) - (24\ h\ sample - 0\ h\ sample)}{0\ h\ negative\ control} \quad (2)$$

Validation of Optimum Condition

The optimal conditions for antimicrobial activity were predicted using the Design-Expert software. The verification of the model was done by hydrolyzing *B. thoracites* protein under these conditions in three tests: pH 9.5, 55°C, 240 minutes of hydrolysis, and 2.05% enzyme-to-substrate ratio. The hydrolysates were then freeze-dried and tested for antimicrobial activity. The predicted and actual activities were compared using t-test and the residual standard error (RSE). If the RSE (Equation 3) was under 5%, the predictions were considered accurate, as per methods from Ngan et al. (2014) and Zakaria et al. (2021).

$$Residual\ standard\ error\ (\%) = \left|\frac{Actual\ value - Predicted\ value}{Predicted\ value}\right| \times 100 \quad (3)$$

Effective Inhibition Concentration

The effectiveness of BTPH against bacteria was tested using methods adapted from Mohamad Asri et al. (2020) and Muhialdin et al. (2020). A bacterial culture was mixed with LB and exposed to different BTPH concentrations to find the least amount that stops bacterial growth. After incubating for 24 hours at 30°C, growth was measured. The smallest BTPH amount preventing visible growth was noted as the MIC. The MBC was determined by placing samples on agar plates before incubating the culture at 37°C for 24 hours, and observing which concentration prevented growth. These tests were repeated three times.

Data Analysis

Data is presented as the average from three repetitions ± standard deviations, using 95% confidence level.

3. Results and Discussion

Model Calibration and Variance Analysis

Table 3 displays the matrix of actual and predicted response values. Each analysis was repeated three times for accuracy. Using Analysis of Variance (ANOVA), the effects of the variables on the responses were examined. The ANOVA results showed that the models were statistically significant, with F-values of 98.68 for Y_1, *P. ananatis*, and 69.49 for Y_2, *P. stewartii*. The data suggested a less than 0.01% likelihood that these values occurred by chance. The models were deemed significant since the probability for each response was below 0.05. Based on the Central Composite Design, the antibacterial activity for *P. ananatis* (Y_1) ranged from -0.978% to 46.748%, while for *P. stewartii* (Y_2), it ranged from -2.947% to 40.768%.

Table 4 shows the quadratic models that link the independent and dependent variables for each outcome. From the ANOVA results, the R^2 values for antibacterial activities against Y_1 (*P. ananatis*) and Y_2 (*P. stewartia*), were 0.9893 and 0.9848, respectively. These values indicate a strong connection between the Central Composite Design (CCD) and the models. Additionally, the predicted R^2 values, which measure how well the model predicts response outcomes, were 0.9599 for Y_1, and 0.9271 for Y_2. This result shows a close match between experimental and model-based predictions. Previous studies, like those by Gottipati et al. (2010) and Bordbar et al. (2018), have shown that an R^2 value above 0.9 indicates a good fit. In this case, the model captures about 99% of the changes in the responses.

Typically, the R^2 value increases whenever an additional variable is incorporated into a model, regardless the significance of the statistic being evaluated. This is true irrespective of the variables number included in the model. Moreover, the adjusted R-square (Adj. R^2) is a crucial measure that determines the model's adequacy. The Adj. R^2 not only communicates the predictive power of the regression models but also considers multiple variables simultaneously. The tally value only increases when a specific variable enhances the model significantly more than what would be expected by chance alone. The adjusted R^2 values for the antibacterial activity were 0.9792 and 0.9706 for *P. ananatis* and *P. stewartii*, respectively. This result validates the models' predictive capacity in defining the optimal conditions necessary for achieving the highest antimicrobial activity of *Bactronophorus thoracites* protein hydrolysates (BTPH) and showing that most of the variability of these responses were explained by the linear model as illustrated in Figure 1. However, there are points that are located further away from this line or clustered region, suggesting discrepancies between predicted and observed values for those data points. The models for antibacterial activities Y_1 (*P. ananatis*) and Y_2 (*P. stewartii*) showed variation coefficients (CV) of 11.86% and 16.70%. Since these are below 20%, the models are therefore considered as consistent. The lack-of-fit test gave p-values of 0.7919 for Y_1 and 0.2797 for Y_2, suggesting a good fit. Thus, these models are reliable in predicting the outcomes.

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Malaysian Journal of Science 43(3): 1-12 (September 2024)

Table 3. Central Composite Design (CCD) and Response Values

Run	Independent variables				Code level				Response variable (%)			
	A (pH)	B (°C)	C (min)	D (% w/v)	A	B	C	D	Y_1		Y_2	
									Actual	Prediction	Actual	Prediction
1	8.5	55	240	2.05	-2	0	0	0	-0.978	-0.503	0.060	0.281
2	9	50	180	1.75	-1	-1	-1	-1	3.788	3.844	1.591	0.623
3	9	50	180	2.35	-1	-1	-1	+1	-1.318	-2.219	17.303	17.885
4	9	50	300	1.75	-1	-1	+1	-1	12.451	13.003	10.021	10.198
5	9	50	300	2.35	-1	-1	+1	+1	7.788	7.693	15.228	16.431
6	9	60	180	1.75	-1	+1	-1	-1	3.121	4.339	-0.659	-1.317
7	9	60	180	2.35	-1	+1	-1	+1	10.249	9.406	17.638	20.243
8	9	60	300	1.75	-1	+1	+1	-1	13.752	11.944	2.382	1.825
9	9	60	300	2.35	-1	+1	+1	+1	18.316	17.764	12.271	12.355
10	9.5	45	240	2.05	0	-2	0	0	18.986	18.899	6.624	5.409
11	9.5	55	120	2.05	0	0	-2	0	1.902	1.897	12.092	10.309
12	9.5	55	240	1.45	0	0	0	-2	7.642	7.150	11.258	13.043
13	9.5	55	240	2.05	0	0	0	0	40.117	42.086	40.768	38.987
14	9.5	55	240	2.05	0	0	0	0	40.405	42.086	40.502	38.987
15	9.5	55	240	2.05	0	0	0	0	41.896	42.086	35.724	38.987
16	9.5	55	240	2.05	0	0	0	0	41.389	42.086	39.777	38.987
17	9.5	55	240	2.05	0	0	0	0	41.959	42.086	37.156	38.987
18	9.5	55	240	2.05	0	0	0	0	46.748	42.086	39.992	38.987
19	9.5	55	240	2.65	0	0	0	+2	12.352	14.268	30.768	26.073
20	9.5	55	360	2.05	0	0	+2	0	13.258	14.687	24.502	23.374
21	9.5	65	240	2.05	0	+2	0	0	18.991	20.502	-1.660	-3.356
22	10	50	180	1.75	+1	-1	-1	-1	10.977	12.320	1.182	2.275
23	10	50	180	2.35	+1	-1	-1	+1	14.024	13.618	2.483	4.774
24	10	50	300	1.75	+1	-1	+1	-1	18.991	20.502	-1.660	-3.356
25	10	50	300	2.35	+1	-1	+1	+1	10.977	12.320	1.182	2.275
26	10	60	180	1.75	+1	+1	-1	-1	14.024	13.618	2.483	4.774
27	10	60	180	2.35	+1	+1	-1	+1	18.122	16.752	24.098	23.227
28	10	60	300	1.75	+1	+1	+1	-1	19.232	18.804	12.863	14.698
29	10	60	300	2.35	+1	+1	+1	+1	5.971	3.851	-2.947	-2.415
30	10.5	55	240	2.05	+2	0	0	0	16.042	16.280	3.383	4.382

A, pH; B, Temperature (°C); C, Hydrolysis time (min); D, Enzyme-to-substrate ratio (% w/v). Response variables for antimicrobial activity (%): Y_1, *P. ananatis* and Y_2, *P. stewartii*.

Table 4. Quadratic polynomial equations for responses Y_1 (*P. ananatis*) and Y_2 (*P. stewartii*) in relation to the coded variables.

Responses	Equations	R^2	R^2 (Adj.)	R^2 (Pred.)	Regression (p-value)	F-Value	Lack of fit
Y_1	42.08 + 2.65A + 0.40B + 3.19C + 1.77D + 2.24AB - 1.18AC - 1.84AD + 0.38BC - 2.78BD + 0.18CD + 9.31 A^2 - 5.59 B^2 - 8.44 C^2 - 7.84 D^2 -	0.9893	0.9792	0.9599	< 0.0001	98.68	0.7919
Y_2	38.99 + 0.707A - 2.19B - 3.27C + 3.26D + 0.68AB - 2.84AC + 3.69AD - 1.61BC - 1.07BD + 2.76CD - 10.03 A^2 - 9.49 B^2 - 5.54 C^2 - 4.86 D^2 -	0.9848	0.9706	0.9271	< 0.0001	69.49	0.2797

Y_1 and Y_2 represent the predicted responses comprising the antimicrobial activity against *P. ananatis* and *P. stewartii*, respectively, while A, B, C, and D are independent variables values, namely pH, temperature (ºC), hydrolysis time (min) and enzyme-to-substrate ratio (% w/v).

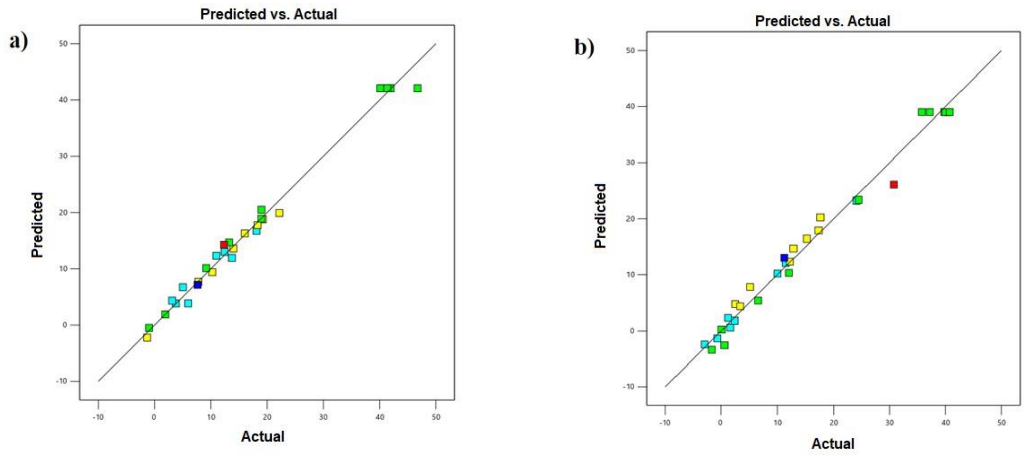

Figure 1. The predicted versus observed values for the antibacterial effect of (a) Y_1, *P. ananatis,* and (b) Y_2, *P. stewartii.*

Interpretation of Response Surface Plot and Influence of Independent Variables on Antimicrobial Activity

The interactive effects between pH (A) and temperature (B) were examined while keeping the hydrolysis duration constant at 2.05% enzyme-to-substrate ratio and 240 minutes. Figure 2(a) and Figure 3(a) depict the visual representation of these interaction effects. The graphs demonstrate that the highest antimicrobial effect was observed at pH of 9.5 and temperature of 55°C. In the pH range of 9 to 10, variations in the temperature did not seem to exert a noteworthy influence on the antimicrobial effect. The activity of Alcalase® enzyme, which is responsible for the antimicrobial effect, is believed to be influenced by the pH of the medium (Jamal et al., 2022a; Bordbar et al., 2018; Sila & Bougatef, 2016). Therefore, maintaining the pH within the optimal range is crucial for achieving maximum antimicrobial potency. Based on this investigation, the optimal pH was determined to be 9.5.

Figure 2(b) and Figure 3(b) show the effects of pH (A) and time spent on hydrolysis (C) at a set temperature of 55°C and enzyme-to-substrate ratio of 2.05%. The data reveal that the Alcalase® enzyme works best at pH of 9.5 and hydrolysis time of 240 minutes, making these the best conditions. This result matches the ideal pH range for Alcalase®, which is between 7.5 and 9.5, as noted by Zainol et al. (2021). Interestingly, the 240-minute hydrolysis time is longer than the previously suggested 60-minute period. The longer time allows the enzyme to break down the protein more effectively, improving its ability to kill bacteria, as mentioned by Haslaniza et al. (2010). However, once past the best pH level, the enzyme's effectiveness drops, likely because it becomes less stable at high pH, as indicated by Amin et al. (2020).

Figure 2(c) and Figure 3(c) show the combined effects of pH (A) and the enzyme-to-substrate ratio (E/S) (D) at a set temperature of 55°C and hydrolysis time of 240 minutes. The data suggests that pH of 9, changes the E/S ratio either to 1.75% or 2.35% and reduces the antimicrobial strength. Earlier studies such as one by Sila & Bougatef (2016) highlighted the role of specific E/S ratios in enhancing antimicrobial effects. Hence, an E/S ratio of 2.05% seems to be the most effective for maximizing antimicrobial activity.

Figure 2(d) and Figure 3(d) demonstrate the interaction effects of temperature and enzyme-to-substrate (E/S) ratio while maintaining a constant pH of 9.5 and hydrolysis time of 240 minutes. Increased E/S ratio leads to higher concentration of the Alcalase® enzyme, resulting in greater number of amino acid peptides being cleaved. However, when the number of substrates in an enzyme reaction is low, the reaction rate is often slower and proportionate to the substrate concentration. The enzyme's affinity for the substrate, as well as the presence of any competitive inhibitors, can further influence this rate (Auwal et al., 2017; Bordbar et al., 2018).

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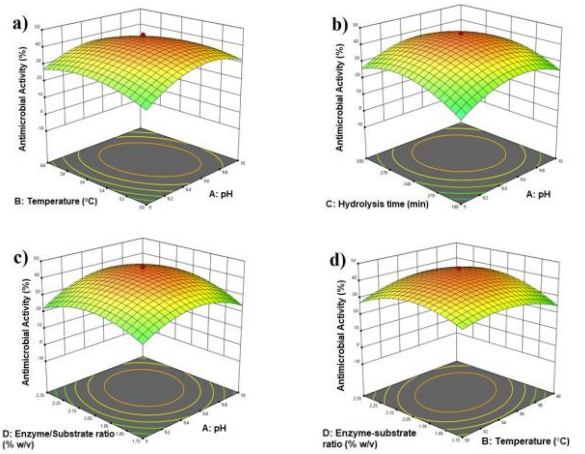

Figure 2. 3D contour plots show the variable parameters' effect on the antimicrobial activity Y_1, *P. ananatis* response. (a) pH vs temperature, (b) pH vs hydrolysis time, (c) pH vs E/S ratio, and (d) temperature vs E/S ratio.

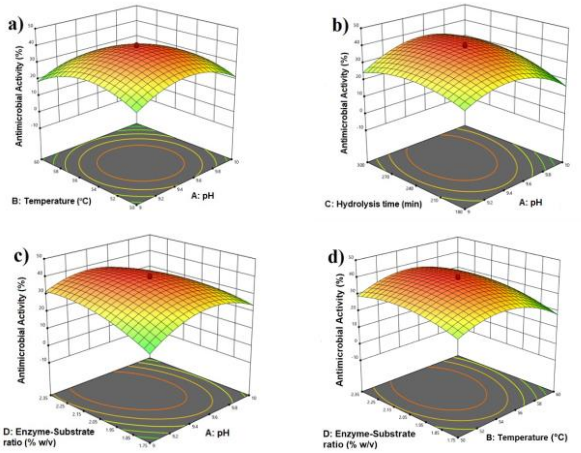

Figure 3. 3D contour plots show the variable parameters' effect on the antimicrobial activity Y_2, *P. stewartii* response. (a) pH vs temperature, (b) pH vs hydrolysis time, (c) pH vs E/S ratio, and (d) temperature vs E/S ratio.

Predicted Optimum Conditions Validation

The reliability of the obtained results was assessed by conducting validation using five independent validation sets, as shown in Table 5. This approach ensured the adequacy and reliability of the final models. The agreement between the actual and the predicted data was assessed by computing the residual standard error (RSE) percentages and contrasting them with the estimates from Equation (3). The models were deemed accurate when the RSE percentages fell below 5%, suggesting that the actual values were in close alignment with the predicted ones. As illustrated in Table 5, all RSE values came under the 5% threshold, underscoring the robustness and precision of the models.

Table 5. Actual and predicted responses.

Set	Variables				Response variables					
					Antimicrobial Activity, Y_1 (%)			Antimicrobial Activity, Y_2 (%)		
	A	B	C	D	Actual	Predicted	RSE	Actual	Predicted	RSE
1	9.1	51	190	1.8	15.952	15.364	3.827	13.419	12.884	4.156
2	9.2	52	200	1.9	27.302	26.159	4.369	27.491	28.164	-2.388
3	9.4	54	220	2	39.311	38.329	2.562	37.681	36.218	4.038
4	9.6	56	250	2.2	42.169	41.663	1.215	36.444	37.953	-3.977
5	9.8	58	270	2.3	34.222	35.184	-2.734	26.307	27.477	-4.257

Y_1 and Y_2 represent the predicted responses comprising the antimicrobial activity against *P. ananatis* and *P. stewartii*, respectively, while A, B, C, and D are independent variables values, namely pH, temperature (ºC), hydrolysis time (min) and enzyme-to-substrate ratio (% w/v).

Effective Inhibition Concentration of BTPH

The findings from this study indicated that BTPH exhibits significant antimicrobial properties. Table 6 shows that BTPH has notable antimicrobial activities, with low MICs against *P. ananatis* and *P. stewartii*. Specifically, the MIC values recorded were 250 µg/mL for *P. ananatis* and 500 µg/mL for *P. stewartii*. Additionally, the MBC for both bacterial strains was found to be 1000 µg/mL. This study's BTPH demonstrated stronger antimicrobial effects compared to those found in the past study on a peptide extracted from the mollusk *Babylonia spirata*, which showed antimicrobial activity against *Staphylococcus aureus* (1000 µg/mL) and *Aspergillus fumigatus* (1000 µg/mL) (Kuppusamy & Ulagesan, 2016). The bactericidal properties of BTPH, with MBC and MIC values of ≤4, aligned with the prior findings suggesting that the antimicrobial efficacy of antimicrobial peptides (AMPs) is concentration-dependent (Arulrajah et al., 2021; Bondaryk et al., 2017). It has been reported that AMPs can disrupt intracellular activities, including inhibiting transcription (Lan et al., 2010; Zhang et al., 2008), translation (Kragol et al., 2001), and the synthesis of large molecules (Hsu et al., 2004; Patrzykat et al., 2002).

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Malaysian Journal of Science 43(3): 1-12 (September 2024)

Table 6. The effects of *Bactronophorus thoracites* protein hydrolysates (BTPH) on antimicrobial activity, MIC, MBC, and bactericidal and bacteriostatic.

Sample	Microorganisms	Antimicrobial activity (%)	MIC (µg/mL)	MBC (µg/mL)	Bactericidal/ Bacteriostatic (MBC/MIC)
BTPH	*P. ananatis*	42.258±2.397	250	1000	Bactericidal
	P. stewartii	40.154±2.181	500	1000	Bactericidal
Control	*P. ananatis*	n.a	n.a	n.a	n.a
	P. stewartii	n.a	n.a	n.a	n.a

n.a = No activity; Control = *B. thoracites* crude extract; Antimicrobial activity were measured in percentage (%) and the values were expressed as mean ± standard error (SEM).

4. Conclusion

The present study effectively used RSM to optimize the enzymatic breakdown of *B. thoracites* protein. The ANOVA outcomes and the R^2 values (0.9893 for *P. ananatis* and 0.9848 for *P. stewartii*) underscored the reliability of our method. The quadratic models developed effectively highlighted the relationships between the factors adjusted and the resulting antimicrobial effects. The optimal conditions (pH 9.5, 55°C temperature, 240-minute hydrolysis time, and 2.05% enzyme/substrate ratio) matched the predictions, as the RSE was under 5%. This study also observed that the minimum concentrations needed to inhibit or kill the bacteria were 250 and 500 µg/mL. This result supports earlier research showing that BTPH's antimicrobial actions vary based on concentration. In conclusion, the produced hydrolysates showed potential as a natural defense against rice leaf blight, suggesting they could aid in disease control.

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