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THE INFLUENCE OF ENZYMATIC HYDROLYSIS ON ANTIMICROBIAL ACTIVITY AGAINST RICE PATHOGENS FROM *Bactronophorus thoracites* (SHIPWORM) PROTEIN HYDROLYSATE

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Abstract

The outbreak of leaf blight disease in Malaysia affects the sustainability of rice production in supporting the growing population. *Bactronophorus thoracites* is a wood-boring shipworm with high protein and possesses numerous bioactive compounds. The present study investigated the effects of enzymatic hydrolysis for generating molluscs protein hydrolysates (MPH) from *B. thoracites* and their efficacy against rice pathogens. The inhibition percentage against rice pathogens was significantly ($p < 0.05$) increased when enzyme concentration was increased from 1.75% to 2.05% (w/v) but slightly decreased at a concentration exceeding 2.35%. The inhibition percentage was also found to increase when the temperature increased to 55°C. However, the hydrolysis at 65°C produced a lower inhibition percentage. Prolonging the incubation time from 120 to 240 minutes significantly ($p < 0.05$) increased the inhibition percentage. The inhibition percentage of hydrolysates was significantly ($p < 0.05$) increased when pH increased from 9.0 to 9.5. However, increasing pH to 10.0 resulted in a reduction in inhibition percentage. Subsequently, the MPH with optimum condition (2.05% w/v, 55°C, 240 min and pH 9.5) was lyophilised and analysed for antimicrobial activities. The MIC and MBC values for MPH were 500 µg/mL and 1000 µg/mL against *P. ananatis* and 250 µg/mL and 1000 µg/mL against *P. stewartii*. The zones of inhibition value for MPH were 20.76 mm (1000 µg/mL) and 11.53 mm (500 µg/mL) for *P. ananatis* and 21.1 mm (1000 µg/mL), 14.03 mm (500 µg/mL) and 8.16 mm (250 µg/mL) for *P. stewartii*. At MIC, 2 MIC, and 4 MIC, the MPH exhibited a longer lag phase. Nonetheless, at a lower MIC concentration, a progressive rise in bacterial growth for *P. ananatis* and *P. stewartii* at 12 hours was observed. This study shows that enzymatic hydrolysis using alcalase can produce potential antimicrobial agent applicable to the agricultural sector.

INTRODUCTION

Leaf blight disease has been recognized as one of the most alarming diseases in the agricultural sector, especially in rice crops. Characteristics of plants infected by leaf blight disease were loss of moisture and brownish lesions along with the leaves. The worst scenario can be is the wilting phase during the early development stage which involved systemic contamination sourced from neighbouring plants [1]. Other than that, leaf blight disease has been reported to be the main cause of approximately 30 to 50% of rice production loss in Sabak Bernam rice field during December 2016 [2]. Recently, there was great attention to *Pantoea* species, a gram-negative bacterium, as this bacterium was suspected to be one of the major sources of leaf blight disease in rice crops, leading to economic losses [3]. Particularly in Malaysia, there was a previous study by Mohammad et al., (2019a, 2019b) [4,5] shows that *Pantoea stewartii* and *Pantoea ananatis* were the plant pathogen that caused leaf blight disease outbreaks in Kedah and Selangor rice granaries. These emerging diseases by rice pathogens tremendously affect the sustainability of rice production especially in meeting the increasing demand for rice production in the growing population. Thus, the search for alternatives to overcome the loss of rice yield by rice pathogen should come to our attention.

Nowadays, marine mollusc has gained interest in the field of antimicrobial discoveries because studies have shown that marine mollusc consisting bioactive peptides that can serve as an antimicrobial agent [6]. This can be supported by looking at the mechanism of molluscs survival in their harsh environment, where they need to defend themselves from microbial and parasitic attacks by producing secondary metabolites and secretion of mucus [7,8].

Bactronophorus thoracites is bivalve mollusc living in the roots and trunks of dead mangrove trees [9,10]. In Malaysia, *B. thoracites* can be found abundantly on the west coast of Peninsular Malaysia, which has an extensive mangrove coastline (approximately 98334 hectares) [11]. This would harbour a large number and species of wood borers having a wide distribution. This large wood storage facility acts as a natural reservoir for larvae, juveniles, and adult marine wood borers.

There are currently just a few studies on mollusc hydrolysates since most researchers prefer to employ proteins derived from fish resources, which may be acquired mainly from fish waste products [12]. Even though mollusc enzymes have been developed to improve seafood industry processing and make additional food items such as fish and shellfish protein hydrolysates and seafood flavourings, mollusc protein hydrolysate is not commercialized [13]. Few studies have been reported on the hydrolyzation of mollusc

protein using alcalase to optimize the protein hydrolysis of Asian hard clam [14], angel wing clam [15,16], razor clam [17], mud clam [18], and freshwater mussel [19].

Bioactive peptides from mollusc protein can be obtained by enzymatic hydrolysis, to serve their functions as antihypertensive agents, antioxidants, anticancer, and antimicrobial properties [20,21]. Antimicrobial properties exhibited by molluscs could kill pathogens as their defense mechanisms against viruses, fungus, marine diatoms, and human hepatocarcinoma cell lines [22,23,24,25]. To gain the optimum inhibition percentage, parameters such as pH, temperature, hydrolysis time, an enzyme to substrate ratio are usually analyzed. Inhibition percentage measures the extent of hydrolyzed protein in a hydrolysate sample and this information was used to relate how hydrolysis can give impact towards functional properties of the product formed [26,27]. Therefore, this study aims to determine the effect of the enzymatic hydrolysis conditions of MPH; enzyme-substrate ratio (E/S, w/w), pH, hydrolysis temperature, and hydrolysis time on antimicrobial activity of protein hydrolysate from *Bactronophorus thoracites* against *P. ananatis* and *P. stewartii*.

MATERIALS AND METHODS

Sample Collection

The sampling was carried out from December 2020 to January 2021. The *B. thoracites* (Figure 1) were collected from mangrove forests near Kelanang Beach in Banting, Selangor (2° 48'44.5", N 101° 22' 08.6 "E) (Figure 2). The samples were placed in a well-sealed plastic bag at approximately 4 °C in an icebox and transferred immediately to the -20°C freezer at Plant Molecular Biology Lab, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia.

Bacterial Culture

Bacterial pathogens used for antimicrobial screening in shipworm hydrolysate were *Pantoea stewartii* and *Pantoea ananatis*. Both bacteria were obtained from Plant Molecular Biology Lab, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, and were cultured in LB broth for 24h at 30°C using an incubator (LabTech, Italy).

Sample Preparation

The molluscs were thoroughly washed to remove the mud and then deshelled to collect their flesh and homogenized at 4 °C with a laboratory-scale blender. 20 ml of precooled, deionized water at 4 °C was used to disperse the homogenate, then stored at -20 °C until subsequent use.



Figure 1. *B.thoracites* from the mangrove forest near Kelanang Beach, Banting, Selangor, Malaysia (2° 48'44.5", N 101° 22' 08.6 "E)



Figure 2. Location of the sampling area: Kelanang Beach, Banting, Selangor, Malaysia (2° 48'44.5", N 101° 22' 08.6 "E)

Freeze-Drying Method

The samples were stored in a freezer (Thermo Scientific) at -80 °C and left overnight. Next, a freeze dryer (Labconco FreeZone, USA) was used to lyophilize the samples, achieving a constant weight. A laboratory-scale blender was used for crushing all the dried samples to a fine powder and sieved using a 200 mm-sized sieve. The prepared samples in fine powder form were stored in screw-capped bottles at -20 °C before subsequent use.

Preparation of Protein Hydrolysate

The protein hydrolysate was prepared according to the method of Amin et al. (2020) with some modifications [35]. Multiple studies (E/S, pH, temperature and hydrolysis time) were carried out to determine the enzymatic hydrolysis conditions of *B.thoracites* using alcalase to obtain the optimal enzymatic hydrolysis parameters according to (Table 1). In a 100 mL conical flask, 6 g of freeze-dried *B.thoracites* was dissolved in 100 mL of phosphate buffers

(0.1 M) and heated at 95°C for 15 minutes. 50 mL of protein solutions were combined with 50 mL of enzyme solutions of different concentrations to obtain the final enzyme concentration, and the combination was incubated in a shaking water bath (Lab Companion, Model BS-21) with continual agitation at 100 rpm. The mixture was heated to 95°C for 15 minutes to deactivate the enzyme and end the procedure. To separate the enzyme and impurities from the

produced hydrolysate, the samples were centrifuged at 2600 g for 15 minutes at 4°C in an Eppendorf 5804 R Refrigerated High-Speed Centrifuge. The supernatant (protein hydrolysate) was collected, and the degree of hydrolysis was determined before freeze drying. Molluscs Protein Hydrolysate (MPH) was kept at -20°C for future research.

Table 1. Parameters and their levels were used to obtain the optimum hydrolysis conditions of *B.thoracites* using the alcalase enzyme

Factors	Units	Symbols	Levels			
			1	2	3	4
Enzyme/Substrate ratio	%	E/S	1.45	1.75	2.05	2.35
pH	pH	pH	8.5	9	9.5	10
Temperature	° C	T	50	55	60	65
Hydrolysis time	min	t	120	180	240	300

Microbial Inhibition Assay

Protein hydrolysates obtained were tested for antimicrobial activity against *P.ananatis* and *P. stewartii* using spectrometer and an unhydrolyzed sample as a control. Briefly, 500 µL of Luria Broth (LB) containing 10⁶ cfu/mL and 500 µL of sample were mix. The mixture was incubated at 30 °C for 24 hours and the absorbance was measured at 600 nm using a spectrometer [28]. The antimicrobial activity was calculated as in the following equation:

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

Finally, the highest antimicrobial activity obtained from each sample was freeze dried (Labconco FreeZone, USA) and characterized.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of MPH were obtained according to the technique described by Mohamad Asri et al. (2020) and Muhialdin et al. (2020), with slight changes [28,30]. Bacterial inoculum from the overnight culture (10⁶cfu/mL) was diluted by inoculating 500 µL of the suspension into LB. The MPH was produced in sterilised distilled water at 1000, 500, 250, 125, 62.5, and 31.25 g/mL to determine the lowest concentration necessary to inhibit bacterial growth. Post incubation of 24 hours at 30°C, the absorbance at 600 nm was determined with a spectrometer. The antimicrobial activity was determined based on Eq. (2).

The MBC was determined by inoculating 100µL aliquots of bacterial suspension from a centrifuge tube containing

MPH onto LB agar before incubating the plates at 37°C for 24 hours. The sample concentration in the agar plates that did not exhibit bacterial growth following incubation was employed to determine the MBC. Meanwhile, the MIC was defined as the lowest MPH concentration that impeded the observable multiplication of the identified pathogens. The MIC and MBC determinations were performed in triplicates.

Agar Well Diffusion Method

The antimicrobial activity of the mollusc protein hydrolysate was determined by the agar well diffusion method [29]. LB agar was poured into the Petri plates and the inoculums were spread onto the agar with a sterile swab moistened with the bacterial suspension. Wells were made using a 5-mm sterile cork borer before the addition of 20 µL protein hydrolysate with different concentrations: 1000, 500 and 250 µg/mL. Distilled water was used as the negative control and chloramphenicol (1000 µg/mL) as the positive control. The plates were incubated at room temperature for 24 h. The inhibitory activity of the compounds was determined by comparing the average sizes of inhibition zones (mm), including well diameter of the different extracts with those of the controls. All analyses were performed in triplicate and the results were reported as the mean ± standard deviation (SD). Significant differences were analysed by one-way ANOVA. Differences at p<0.05 were considered significant.

Turbidimetric Growth Inhibition Assay

The present study conducted a turbidimetric inhibition assay [31]. A 96- well microplate was filled with 225µL MPH (0.5, 1, 2, and 4 MIC) and 25µL LB incorporated with 10⁶cfu/mL bacterial culture and incubated briefly at 30°C. Subsequently, at two-hourly, the optical density at 600nm was measured for 32 hours. The bacterial growth, expressed as absorbance, was plotted against time to assess the

inhibitory activity against *Pantoea stewartii* and *Pantoea ananatis*.

Statistical Analysis

In the present study, the data obtained were expressed as means of triplicates \pm SD and analysed with IBM SPSS, Version 27.0.

RESULTS AND DISCUSSION

Effect of Enzyme/Substrate Ratio

The effect of the E/S ratio on microbial inhibition percentage was examined, as shown in Figure 3(A). As found, inhibition percentage was only negative at an enzyme concentration below 2.05%, mainly attributed to an inadequate number of catalytic sites necessary to accelerate the hydrolysis process. The inhibition percentage steadily increased to 20.43% for *P. ananatis* and 9.45% for *P. stewartii* as the enzyme-substrate ratio increased from 1.75% to 2.05% (w/w). At 2.05% of E/S, the greatest inhibition percentage was obtained. However, when the enzyme-substrate ratio was increased over than 2.05% (w/w), the inhibition percentage was decreased. This decrease can be appropriately attributed to the increase in amino acids and the smaller peptides present in the hydrolysate. Some of the released peptides were severely hydrolyzed as the alcalase concentration increased [32]. It was discovered that increasing the enzyme concentration above its optimal value had no significant effect on inhibition percentage. This result is most likely due to enzyme aggregation, which increases substrate diffusion inhibition, resulting in reaction rate saturation. As a result, a concentration of 2.05% (w/w) of the enzyme was chosen for future research studies.

Effect of pH

The pH effect on inhibition percentage was investigated at 8.5, 9.0, 9.5 and 10. As shown in Figure 3(B), as the pH increased from 9.0 to 9.5, the inhibition percentage increased from -20.01% to 7.35% for *P. ananatis* and -16.53% to 17.47% for *P. stewartii*. However, increasing pH to 10 resulted in an apparent reduction in inhibition percentage. This decrease is most likely due to the disruption of the substrates' ionic nature, impairing the substrate's capacity to bind the enzyme [33,34]. Amin et al. (2020) and Shahidi et al. (1995) were reported similar findings, indicating that the optimal hydrolysis pH was between 8.5 and 9.5 when utilizing the alcalase enzyme [35,36].

Effect of Temperature

The reaction temperature considerably impacts the inhibition percentage of MPH. To elucidate the influence of reaction temperature on the inhibition percentage, the reaction

temperature was varied to 50, 55, 60, and 65 °C, as shown in Figure 3(C). When the reaction temperature increased from 50 to 55 °C, the inhibition percentage increased from -64.33% to 1.59% for *P. ananatis* and -48.11% to 13.75% for *P. stewartii*. However, raising the reaction temperature to 60 °C resulted in a slight decrease in inhibition percentage. This drop-in inhibition percentage was most likely caused by heat denaturation of the enzyme, which resulted in a reduction of inhibition percentage [37]. As a result, 55 °C was chosen as the optimal reaction temperature in the current investigation. The observed effects were consistent with Amin et al. (2019), who discovered that the greatest hydrolysis of angel wing clam (*P. orientalis*) could be produced at 55–65°C with a greater yield when alcalase enzyme was used [15].

Effect of Hydrolysis Time

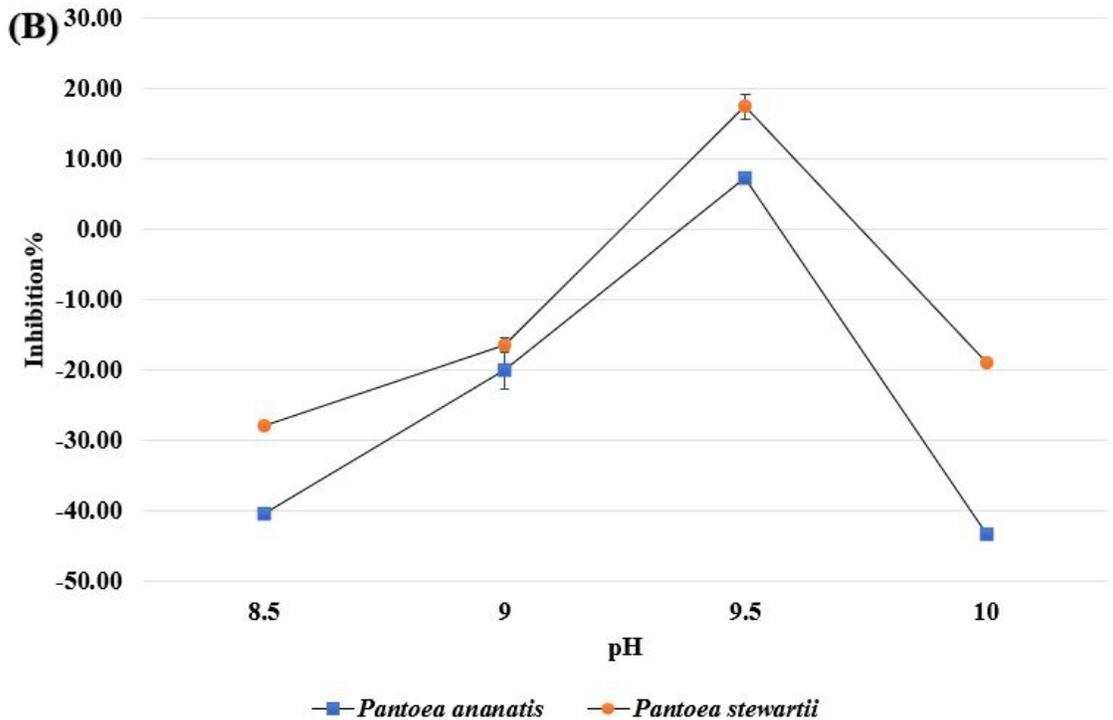
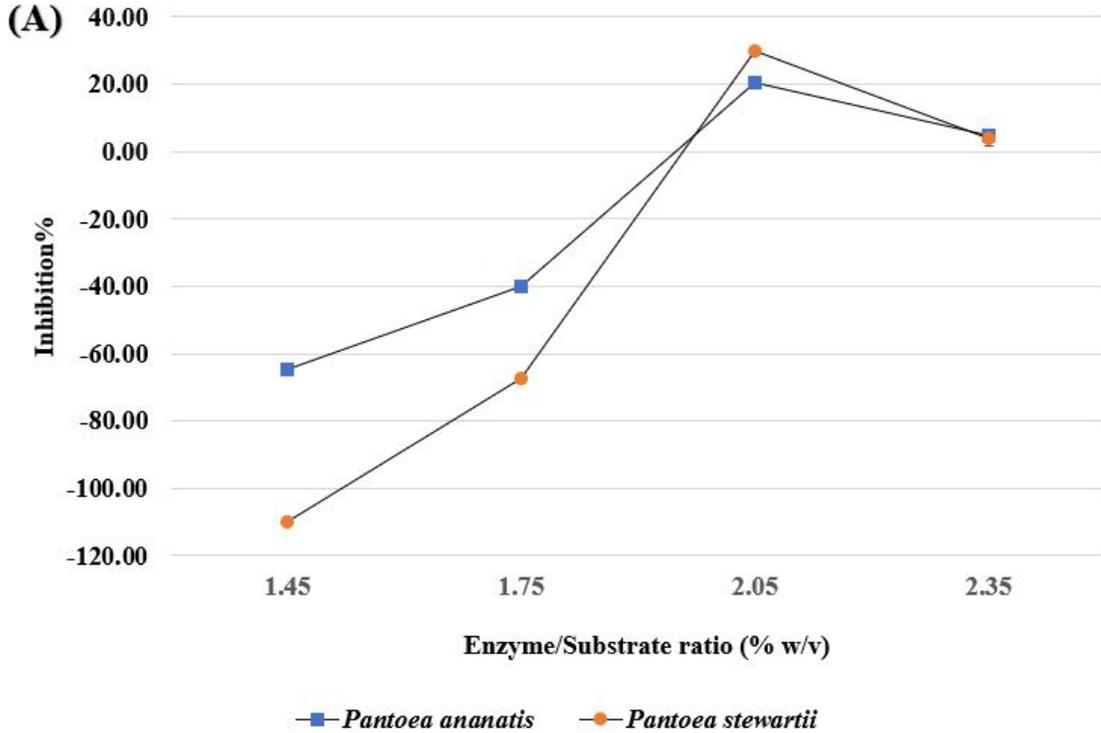
Hydrolysis time on the inhibition percentage was determined between 120 and 300 minutes. As seen in Figure 3(D), the inhibition percentage level rapidly decreased as the incubation time increased. According to Haslaniza et al. (2010), increasing the incubation period enables the enzyme to operate more extensively on the protein [33]. Additionally, Montecalvo et al. (1984) showed that the rising of hydrolysis was caused by enhanced peptide bond breaking, enhancing the peptide's solubility in TCA [38]. When the reaction time was prolonged from 180 to 240 minutes, the inhibition percentage increased significantly, from -17.49% to 11.40% for *P. ananatis* and -28.61% to 19.51% for *P. stewartii*. 240 min was determined as the optimum reaction time based on the collected data. The acquired findings were consistent with those reported by Amin et al. (2019), who generated protein hydrolysates from angel wing clam (*P.orientalis*) using alcalase enzyme and an incubation duration of 60 to 180 min [15]. Numerous authors have reported similar results, which the hydrolysis rose as the incubation duration increased [39,40].

Effective Inhibition Concentration of MBH

The MIC and MBC values were essential for determining the most effective concentrations to suppress bacterial growth. Lower MIC and MBC values implied significant antimicrobial activity and necessitated lowered doses to inhibit specific microorganisms. The data obtained in the current study suggested that MPH had potential antimicrobial activity. The MPH recorded high antimicrobial activities as denoted by the low MICs against *P. ananatis* and *P. stewartii* (Table 2). MPH recorded MIC at 500µg/mL for *P. ananatis* and 250µg/mL for *P. stewartii*, while MBC at 1000 µg/mL for both bacteria, respectively. The MPH in the present study exhibited more vigorous antimicrobial activities compared to a previous investigation on peptide acquired from mollusc *Babylonia spirata* against *Staphylococcus aureus* (1000 µg/mL) and *Aspergillus fumigatus* (1000 µg/mL) [29]. The bactericidal effects

exhibited by MPH with MBC and MIC ≤ 4 (Table 2) agreed with previous reports where the antimicrobial effects of antimicrobial peptides (AMPs) were concentration-dependent [31, 41]. The AMPs were able to target intracellular contents to inhibit transcription [42,43],

translation [44], and macromolecular synthesis [45,46]. Furthermore, the AMPs hinder precursors and or essential intermedia in peptidoglycan, LPS, or other biosynthetic pathways to interfere with cell wall functional synthesis and subsequent bacterial replication [47].



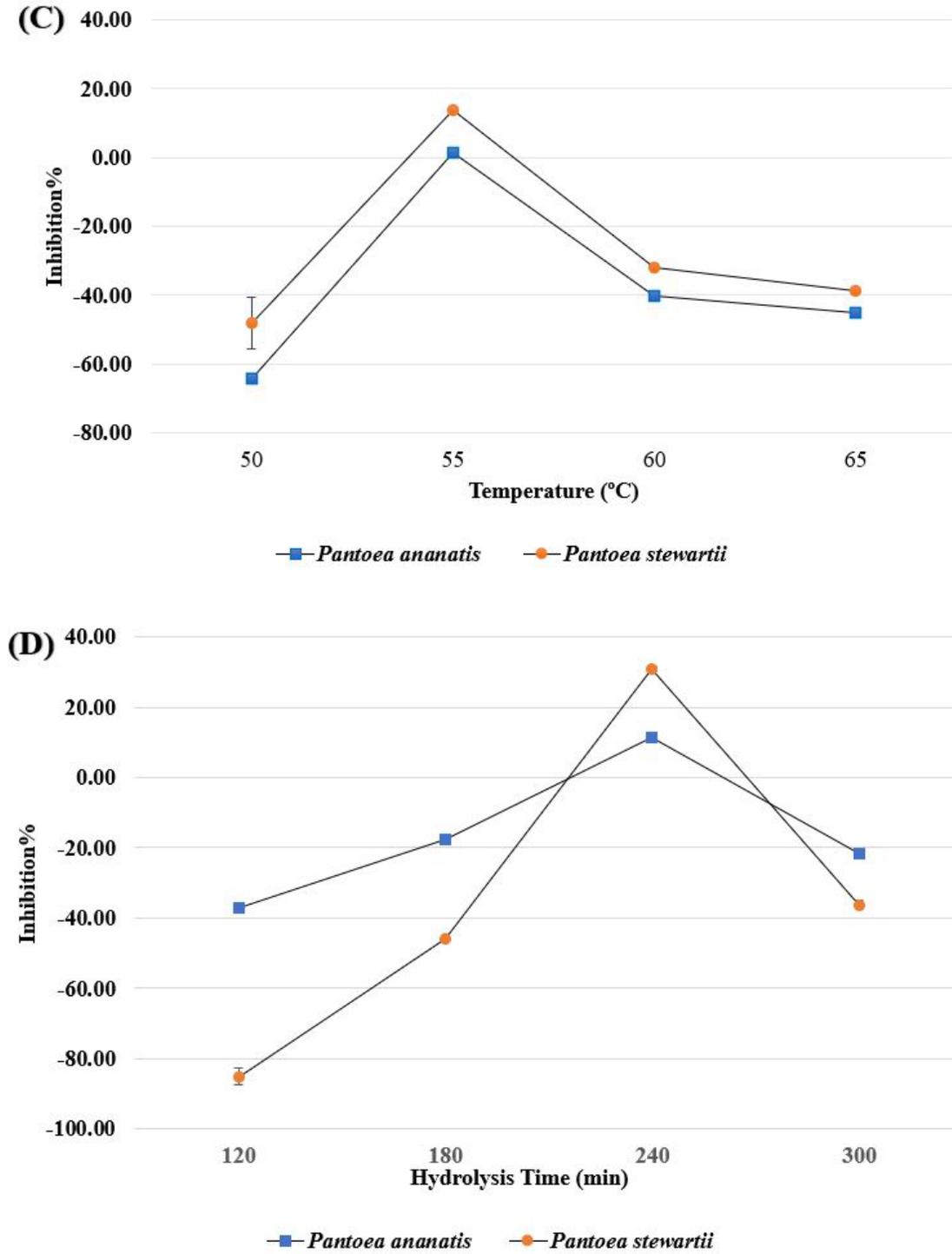


Figure 3. Effects of different conditions on the inhibition percentage: (A) E/S; (B) pH, (C) temperature; and (D) hydrolysis time. Means in the same form with various characters have significant differences ($p < 0.05$). Data are expressed as mean \pm S.D. of triplicate determinations

Table 2. The effects of mollusc protein hydrolysates (MPH) on antimicrobial activity, MIC, MBC, and bactericidal and bacteriostatic

Sample	Microorganisms	Antimicrobial activity (%)	MIC (µg/mL)	MBC (µg/mL)	Bactericidal/ Bacteriostatic (MBC/MIC)
MPH	<i>P. ananatis</i>	13.239±2.079	500	1000	Bactericidal
	<i>P. stewartii</i>	13.169±2.353	250	1000	Bactericidal
Control	<i>P. ananatis</i>	n.a	n.a	n.a	n.a
	<i>P. stewartii</i>	n.a	n.a	n.a	n.a

MPH = molluscs protein hydrolysates; 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).

Agar Well Diffusion

The agar well diffusion approach was employed to determine the antimicrobial activity MPH in the present study. The MPH inhibition zones against rice pathogens are listed in Table 3.

The findings revealed a notably heightened impact on bacterial growth inhibition ($P < 0.05$), with increasing MPH (Figure 4) concentrations. The highest inhibition zones recorded were 20.76 ± 0.25 mm for *P. ananatis* and 21.1 ± 1.15 mm for *P. stewartii* at 1000 µg/ml of MPH. Nevertheless, no inhibition zones were observed when *P. ananatis* were treated with 250 µg/ml. A previous study evaluated the protein hydrolysate of mollusc *Babylonia spirata* for antimicrobial activity. At 1000 g/ml, the most significant inhibition zone against *Staphylococcus aureus* was $22.16 + 1.04$ mm, while at 1000 g/ml, the highest zone of inhibition was observed in *Aspergillus fumigatus* $13.5 + 0.5$ [29]. In another study, the antibacterial activities of horse mussels were effective at 200 g/mL against all bacterial strains tested, with a substantially stronger antibacterial activity against *Escherichia coli* (9 mm) and *Bacillus subtilis* (8 mm) [48]. Moreover, the largest zone of inhibition against *Staphylococcus aureus*, 23 mm, was observed at 100 g/ml of crude extract and 20 mm at 100 g/ml of protein hydrolysate from the *Clithon oualaniense* molluscs [49]. The findings in

the present study indicated that the antibacterial activities of the MPH were within an acceptable range and equivalent to bioactive peptides derived from other molluscs extracts.

The Turbidimetric Growth Inhibition Assay

Figure 5 demonstrates that MPH inhibited the growth of *P. ananatis* and *P. stewartii*. Generally, a prolonged lag phase is an essential element in characterising the efficacy of antimicrobial agents [50]. At 1, 2, and 4 MPH MIC, the growth rate of *P. ananatis* and *P. stewartii* exhibited a prolonged lag phase, but at a lower concentration of 1/2 MIC, a progressive rise in bacterial growth at 12 hours for *P. ananatis* and *P. stewartii*, respectively. The MPH demonstrated an antimicrobial effect at higher MIC and concentrations, although a bacteriostatic impact was observed at concentrations below the MIC. Compared to chloramphenicol (1000 µg/ml), MPH FBTP recorded a significant antibacterial action from the initial dose within 2–4 hours. According to [31], the antimicrobial activity of peptides might be attributed to the rupture of the fungal membrane or targeting the intracellular contents of fungal cells, or a combination of the two effects. Consequently, the bacteria could not grow since all their energy was consumed on overcoming the disruptive impact, causing them to remain in the lag phase or die [51].

Table 3. The inhibition zones of mollusc protein hydrolysates (MPH) against *P. ananatis* and *P. stewartii*

Conc. (µg/ml)	Microorganisms	
	<i>Pantoea ananatis</i>	<i>Pantoea stewartii</i>
1000	20.76 ± 0.25^a	21.1 ± 1.15^a
500	11.53 ± 0.95^b	14.033 ± 0.45^b
250	n.a	8.16 ± 0.28^c
Positive Control	28.267 ± 0.68^d	30.333 ± 0.76^d
Negative Control	n.a	n.a

n.a = No activity, positive control = chloramphenicol 1000 µg/ml, negative control = sterilised distilled water, and n = three in each group. Inhibition zones were measured in mm and the values were expressed as mean ± standard error (SEM). Means in the same row within groups not followed by the same superscript are significantly different; one-way ANOVA and Tukey's test were conducted ($P < 0.05$).

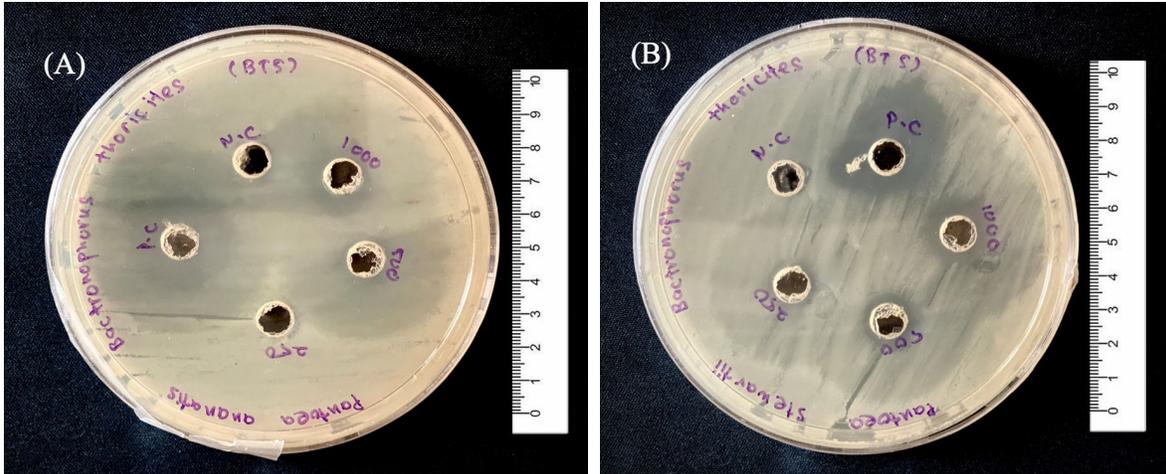


Figure 4. Agar well diffusion assay of molluscs protein hydrolysates (MPH). (a) MPH in *P. ananatis* and (b) MPH in *P. stewartii*. P.C = Positive control, N.C = Negative control, 1000 = 1000 µg/mL, 500 = 500 µg/mL, 250 = 250 µg/mL

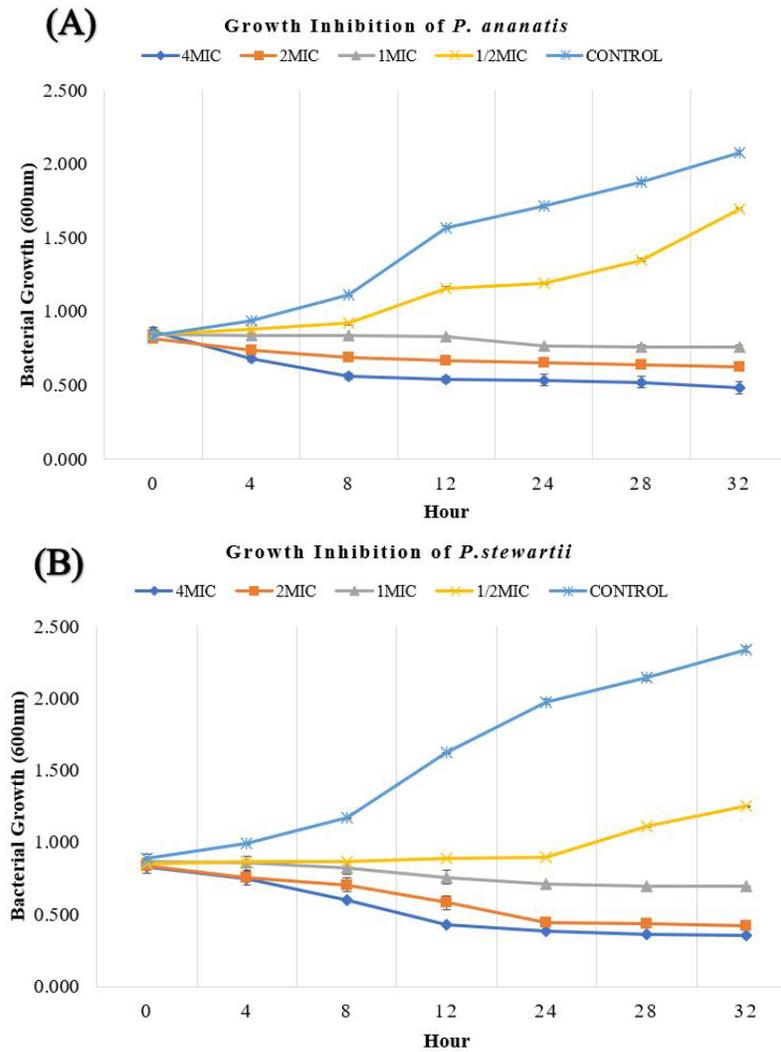


Figure 5. The growth inhibition curves of (A) *P. ananatis* and (B) *P. stewartii* at different MIC of mollusc protein hydrolysates (MPH). Control = LB broth inoculated with bacteria without MPH

CONCLUSION

To summarise, this study revealed the potential of antimicrobial protein hydrolysates from *B. thoracites*. According to the results, optimal conditions (E/S, 2.05%; pH, 9.5; temperature, 55 °C; hydrolysis time, 240 min) significantly affected the antimicrobial activity of MPH. The results indicated that enzymatic hydrolysis using alcalase resulted in bioactive peptides with antimicrobial activity against rice pathogens. However, utilising the generated protein hydrolysates in agricultural industries would require more research to optimise the hydrolysis conditions to achieve the optimum level of antimicrobial activity. Additionally, further study is necessary, especially in emphasising the fractionation and potential applications of protein hydrolysates.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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