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Optimization of Lactic Acid Fermentation Conditions for the Production of Antibacterial Peptides Targeting *Pantoea* spp. for Rice Leaf Blight Control

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ABSTRACT

This study aimed to optimize the production of antibacterial peptides from *Bactronophorus thoracites* via lactic acid fermentation, specifically focusing on *Pantoea* species to manage rice leaf blight. The main goal was to investigate sustainable and environmentally friendly approaches to address this agricultural disease using bioactive compounds derived from marine sources. The fermentation process was refined using Response Surface Methodology (RSM), producing highly reliable results confirmed by the analysis of variance (ANOVA) and strong determination coefficients (R^2 = 0.9952 for *Pantoea ananatis* and R^2 = 0.9967 for *Pantoea stewartii*). The optimized parameters included a 4-day fermentation duration, a 3% (w/v) glucose concentration, and a 0.92% (w/v) solid-to-water ratio. These conditions closely matched predictive models and were further validated by a residual standard error (RSE) of less than 5%. The study identified the minimum inhibitory concentration (MIC) of the bioactive peptides, determining that 125 µg/ml was effective against the target bacteria. The hydrolysates produced in this study show promise as a natural method to control rice leaf blight and may have broader applications in agricultural disease management. This research highlights the potential of optimized lactic acid fermentation to produce effective antimicrobial agents, contributing to sustainable agriculture and offering new biotechnological strategies for plant disease control.

Keywords: Antimicrobial peptides, bioactive molecules, lactic acid fermentation, *Pantoea* spp., rice disease control

INTRODUCTION

Rice leaf blight, mainly attributed to different strains of *Pantoea* spp., represents a major agricultural challenge affecting rice production worldwide. First reported in Venezuela in 2002 (González *et al*., 2015), the disease has since been identified in several Asian countries, manifesting as water-soaked lesions on rice leaves and resulting in dramatic yield losses, sometimes up to 70% (Azizi & Zulperi, *et al*., 2019; Toh *et al*., 2019). The emergence of different Pantoea species, including *P. stewartii* and *P. ananatis*, in regions like Malaysia has heightened the need for effective and sustainable control measures (Azizi & Ismail, *et al*., 2019; Azizi & Zulperi, *et al*., 2019; Toh *et al*., 2019). Conventional strategies, particularly the use of agrochemicals, have faced criticism due to their environmental impacts and the increasing resistance of pathogens to these chemicals (Khan *et al*., 2012; Naqvi, 2019; Abdallah *et al*., 2020; Sopialena *et al*., 2021). This situation has redirected attention towards the investigation of new antimicrobial agents, particularly antimicrobial peptides (AMPs), which not only play a crucial role in innate immune defense against a wide range of pathogens but also show promising potential in managing plant diseases, such as defensins and cyclotides, which inhibit fungal pathogens like Fusarium and Magnaporthe species in crops like wheat and rice (De Zoysa, 2013; Tassanakajon *et al*., 2015; Yuan *et al*., 2022).

In this context, molluscs, particularly bivalves like *Bactronophorus thoracites*, are recognized for their rich protein content and essential amino acids, making them a viable source for AMP production (Lee *et al*., 2019; Jamal *et al*., 2022). Lactic acid fermentation has emerged as an innovative technique for

converting high molecular weight proteins into bioactive peptides with reduced molecular weights, offering several advantages over other hydrolysis methods. Compared to enzymatic hydrolysis, lactic acid fermentation is more cost-effective as it utilizes naturally occurring lactic acid bacteria (LAB) rather than expensive enzymes, and unlike chemical hydrolysis, it avoids harsh chemicals and extreme conditions, preserving the structural integrity and bioactivity of the peptides.

Additionally, lactic acid fermentation enhances bioactivity by producing synergistic bioactive compounds like organic acids and bacteriocins, selectively hydrolyzes proteins to release targeted bioactive peptides, and improves safety by reducing anti-nutritional factors and producing antimicrobial compounds that extend shelf life. Its natural, sustainable process is versatile and easily integrated into food systems, making it a superior method for producing functional peptides from diverse raw materials. This process is facilitated by LAB strains, which secrete proteolytic enzymes to break down protein substrates, liberating peptides and generating various bioactive compounds (Muhialdin *et al*., 2020; Jamal *et al*., 2022). The effectiveness of this hydrolysis depends on factors such as the LAB strain used, the type of protein involved, and the duration of fermentation (Muhialdin *et al*., 2020; Jamal *et al*., 2022).

Considering the varying specificities and abilities of different LAB strains, especially *Lactobacillus casei*, it is crucial to examine the factors affecting the lactic acid fermentation of *Bactronophorus thoracites*. Traditionally, the pH-stat method has been used to assess the impact of reaction parameters (Arulrajah *et al*., 2020). Additionally, RSM has proven effective in optimizing parameters that affect the hydrolysis of various protein types (Harun *et al*., 2017; Amin & Cheng, 2019; Arulrajah *et al*., 2020).

This study aims to bridge a crucial gap by exploring the optimal conditions for lactic acid fermentation in producing bioactive peptides from *B. thoracites*. By highlighting the sustainable and eco-friendly aspects of this method, the research seeks to leverage the antimicrobial properties of these peptides, especially against *Pantoea* spp., as a promising solution for managing rice leaf blight. This effort not only advances agricultural sustainability but also paves the way for new biotechnological research, investigating the potential of marine-derived bioactive compounds in plant disease management.

MATERIALS & METHODS

Sampling

In December 2020, *Bactronophorus thoracites* specimens were obtained from a mangrove forest near Kelanang Beach in Banting, Selangor, Malaysia, at the coordinates 2° 48'44.5" N and 101° 22'08.5" E. The shipworms were carefully packaged in plastic bags and kept refrigerated during transport to the Plant Molecular Biology Laboratory (PMB Lab) at the Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia (UPM), in Serdang, Selangor, Malaysia.

Preparation of Crude Extract

The collected shipworms were thoroughly cleaned and homogenized at 4 °C using a Waring laboratory blender. The resulting homogenate was mixed with 20 ml of chilled, deionized water, also maintained at 4 °C, and divided into small polyethylene bags (10 cm by 15 cm). The samples were initially frozen overnight at −80 °C in a Thermo Scientific freezer. Once frozen, they underwent freeze-drying in a Labconco FreeZone freeze dryer until a stable weight was achieved. After freeze-drying, the samples were ground into a fine powder using a Waring laboratory-grade blender and passed through a 200 μm mesh sieve. The finely milled powder was collected in screw-capped bottles and stored at −20 °C for future use.

Lactic Acid Fermentation

Optimisation of lactic acid fermentation conditions was carried out using RSM and Central Composite Design (CCD) in Design-Expert® software, following the protocol of Muhialdin *et al*. (2020) with slight modifications. The fermentation process took place in a 100 ml conical flask, which was kept in a shaking water bath at 37 °C with continuous agitation at 100 rpm. The fermentation began by adding 2% (v/v) starter culture, and the mixture was incubated for periods of 2, 4, 6, and 8 days

at 37 °C. Cell count analysis was conducted at each fermentation stage using the spread plate method. Approximately 0.1 ml of each fermented sample was plated on fresh MRS agar and incubated at 37 °C for 48 hours. The colonies were counted, and results were expressed as colony-forming units per milliliter (CFU/ml). To stop the fermentation process, the mixture was heated to 100 °C for 30 minutes with intermittent stirring, rapidly cooled in an ice bath, and centrifuged at 14,000 × g for 20 minutes. The resulting *Bactronophorus thoracites* Fermented Protein (BTFP) was collected, and inhibition percentages were recorded before freeze-drying. The BTFP was then stored at −20 °C for further analysis.

Experimental Design, Modeling, and Statistical Analysis

The experiments were designed, and data were analyzed using Design-Expert® software, version 13.0. A Central Composite Design (CCD) approach was applied to assess the effects of three variables across five levels, focusing on their influence on antimicrobial activity against *P. ananatis* and *P. stewartii*. The variables and their respective levels are detailed in Table 1, with the experimental design provided in Table 2. A total of 20 experiments were conducted, including three replicates at the central point. Randomization was employed to minimize potential bias from unforeseen factors. The data were modeled using a second-order polynomial equation or its simplified form, as illustrated in Eq.(1):

$$\boldsymbol{Y = z_0 + \sum z_1\ A + \sum z_{12}\ AB + \sum z_{123}\ AA} \quad \text{Eq.(1)}$$

In this equation, "Y" represents the response variable, specifically the antibacterial effect against *P. ananatis* and *P. stewartii*. The term "z□" denotes the model's intercept, while "z□" corresponds to the coefficient linked to variable A. The coefficients "z□□" and "z□□□" reflect the interaction effects between variables AB and AA, respectively. This equation accounts for the quadratic, linear, and interaction effects of the process variables on the antibacterial activity. The significance of each variable's coefficient was assessed using the Student's t-test. The relevance, accuracy, and impact of the process variables on the results were also evaluated through Analysis of Variance (ANOVA).

Antimicrobial Activity

The antimicrobial effectiveness of BTFP from each experimental trial was evaluated against *P. ananatis* and *P. stewartii*, with a non-hydrolyzed sample serving as a control. The procedure involved mixing 500 µL of Luria Broth (LB) containing 10^6 CFU/ml with 500 µL of the BTFP sample. The mixture was then incubated at 30°C for 24 hours. After incubation, the absorbance was measured at 600 nm using a spectrophotometer, following the method described by Mohamad Asri *et al*. (2020). This absorbance reading was essential for determining antimicrobial activity. The level of antimicrobial efficacy was calculated using a specific formula, as shown in Eq.(2), based on the absorbance values obtained. The sample showing the highest antimicrobial activity was then freeze-dried (Labconco FreeZone, USA) and further characterized.

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

Validation of optimum condition

Design-Expert® software was employed to predict the optimal conditions for maximizing antimicrobial activity. To validate the model's accuracy, BTFP was hydrolyzed under various randomly selected conditions. The resulting hydrolysates were freeze-dried and evaluated for antimicrobial activity. Predicted outcomes were then compared with actual experimental results using a t-test and residual standard error (RSE), as outlined in Eq. (3). Following the methodologies of Arulrajah *et al*. (2021) and Zakaria *et al*. (2021), predictions were deemed accurate if the RSE value remained below 5%.

$$RSE\ (\%) = \left|\frac{Actual\ value - Predicted\ value}{Predicted\ value}\right| \times 100\% \quad \text{Eq.(3)}$$

Effective Inhibition Concentration

The antibacterial effectiveness of BTFP was evaluated following modified methods from Jamal *et al*. (2022). Bacterial cultures were mixed with LB, and varying concentrations of BTFP were introduced to identify the minimum concentration necessary to inhibit bacterial growth. The cultures were incubated at 30 °C for 24 hours, after which inhibition of growth was assessed. The lowest concentration of BTFP that

successfully prevented visible bacterial growth was recorded as the MIC. To determine the Minimum Bactericidal Concentration (MBC), samples were plated on agar and incubated at 37 °C for 24 hours. The concentration at which no bacterial growth was observed was recorded as the MBC. The procedure was repeated three times to ensure accuracy and consistency.

Data Analysis

The data are reported as the mean of three replicates, with standard deviations calculated within a 95% confidence interval.

Table 1. The four independent variables and their coded levels of independent variables for the lactic acid fermentation optimization

Symbol	Independent variable	Coded level				
		-1.68	-1	0	+1	+1.68
A	Fermentation day	0.6	2	4	6	7.4
B	Glucose concentration (%)	1.3	2	3	4	4.7
C	S/W ratio (% w/v)	0.85	0.88	0.92	0.96	0.99

Table 2. Central Composite Design (CCD) design for optimizing lactic acid fermentation conditions under different levels of process variables

Run	Independent variables			Code level		
	A (day)	B (% v/v)	C (% w/v)	A	B	C
1	0.6	3	0.92	-1.68	0	0
2	2	2	0.88	-1	-1	-1
3	2	2	0.96	-1	-1	1
4	2	4	0.88	-1	1	-1
5	2	4	0.96	-1	1	1
6	4	1.32	0.92	0	-1.68	0
7	4	3	0.85	0	0	-1.68
8	4	3	0.92	0	0	0
9	4	3	0.92	0	0	0
10	4	3	0.92	0	0	0
11	4	3	0.92	0	0	0
12	4	3	0.92	0	0	0
13	4	3	0.92	0	0	0
14	4	3	0.99	0	0	1.68
15	4	4.68	0.92	0	1.68	0
16	6	2	0.88	1	-1	-1
17	6	2	0.96	1	-1	1
18	6	4	0.88	1	1	-1
19	6	4	0.96	1	1	1
20	7.4	3	0.92	1.68	0	0

Note: A = Fermentation Day; B = Glucose concentration (% v/v); C = Substrate/water ratio (% w/v)

RESULTS & DISCUSSION

Model Fitting and Variance Analysis (ANOVA)

A three-level factorial design was employed to evaluate the impact of fermentation time, glucose concentration, and solid-to-water (S/W) ratio on the antimicrobial activity of BTFP produced through lactic acid fermentation. Table 3 outlines the experimental design, along with both the actual and predicted values for each variable. Statistical analysis was performed using ANOVA to assess the significance of the variables. The results demonstrated that all independent variables significantly influenced their respective responses in the fermentation process. Specifically, the F-values for BTFP's antimicrobial inhibition against *Pantoea*

ananatis (Y1) and *Pantoea stewartii* (Y2) were 230.89 and 339.64, respectively.

The statistical data revealed that the probability of the observed effects occurring by random chance was just 0.01%, confirming the robustness of the findings. Furthermore, the p-values for each response were extremely low ($p< 0.05$), reinforcing the relevance of the models. The actual inhibition percentages ranged from 3.105% to 63.759% for Y1 and from 0.550% to 57.132% for Y2, reflecting the real data obtained from the experimental design matrix and the predicted responses calculated using CCD, as shown in Table 3.

Table 4 presents the quadratic polynomial models. Equations 4 and 5 describe the empirical relationships between the independent and dependent variables for each response. The coefficient of determination R^2 for Y1 (0.9952) and Y2 (0.9967) revealed a strong alignment between the experimental data and the quadratic polynomial models, as confirmed by ANOVA. Although R^2 tends to increase with the inclusion of additional variables, regardless of their statistical significance, it remains a valuable indicator of model fit. Moreover, the predicted R^2 (pred R^2) values of 0.9733 for Y1 and 0.9881 for Y2 indicated that the regression model had high predictive accuracy in estimating the response values, consistent with the findings of Che Sulaiman *et al*. (2017).

According to Zainol *et al*. (2021) and Zakaria *et al*. (2021), an R^2 value exceeding 0.9 denotes a strong fit and correlation between experimental results and the regression model. Therefore, the model developed in this study successfully captured nearly 99% of the variability in the response variables. Additionally, the adjusted R^2 (Adj. R^2) serves as a critical metric in evaluating the model's suitability, reflecting the explanatory power of the regression models when multiple variables are considered. The adjusted R^2 values for antimicrobial inhibition were 0.9909 (Y1) and 0.9938 (Y2), affirming the models' capability to predict the optimal conditions for maximizing antimicrobial inhibition by the protein hydrolysates.

Table 3. CCD, predicted, and response values of two dependent variables under different lactic acid fermentation conditions

Run	Independent variables			Code level			Response variable (%)			
							Y_1		Y_2	
	A	B	C	A	B	C	Act.	Pred.	Act.	Pred.
1	0.6	3	0.92	-1.68	0	0	3.105	5.189	3.117	4.252
2	2	2	0.88	-1	-1	-1	5.600	5.600	0.550	0.272
3	2	2	0.96	-1	-1	1	8.298	7.140	8.870	8.621
4	2	4	0.88	-1	1	-1	13.947	12.309	11.365	10.325
5	2	4	0.96	-1	1	1	19.370	19.240	16.788	16.748
6	4	1.32	0.92	0	-1.68	0	21.210	20.343	13.690	13.099
7	4	3	0.85	0	0	-1.68	14.811	14.514	13.668	14.017
8	4	3	0.92	0	0	0	59.675	61.509	53.359	55.377
9	4	3	0.92	0	0	0	63.759	61.509	52.759	55.377
10	4	3	0.92	0	0	0	60.861	61.509	55.924	55.377
11	4	3	0.92	0	0	0	60.276	61.509	56.107	55.377
12	4	3	0.92	0	0	0	63.683	61.509	57.132	55.377
13	4	3	0.92	0	0	0	60.624	61.509	56.893	55.377
14	4	3	0.99	0	0	1.68	23.240	22.528	23.526	22.650
15	4	4.68	0.92	0	1.68	0	23.417	23.274	17.104	17.169
16	6	2	0.88	1	-1	-1	18.252	19.095	13.645	14.057
17	6	2	0.96	1	-1	1	19.343	21.694	16.487	17.900
18	6	4	0.88	1	1	-1	8.608	10.481	10.149	10.770
19	6	4	0.96	1	1	1	17.757	18.471	12.037	12.688
20	7.4	3	0.92	1.68	0	0	18.983	15.890	14.091	12.430

Note: A = Fermentation Day (Day); B = Glucose concentration (% v/v); C = Substrate/water ratio (% w/v); Y_1 = *P. ananatis*; Y_2 = *P. stewartii*; Act. = Actual; Pred = Prediction

Table 4. The quadratic polynomial equations for the two responses based on the coded factors of lactic acid fermentation

Response	Equation	R^2	Adj. R^2	Pred. R^2	Regression (p-value)	Lack-of-fit
Y_1	61.51 + 3.18A + 0.87B + 2.38C + 3.83AB - 0.2649AC + 1.35BC + 18.02A^2 - 14.04B^2 - 15.2C^2	0.9952	0.9909	0.9733	< 0.0001	0.2554
Y_2	55.38 + 2.43A + 1.21B + 2.57C + 3.33AB- 1.13AC- 0.4814BC - 16.63A^2 - 14.23B^2 - 13.1C^2	0.9967	0.9938	0.9881	< 0.0001	0.7338

Note: A = Fermentation Day (Day); B = Glucose concentration (% v/v); C = Substrate/water ratio (% w/v); Y_1 = *P. ananatis*; Y_2 = *P. stewartii*; Adj. = Adjusted; Pred = Prediction

Response Surface Analysis and the Impact of Fermentation Variables

This research explored how three key fermentation factors—fermentation duration, glucose concentration, and the solid-to-water (S/W) ratio—influenced the antimicrobial properties of BTFP during lactic acid fermentation, using *Lactobacillus casei* as the starter culture. Three-dimensional response surface plots were generated to visually represent the interactions between these variables and their effect on antimicrobial activity against *Pantoea ananatis* (Y1) and *Pantoea stewartii* (Y2). These plots, shown in Figures 1 and 2, were instrumental in identifying the optimal conditions for maximizing antimicrobial effectiveness. The construction of these plots was based on the significant interaction effects revealed by ANOVA.

Figure 1(a) and 2(a) illustrate the combined effects of fermentation time and glucose concentration, with the S/W ratio held constant at 0.92%. The data showed that antimicrobial activity against Y1 and Y2 increased with higher glucose levels, up to 3%, and fermentation durations of up to 4 days. However, reducing either the glucose concentration or the fermentation time led to a decline in antimicrobial efficacy. This finding is consistent with the results of Sánchez-Clemente *et al*. (2020), who observed that adding a carbon source like glucose enhances the growth of lactic acid bacteria (LAB) and boosts their antimicrobial properties. Additionally, moderate glucose levels can accelerate fermentation, leading to increased carbon dioxide production.

Figure 1(b) and 2(b) highlight the effects of fermentation duration and the S/W ratio, while maintaining glucose concentration at 3%. Extending the fermentation period to 4 days and keeping the S/W ratio at 0.92% improved antimicrobial performance against Y1 and Y2. However, further increases in these variables resulted in decreased antimicrobial activity. This pattern aligns with the findings of Muhialdin *et al*. (2020), who reported that antimicrobial activity peaked after 48 hours of fermentation but declined after 60-72 hours. Similarly, Rodríguez *et al*. (2017) observed a reduction in *Limosilactobacillus fermentum* viability after 24 hours of fermentation, which may explain the drop in antimicrobial effectiveness during extended fermentation periods. Prolonged fermentation could decrease the viability of *L.casei*, impairing its ability to hydrolyze BTCP and thereby reduce antimicrobial activity. This reduction in antimicrobial activity over time can be attributed to the extended fermentation period often leads to the accumulation of organic acids, particularly lactic acid, which lowers the pH of the environment. A significantly reduced pH can negatively impact the survival of *L. casei* and its metabolic activity (Muhialdin *et al*.; 2020).

Figure 1(c) and 2(c) show the combined influence of glucose concentration and S/W ratio during a 4-day fermentation period. Optimal antimicrobial activity against Y1 and Y2 was achieved with a 0.92% S/W ratio and a glucose concentration of 3%. Arulrajah *et al*. (2021)

noted that substrates with higher moisture content are more readily fermented by certain *Lactobacillus* species, likely due to their need for ample water availability. The optimal S/W ratio identified in this study indicates that *L. casei* requires sufficient water to effectively break down BTCP into smaller peptides during fermentation. Overall, the antimicrobial activity of BTFP against Y1 and Y2 increased to a peak, then declined, in response to varying fermentation durations, glucose concentrations, and S/W ratios.

Validation of Predicted Optimal Conditions

To assess the accuracy and reliability of the results, five independent validation tests were performed, as outlined in Table 5. This step was crucial for verifying the effectiveness and robustness of the final models established in this study. The comparison between the actual experimental results and the predicted outcomes was conducted by calculating the residual standard error (RSE) percentages, which were then evaluated against the estimates provided by Eq. (3). The models were deemed precise if the RSE percentages were below 5%, reflecting a close alignment between observed and predicted values. Table 5 illustrates that all RSE percentages were within this 5% threshold, confirming the precision and dependability of the models used.

Effective Inhibition Concentration of BTFP

The findings from this study reveal that BTFP demonstrates considerable antimicrobial efficacy. As shown in Table 6, BTFP achieved notable antimicrobial activity, with MICs of 125 µg/ml against both *P. ananatis* and *P. stewartii*. This performance is particularly significant when compared to other antimicrobial peptides (AMPs) tested against these pathogens, which typically exhibit MIC values ranging between 200–500 µg/ml (Arulrajah *et al*., 2020; Muhialdin *et al*., 2021). The lower MIC of BTFP highlights its superior potency and efficiency in inhibiting these bacterial strains, positioning it as a highly effective alternative for combating these plant pathogens.

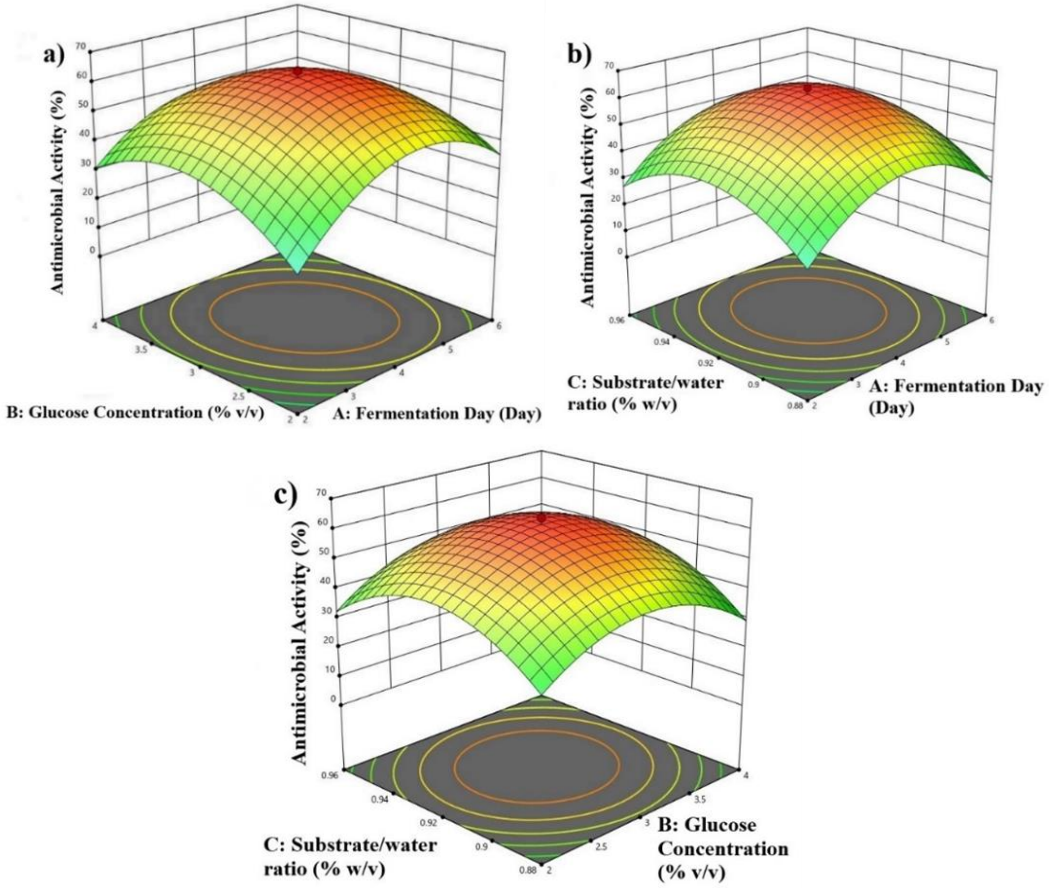

Figure 1. The 3D contour plots showing the influence of variable parameters on the antimicrobial activity of BTFP against Y_1 (*P. ananatis*). (a) Day vs glucose concentration, (b) Day vs S/W ratio, and (c) Glucose concentration vs S/W ratio

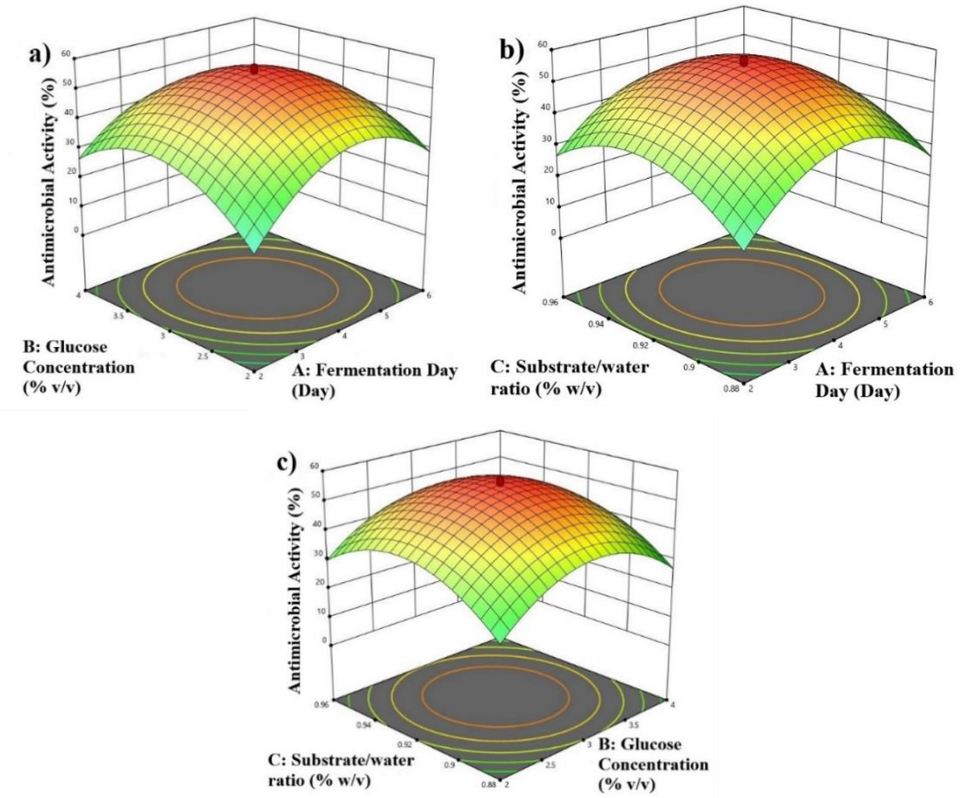

Figure 2. The 3D contour plots showing the influence of variable parameters on the antimicrobial activity of BTFP against Y_2 (*P. stewartii*). (a) Day vs glucose concentration, (b) Day vs S/W ratio, and (c) Glucose concentration vs S/W ratio

Table 5. Actual and predicted responses for the model verification of lactic acid fermentation

	Variables			Y_1			Y_2		
				Inhibition (%)		RSE (%)	Inhibition (%)		RSE (%)
	A	B	C	Actual	Predicted		Actual	Predicted	
1	2.5	2.1	0.91	31.85	33.054	-3.643	26.77	27.554	2.845
2	3.5	2.5	0.93	52.42	54.624	-4.035	51.331	49.105	4.533
3	4.5	3.5	0.94	56.289	55.387	1.629	48.217	49.325	2.246
4	5.0	3.9	0.95	41.522	40.532	2.443	33.646	34.301	1.910
5	5.5	3.8	0.95	36.221	37.369	-3.072	30.028	31.343	4.196

Note: A = Fermentation Day; B = Glucose concentration (% v/v); C = Substrate/water ratio (% w/v). Response variables for antimicrobial activity (%): Y_1 = *P. ananatis*; Y_2 = *P. stewartii*. Data are expressed as the mean ± S.D. of triplicate samples

Furthermore, the MBCs for these bacterial strains were found to be 500 µg/ml. These levels of antimicrobial efficacy exceed those reported in previous research; for instance, a study on a peptide derived from the mollusc *Babylonia spirata* found MICs of 1000 µg/ml against *Staphylococcus aureus* and *Aspergillus fumigatus* (Kuppusamy & Ulagesan, 2016). The potent bactericidal properties of BTFP, characterized by MBC and MIC values of ≤ 500 µg/ml, align with earlier studies highlighting the concentration-dependent nature of AMP effects (Buda De Cesare *et al*., 2020; Sanchez Armengol *et al*., 2021). Research indicates that AMPs can interfere with various intracellular processes, such as inhibiting transcription, disrupting translation, , and hindering macromolecule synthesis, thereby contributing to their antimicrobial efficacy (Erdem Büyükkiraz & Kesmen, 2022; Naiel *et al*., 2023; Seyfi *et al*., 2020).

Table 6. The effects of BTFP on antimicrobial activity, MIC, MBC, and bactericidal and bacteriostatic

Sample	Microorganisms	Antimicrobial activity (%)	MIC (µg/ml)	MBC (µg/ml)	Bactericidal/ Bacteriostatic (MBC/MIC)
BTFP	*P. ananatis*	70.136 ± 0.273%	125	500	Bactericidal
	P. stewartii	70.024 ± 0.244%	125	500	Bactericidal
Control	*P. ananatis*	n.a	n.a	n.a	n.a
	P. stewartii	n.a	n.a	n.a	n.a

Note: MIC = Minimum inhibitory concentration; MBC = Minimum Bactericidal concentration; n.a = No activity; Control = *B. thoracites* crude extract; Bactericidal = MBC/MIC ≤ 4; Bacteriostatic = MBC/MIC > 4. Antimicrobial activity was measured in percentage (%) and the values were expressed as mean ± standard error (SEM).

CONCLUSION

This study effectively employed Response Surface Methodology (RSM) to refine the lactic acid fermentation process for proteins from *B. thoracites*. The ANOVA results, which showed high coefficients of determination (R^2) of 0.9952 for *P. ananatis* and 0.9967 for *P. stewartii*, affirm the validity and reliability of our methodology. The quadratic models successfully mapped the relationships between the adjusted variables and their effects on antimicrobial activity. The identified optimal fermentation parameters—4 days, a glucose concentration of 3% (w/v), and a solid-to-water (S/W) ratio of 0.92% (w/v)—aligned with our predictions and were confirmed by a residual standard error (RSE) of less than 5%. Moreover, the minimum concentrations necessary to achieve inhibitory or bactericidal effects were 125 µg/ml, consistent with previous findings that BTFP's antimicrobial efficacy is dependent on concentration. Overall, the hydrolysates generated in this study exhibit significant potential as natural agents against rice leaf blight, suggesting their practical use in managing this agricultural issue. This underscores the effectiveness of our optimised fermentation process in producing potent antimicrobial agents.

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