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# The effect of lactic acid fermentation of *Bactronophorus thoracites* on antimicrobial activity against rice pathogens

**Siti Norazura Jamal[1,2], Belal J. Muhialdin[3], Noor Baity Saidi[1], Lai Kok Song[4], Mohd Termizi Yusof[5] and Dhilia Udie Lamasudin[1]***

[1]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
[2]Faculty of Applied Sciences, Universiti Teknologi MARA, Kampus Kuala Pilah, Pekan Parit Tinggi, 72000 Kuala Pilah, Negeri Sembilan, Malaysia.
[3]Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Ave, Saint Paul, MN 55108, USA.
[4]Health Sciences Division, Abu Dhabi Women's College, Higher Colleges of Technology, 41012, Abu Dhabi, UAE.
[5]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
Email: dhilia@upm.edu.my

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## ABSTRACT

**Aims:** Leaf blight disease caused by *Pantoea* spp. reduces rice yields in numerous nations. However, the exact strategy to combat *Pantoea* spp. has yet to be determined. *Bactronophorus thoracites* is a promising source of natural antimicrobial agents due to their potential as a substrate to generate peptides with high antimicrobial activity. This study determined the effects of lactic acid fermentation using *Lactobacillus casei* ATCC334 as a starter culture on antimicrobial activity against rice pathogens, proximate composition, and amino acid profiles from *B. thoracites* crude extract.
**Methodology and results:** *Bactronophorus thoracites* was washed and deshelled to collect the flesh and homogenised at 4 °C before freeze-drying. The freeze-dried samples were fermented with *L. casei* for 4 to 8 days at 37 °C. The antimicrobial activity, MIC and MBC were determined using a spectrometer. The fermented protein was subjected to proximate and amino acid analyses. The antimicrobial activity of fermented *B. thoracites* protein (FBTP) was significantly ($p$<0.05) decreased with the increased fermentation days (from 4 to 8 days). The antimicrobial activity was also increased when the glucose concentration increased from 2% to 3%. However, raising the glucose concentration to 4% decreased the antimicrobial activity. The antimicrobial activity was significantly ($p$<0.05) increased when the substrate-water (S/W) ratio increased from 0.84% to 0.96%. The FBTP (4 days, 3% glucose concentration and 0.96% S/W ratio) showed high antimicrobial activity against *Pantoea ananatis* and *P. stewartii*. The MIC and MBC values for FBTP were 500 µg/mL and 250 µg/mL against *P. ananatis* and *P. stewartii*. The zones of inhibition value for FBTP were 16.0 ± 0.5 mm (1000 µg/mL) and 9.33 ± 0.57 mm (500 µg/mL) for *P. ananatis*, and 11.7 ± 0.61 mm (1000 µg/mL), 9.33 ± 0.58 mm (500 µg/mL) and 7.17 ± 0.77 mm (250 µg/mL) for *P. stewartii*. The proximate composition and amino acid profiles of the freeze-dried protein hydrolysate powder were characterised. FBTP produced a higher value of protein (61.56%) and ash (32.38%) and a lower value of total fat (0.273%) and carbohydrates (6.27%) than the *B. thoracites* crude extract. Total amino acid content was 39.480 g/100 g in *B. thoracites* crude extract and 155.442 g/100 g in FBTP. The essential amino acid glutamine was the most abundant in *B. thoracites* crude extract and methionine in FBTP.
**Conclusion, significance and impact of study:** This study showed that lactic acid fermentation could produce FBTP using *L. casei* with improved functional characteristics and as a source of a natural antimicrobial agent against rice pathogens.

*Keywords: Bactronophorus thoracites*, lactic acid fermentation, proximate analysis, rice pathogens, antimicrobial

## INTRODUCTION

Leaf blight is a significant problem in the rice agroecosystems of many nations, resulting in substantial economic losses in rice productivity (Chukwu *et al*., 2019). The impact of leaf blight on rice farming systems is likely to increase, with output losses of up to 70% in some regions (IRRI, 2019). Bacteria belonging to the genus

***Corresponding author**


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*Pantoea* have been documented to cause leaf blight disease in various rice-growing regions worldwide (Lee *et al*., 2010; Mondal *et al*., 2011; González *et al*., 2015; Azizi *et al*., 2019a, 2019b; Toh *et al*., 2019).

The marine environment is a treasure for the discovery of bioactive natural compounds. The number of natural products extracted from marine species has expanded and has now surpassed a thousand, with hundreds of new compounds identified each year (Proksch *et al*., 2002; Benkendorff *et al*., 2015; Gogineni and Hamann, 2018). However, bioactive peptides in the natural compounds of molluscs have not been explored in nearly half of the natural compounds discovered so far, and less than 1% of the molluscan species found were analysed for the presence of bioactive peptides (Benkendorff *et al*., 2015; Khan and Ahmad, 2019).

Shipworms are molluscs of the family Teredinidae comprising three subfamilies. They are obligatory wood borers, with certain species boring mudstones, sediments, rock and rhizomes (Turner, 1966; Morley *et al*., 2007; Shipway *et al*., 2016). However, the study of the distribution and the abundance of shipworm is still lacking worldwide (Filho *et al*., 2008; Appelqvist *et al*., 2015; Velásquez and Shipway, 2018). In Malaysia, several studies on shipworms have been conducted, primarily focusing on the distribution and ecological aspects of shipworms (Sing and Sasekumar, 1994; Roszaini and Salmiah, 2015; Lee *et al*., 2019). In general, less critical studies on the antimicrobial proteins of molluscs have been conducted with whole-body homogenates of several marine molluscs and identified as having antimicrobial substances.

The lactic acid bacteria (LAB) fermentation method is a revolutionary biotransformation technology for converting high molecular weight proteins to low molecular weight peptides (Aguirre *et al*., 2008). LABs are used as the main culture in food and drink fermentation. They are referred to as the “friendly bacteria” and several strains, including those belonging to the genus *Lactobacillus*, have been designated as Generally Recognised as Safe (GRAS). These properties have piqued the interest of researchers in using LAB strains to generate bioactive peptides. Bioactive peptides are well-known among natural compounds for their broad spectrum of antibacterial action. Thus, such a broad spectrum of antimicrobial activity may impart resistance to plant species against fungal and bacterial diseases (Sathoff *et al*., 2019).

Nonetheless, although most studies demonstrated the production of bioactive peptides from plant-based source materials employing LAB strains (Arulrajah *et al*., 2020; Asri *et al*., 2020; Muhialdin *et al*., 2020, 2021), no studies have reported on mollusc raw materials. No study has been published describing the production of bioactive peptides from *B. thoracites* by lactic acid fermentation. Thus, this work aimed to measure the effect of lactic acid fermentation of *B. thoracites* using the *L. casei* strain and assess the antimicrobial activity against rice pathogens, proximate analysis and amino acid contents of the resultant product.

## MATERIALS AND METHODS

### Materials and chemicals

*Bactronophorus thoracites* (Figure 1) was collected from mangrove forests near Kelanang Beach in Banting, Selangor (2°48’44.5’’N, 101°22’08.6’’E). Meanwhile, the rice pathogens, *P. ananatis* and *P. stewartii* were obtained from the Plant Molecular Biology Lab, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia (UPM) and used for antimicrobial activity testing. *Lactobacillus casei* ATCC334 was obtained from the Biotechnology Lab, Faculty of Food Science and Technology, Universiti Putra Malaysia (UPM).


**Figure 1:** *Bactronophorus thoracites* from the mangrove forest near Kelanang Beach, Banting, Selangor, Malaysia.

### Preparation of crude extract

The *B. thoracites* was washed thoroughly to remove mud, deshelled to collect the flesh and homogenised at 4 °C with a laboratory-scale blender. The homogenate was dispensed in 20 mL precooled, deionised water at 4 °C and stored at -20 °C until further use.

### Freeze-drying method

The samples were subjected to overnight freezing at -80 °C (Thermo Scientific), followed by lyophilising in a freeze dryer (Labconco FreeZone, USA) until a constant weight was attained. All dry samples were subsequently ground into a fine powder with a laboratory-scale blender and sieved using a 200 mm sieve. The fine powder was collected in screw-capped bottles and stored at -20 °C until further use.

### Starter culture preparation

*Lactobacillus casei* ATCC334 was selected based on its antibacterial activity (Arulrajah *et al*., 2020). *Lactobacillus casei* was grown in 10 mL of De Man, Rogosa and the cells were washed twice with 0.1% saline water before being suspended in 10 mL saline, centrifuged (Refrigerated High-Speed Centrifuge Eppendorf, Model 5804 R) at 5000 rpm for 10 min and washed twice with peptone water (0.1%; Oxoid, Hampshire, England) and

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**Table 1:** The parameters and their levels to obtain optimum lactic acid fermentation.

| Factors | Units | Symbols | Levels | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| Day | day | day | 2 | 4 | 6 | 8 |
| Glucose concentration | % | % | 2 | 3 | 4 | 5 |
| Substrate-water ratio | % w/v | % w/v | 0.84 | 0.88 | 0.92 | 0.96 |

**Table 2:** Selected range values of each factor based on the highest inhibition percentage values.

| Fermentation day | Glucose concentration (%) | Substrate-water ratio (% w/v) (mg/mL) |
|---|---|---|
| 2-4 | 2-3 | 0.92-0.96 |

maintained at -80 °C in 10 mL peptone water supplemented with 12.5% glycerol (v/v). The final cell concentration was $10^7$ cfu/mL.

**Lactic acid fermentation of *B. thoracites* protein**

The lactic acid fermentation process was chosen according to Muhialdin *et al*. (2020) with modifications. The fermentation was performed in a 100 mL conical flask and incubated in a shaking water bath at 37 °C (Lab companion, Model BS-21) with constant agitation at 100 rpm. A screening experiment was performed to determine the appropriate ranges of independent variables such as fermentation day, glucose concentration v/v (%) and substrate-water (S/W) ratio (% w/v) to be used in the designed experiment for optimal lactic acid fermentation parameters (Table 1).

The effect of each parameter was evaluated at varying values, while other parameters were fixed at certain values. For instance, to determine optimal fermentation duration, the following parameters were fixed; S/W ratio at 0.96% (w/v), glucose concentration 3% (v/v) for different fermentation days from days 2, 4, 6 and 8, and incubation at a fixed temperature of 37 °C. Meanwhile, the S/W ratio was evaluated at 0.84%, 0.88%, 0.92% and 0.96% (w/v), while other parameters were fixed at 3% (v/v) glucose concentration and incubation at 37 °C for 4 days. Finally, glucose concentration (% v/v) was evaluated at the values of 2.0%, 3.0%, 4.0% and 5.0%, while other parameters were fixed at 0.96% (w/v) for S/W ratio, 3% (v/v) glucose concentration and incubation at 37 °C for 4 days.

According to the S/W ratio, the freeze-dried *B. thoracites* crude extract was weighed and mixed with glucose solution. The mixture was autoclaved at 121 °C for 15 min to kill all the microorganisms before fermentation.

The fermentation was conducted by inoculating 2% (v/v) of the starter culture and incubating at 37 °C for 2, 4, 6 and 8 days. The cell count for the 0-day sample and 8-day fermented sample was performed using the spread plates technique on De Man, Rogosa, and Sharpe (MRS) agar. About 0.1 mL of fermented samples were inoculated on the MRS agar plates, and the plates were incubated at 37 °C for 48 h. The colonies were counted and the results were expressed as cfu/mL.

The fermentation reaction was terminated by heating at 100 °C for 30 min occasional agitation. The mixture was cooled immediately in chilled water before being centrifuged using the refrigerated centrifuge (Eppendorf Model 5804 R) at 14000 rpm for 20 min. The supernatant was collected, and the inhibition percentage was determined (Equation 1). The selected range values of each factor are based on the highest inhibition percentage values obtained (Table 2).

Inhibition % = [(24 h negative control - 0 h negative control) - (24 h sample - 0 h sample)]/0 h negative control (1)

**Effective inhibition concentration**

The minimum inhibition concentration (MIC) and the minimum bactericidal concentration (MBC) of fermented *B. thoracites* protein (FBTP) were determined according to previous studies with some modifications (Asri *et al*., 2020; Muhialdin *et al*., 2020). Bacterial inoculum from the overnight culture ($10^6$ cfu/mL) was diluted by inoculating 500 µL of the suspension into Luria-Bertani (LB) broth. FBTP at 1000, 500, 250, 125 and 50 µg/mL was prepared in sterilised distilled water and used to determine the lowest concentration of sample required to inhibit bacterial growth. The mixture was incubated at 30 °C for 24 h and the absorbance was measured at 600 nm using a spectrometer (Amersham Bioscience Corp, model: Ultrospec 3100 Pro).

MBC was determined by inoculating a 100 µL aliquot of bacterial suspension from a centrifuge tube containing FBTP onto LB agar plates and incubation at 37 °C for 24 h. MBC was determined based on the lowest concentration of the sample at which bacteria did not grow on the agar plate during the fermentation period. In contrast, MIC was determined as the lowest concentration of FBTP that prevented the visible growth of the selected pathogens. MIC and MBC determination was performed in triplicate.

**Agar well diffusion method**

The antimicrobial activity of the mollusc protein hydrolysate was determined by the agar well diffusion method (Kuppusamy and Ulagesan, 2016). LB agar was

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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.

**Proximate chemical composition**

The chemical compositions of *B. thoracites* and FBTP were evaluated using the Association of Official Analytical Chemists (AOAC) technique (AOAC, 2005). The Kjeldahl method (N6.25) was used to determine the crude protein content. The Soxhlet technique was used to determine the total fat content using petroleum ether. The entire ash content was calculated by incinerating the sample at 550 °C until it was converted to ash. The carbohydrate content was calculated by subtracting the total protein, fat and ash from 100%. All experiments were replicated three times.

**Amino acid composition**

Amino acid compositions were determined using the AOAC method (AOAC, 2005). About 300 mg of the solid sample were digested with 8 mL of 6 M hydrochloric acid at 110 °C for 22 h in a nitrogen environment. Once the solution had cooled, 4.8 mL of 10 M NaOH was added, and the volume was increased to 25 mL with distilled water. Then, it was filtered through two layers of filter paper No. 40 and centrifuged at 10,000× *g* for 10 min. Reverse-phase high-performance liquid chromatography was used to examine amino acids in the samples (Agilent 1100 HPLC; Agilent Ltd., Palo Alto, CA, USA). Approximately 1 mL of sample was injected into a Zorbax 80 A C-18 column (column size: 4.0250 mm, particle size: 5 mm; Agilent, USA) at 40 °C with detection at 338 nm for each of the three samples. Sodium acetate, triethylamine and tetrahydrofuran (500:0.12:2.5, v/v/v) were used in mobile phase A, which was adjusted to pH 7.2 using acetic acid. Meanwhile, mobile phase B (pH 7.2) was made of sodium acetate, methanol and acetonitrile (1:2:2, v/v/v). The amino acid composition was given as g of amino acids per 100 g of protein, representing one amino acid.

**Data analysis**

All data analyses were performed using the Social Science Statistics Package (IBM SPSS, Version 27). Data were measured in triplicates, and normality was tested using the level of skewness and kurtosis. Since the data were normally distributed, they were presented as mean ± SD. A one-way analysis of variance (ANOVA) and paired samples t-test were performed to compare the means. Further analyses were performed using the Tukey post hoc test when significant differences ($p<0.05$) were detected between the different processing stages (IBM SPSS statistic 27).

## RESULTS AND DISCUSSION

**Effect of fermentation day**

The effect of the fermentation days on the inhibition percentage for *P. ananatis* and *P. stewartii* were examined, as shown in Figure 2(a). The most significant inhibition percentage for *P. ananatis* (20.05 ± 1.06%) and *P. stewartii* (13.82 ± 0.36%) were obtained on day 4 of the fermentation period. However, the inhibition percentage steadily decreased as the fermentation increased from 4 to 8 days. This might be due to the culture reaching the stationary phase, and as a result of metabolism, microorganisms constantly alter the properties of the medium and surroundings (Panesar *et al*., 2010). Additionally, the decrease in fermentation time benefits the cost of the process. Hence, the 2 to 4 days fermentation period was chosen for future research studies.

**Effect of glucose concentration**

The glucose concentration considerably impacted the inhibition percentage of *B. thoracites* samples. To elucidate the influence of glucose concentration on the inhibition percentage, the glucose concentration was varied to 2%, 3%, 4% and 5%, as shown in Figure 2(b). When the glucose concentration increased from 2% to 3%, the inhibition percentage of both bacteria increased, with *P. ananatis* from 32.99 ± 0.15% to 35.01 ± 0.21% and *P. stewartii* from 29.18 ± 0.22% to 35.98 ± 0.23%. However, raising the glucose concentration to 4% resulted in an extreme decrease in inhibition percentage. This is because high glucose content in the fermentation media caused the surrounding environment to become hypertonic. Consequently, osmosis occurs, in which water molecules migrate from within the LAB cells to the surrounding solution. Cell shrinkage (plasmolysis) occurs due to the reduced water availability in the cells, limiting the number of viable cells in the fermentation media and affecting the hydrolysis rate of *B. thoracites* (Arulrajah *et al*., 2021).

**Effect of substrate-water (S/W) ratio**

The effect of the S/W ratio on the inhibition percentage was determined between 0.84% and 0.96%. As seen in Figure 2(c), the inhibition percentage of *P. ananatis* rapidly increased from 10.36 ± 1.35% to 15.21 ± 0.99% as the S/W ratio increased from 0.88% to 0.96%.

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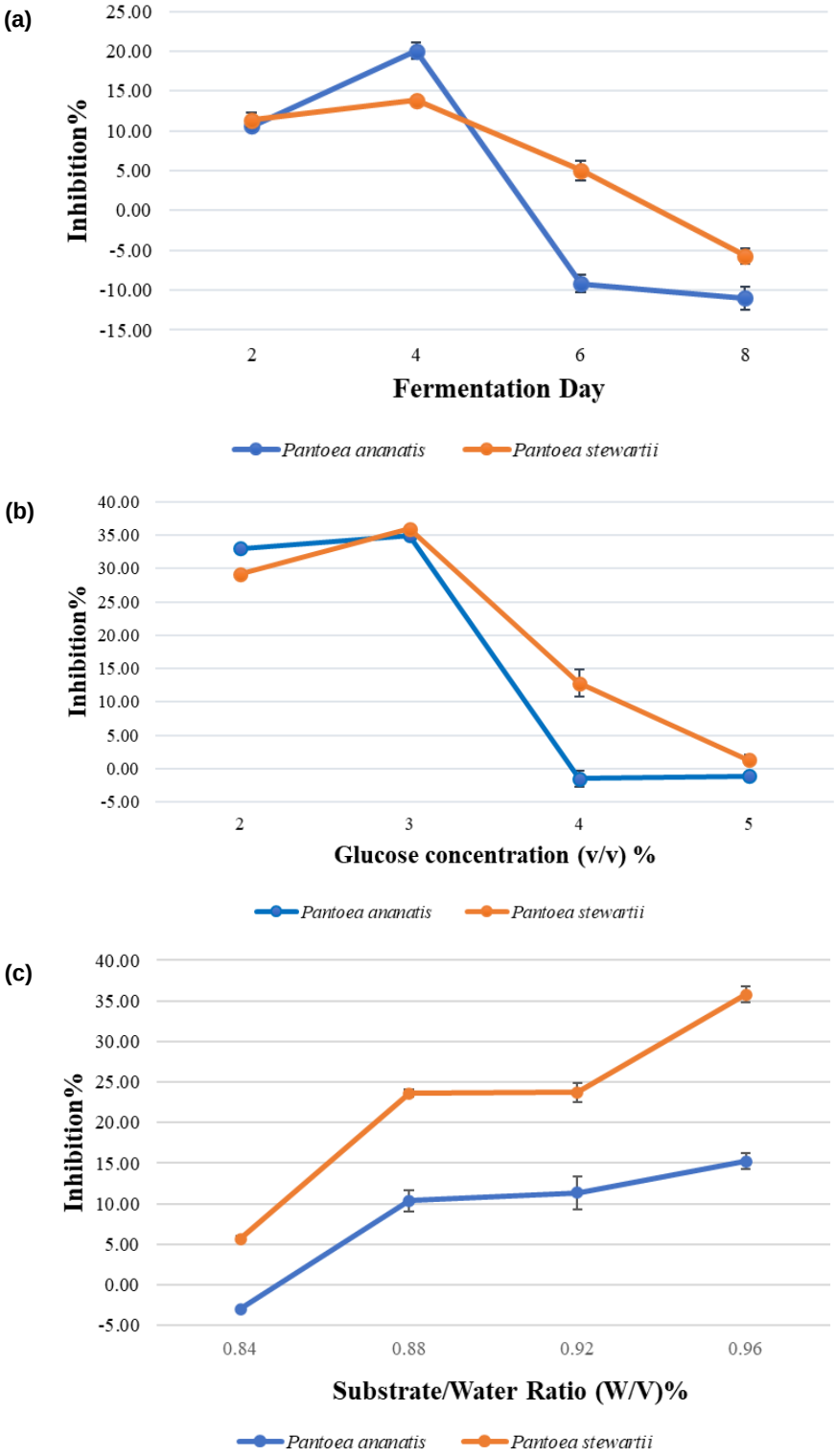


**Figure 2:** Effects of different conditions on the lactic acid fermentation of *B. thoracites* using *L. casei* as a starter culture. Inhibition percentage versus (a) fermentation day, (b) glucose concentration and (c) S/W ratio. Means in the same form with various characters have significant differences ($p$<0.05). Data were expressed as mean ± SD of triplicate determinations.

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**Table 3:** Antimicrobial activity, MIC and MBC against *P. ananatis* and *P. stewartii*.

| Sample | Microorganisms | Antimicrobial activity (%) | MIC (µg/mL) | MBC (µg/mL) |
|---|---|---|---|---|
| FBTP | *Pantoea ananatis* | 30.011 ± 0.21 | 250 | 250 |
| | *Pantoea stewartii* | 31.981 ± 0.23 | 500 | 500 |
| Control | *Pantoea ananatis* | n.a | n.a | n.a |
| | *Pantoea stewartii* | n.a | n.a | n.a |

FBTP: Fermented *B. thoracites* protein; Control: *B. thoracites* crude extract; n.a: No activity.

**Table 4:** Antimicrobial activity subjected to different treatments against *P. ananatis* and *P. stewartii*.

| Sample | Microorganisms | Zone of inhibition in mm | | | | |
|---|---|---|---|---|---|---|
| | | 1000 µg/mL | 500 µg/mL | 250 µg/mL | Positive control | Negative control |
| FBTP | *Pantoea ananatis* | $14.67 \pm 0.57^{a}$ | $9.33 \pm 0.58^{b}$ | $4.67 \pm 0.58^{c}$ | $20.33 \pm 0.58^{d}$ | n.a |
| | *Pantoea stewartii* | $12.33 \pm 0.58^{a}$ | $7.67 \pm 0.58^{b}$ | n.a | $15.67 \pm 0.58^{d}$ | n.a |

FBTP: Protein hydrolysates with high inhibition percentage; *P. ananatis* (30.011 ± 0.21) and *P. stewartii* (31.981 ± 0.23); n.a: No activity; Inhibition zones were measured in mm; Positive control (chloramphenicol 1000 µg/mL); Negative control (sterilised distilled water); Values expressed as mean ± standard error (SEM); n=3 in each group; Means in the same raw within groups not followed by the same superscript are significantly different; one-way ANOVA and Tukey's test were conducted ($p$<0.05).

Likewise, the inhibition percentage of *P. stewartii* rapidly increased from 8.67 ± 0.29% to 20.59 ± 0.98% as the S/W ratio increased from 0.84% to 0.96%.

de Olmos *et al*. (2017) reported that specific *Lactobacillus* spp. fermented substrates with more excellent moisture content are more efficient, probably because of their stringent demand for accessible water. As a result of the optimal S/W ratio determined in this work, *L. casei* required additional accessible water to break down *B. thoracites* into peptides during fermentation.

**Effective inhibition concentration**

The MIC and MBC values are required to determine the concentrations that effectively inhibit bacterial growth. Smaller MIC and MBC values imply high antibacterial activity and the consequent requirement for lower doses to inhibit specific microorganisms.

The data obtained from the lactic acid fermentation suggested that FBTP (4 days, 3% v/v glucose concentration and 0.96% S/W ratio) exerted potential antimicrobial activity against *P. ananatis* and *P. stewartii*. FBTP yielded the MIC and MBC values of 250 µg/mL and 500 µg/mL for *P. ananatis* and *P. stewartii* (Table 3). In previous studies, antibacterial activities have been described in several molluscan species, such as sea snails (Kuppusamy and Ulagesan, 2016), mussels (Romanenko *et al*., 2008) and oysters (Zasloff, 2019). The maximum antimicrobial activity of FBTP was observed against *P. ananatis* (30.011 ± 0.21%) and *P. stewartii* (31.981 ± 0.23) at 1000 µg/mL.

The agar well diffusion approach determines the antimicrobial activity of FBTP. The inhibition zone of FBTP against rice pathogens is shown in Table 4. The results obtained from the agar well diffusion assay indicated a significant increasing effect on bacterial growth inhibition ($p$<0.05) with the increasing concentration of FBTP (Figure 3). *P. ananatis* showed the highest sensitivity when treated with 1000 µg/mL of FBTP, with an inhibition zone of 14.67 ± 0.578 mm. However, no inhibition zones are recorded for *P. ananatis* when treated with 250 µg/mL of FBTP. Meanwhile, the highest sensitivity when *P. stewartii* is treated with 1000 µg/mL of FBTP is 12.33 ± 0.58 mm. No inhibition zones are recorded for *P. stewartii* when treated with 250 µg/mL of FBTP.

In a previous study, the antimicrobial activity of protein hydrolysate of *Babylonia spirata* mollusc was evaluated, and the maximum zones of inhibition against *Staphylococcus aureus* and *Aspergillus fumigatus* were 22.16 ± 1.04 mm and 13.5 ± 0.5 mm at 1000 µg/mL concentration (Kuppusamy and Ulagesan, 2016).

In another study, Varma and Vasudevan (2020) reported the efficient antibacterial activity of horse mussels at the concentration of 200 µg/mL against all bacterial strains tested, with a comparatively higher antibacterial activity against *Escherichia coli* (9 mm) and *Bacillus subtilis* (8 mm). Agneswari *et al*. (2021) reported the maximum zone of inhibition against *S. aureus* (23 mm) at 100 µg/mL concentration of crude extract and 20 mm at 100 µg/mL concentration of protein hydrolysate from the mollusc *Clithon oualaniense.* Hence, the present study results showed that the antimicrobial activity of FBTP is within an acceptable range and comparable to bioactive peptides from other mollusc extracts.

**Proximate chemical composition**

The change in proximate composition obtained from the lactic acid fermentation of *B. thoracites* using *L. casei* as a starter culture is shown in Table 5. Table 5 represents the data obtained after 4 days of fermentation, which records the optimal lactic acid fermentation conditions investigated during the study.

The protein content ranged from 48.7567 ± 0.0702% to 61.56 ± 0.47%, with *B. thoracites* crude extract exhibiting the lowest content and FBTP the highest. The

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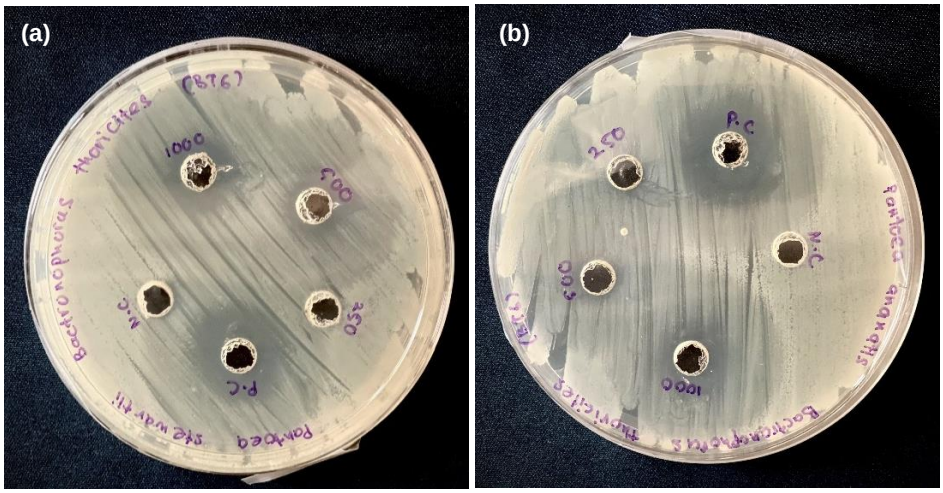


**Figure 3:** Agar well diffusion assay of FBTP. (a) FBTP in *P. ananatis* and (b) FBTP in *P. stewartii*. P.C = Positive control, N.C = Negative control, 1000 = 1000 µg/mL, 500 = 500 µg/mL, 250 = 250 µg/mL.

**Table 5:** Effect of lactic acid fermentation on proximate composition contents (% dry matter basis) of *B. thoracites* and fermented *B. thoracites* protein (FBTP).

| Proximate composition (%) | *B. thoracites* | FBTP |
|---|---|---|
| Protein | 48.76 ± 0.07 | 61.56 ± 0.47 |
| Total fat | 7.23 ± 0.03 | 0.27 ± 0.02 |
| Ash | 17.45 ± 0.25 | 32.38 ± 0.56 |
| Carbohydrates | 25.75 ± 0.06 | 6.27 ± 0.05 |

FBTP used to estimate the proximate composition was obtained under optimal lactic acid fermentation conditions; Proximate analysis data were expressed as mean ± standard deviation (n=3); Different superscripts within each row indicate significant differences ($p$<0.05).

highest value of fat content obtained is from *B. thoracites* crude extract, i.e., 7.2333 ± 0.032%, while FBTP has the lowest fat content of 0.273 ± 0.021%. The FBTP has the highest ash content of 38.38 ± 0.56% and *B. thoracites* crude extract has 17.4533 ± 0.251%. The carbohydrate level decreased from 25.75 ± 0.057% in *B. thoracites* crude extract to 6.27 ± 0.05% of FBTP after lactic acid fermentation.

The increase in microorganism production is due to the presence of essential nutrients that support the growth of the microorganisms, indicating the possibility that the microorganisms must have utilised the nutrients, thereby releasing some substances through extracellular activities (Ojokoh and Eromosele, 2015). Among the microorganisms produced, the *Lactobacillus* is the major species responsible for the fermentation of the sample. Protein content increased after fermentation from 48.7567 ± 0.0702% to 61.56 ± 0.47%. It could be the results of metabolic activities of the microorganisms that resulted in the release of extracellular enzymes into the samples, as reported by Oboh and Akindahunsi (2003). Nonetheless, the fat content decreased after the fermentation process of *B. thoracites* from 7.2333 ± 0.032% to 0.273 ± 0.021%, which is similar to the findings of Ojokoh (2005).

Since the ash content is a measure of the total amount of minerals present, its increase during lactic acid fermentation could result from the incomplete utilisation of minerals by fermenting organisms during their metabolism (Ojokoh, 2005). The carbohydrate level of FBTP decreased from 6.27 ± 0.05 to 25.75 ± 0.057. The reduction in carbohydrate content is due to the utilisation of starch to glucose by the microorganisms, which served as the carbon source for protein-rich biomass synthesis. The reduced carbohydrate level results in this study agree with the study conducted by Odunfa (1983). He reported that the reduction of carbohydrate levels during fermentation is due to the activities of fermenting microbes, which is also observed in this study.

**Amino acid analysis**

The changes in 17 amino acid contents determined in the *B. thoracites* crude extract and FBTP are given in Table 6. Lactic acid fermentation showed a significant ($p$<0.05) increase in all amino acids compared to the *B. thoracites* crude extract. The increments are observed more in methionine, leucine, valine, glutamine and cysteine in FBTP, at 13.434 ± 0.755, 12.549 ± 1.252, 12.227 ± 0.588, 11.763 ± 0.48 and 11.172 ± 0.2 g/100 g. The increment of amino acids in fermented plant protein has been reported in previous studies (Dairo and Fasuyi, 2008; Jannathulla *et al*., 2017).

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**Table 6:** Effect of lactic acid fermentation on amino acid contents (% dry matter basis) of *B. thoracites* and fermented *B. thoracites* protein (FBTP).

| Amino acids (g/100 g) | *B. thoracites* | FBTP |
|---|---|---|
| Alanine** | 1.597 ± 0.0751 | 5.214 ± 0.5760 |
| Arginine | 1.327 ± 0.0569 | 6.463 ± 0.0590 |
| Asparagine | 5.187 ± 0.2702 | 9.244 ± 0.0410 |
| Cysteine | 1.377 ± 0.0416 | 6.700 ± 0.0220 |
| Glutamine | 8.723 ± 0.0702 | 11.763 ± 0.4800 |
| Glycine** | 1.507 ± 0.0306 | 7.368 ± 0.3670 |
| Histidine* | 1.930 ± 0.0361 | 11.172 ± 0.2000 |
| Isoleucine*** | 1.187 ± 0.0451 | 8.543 ± 0.2150 |
| Leucine*** | 2.543 ± 0.0757 | 12.549 ± 1.2520 |
| Lysine* | 1.677 ± 0.0451 | 4.930 ± 0.5380 |
| Methionine* | 0.817 ± 0.0702 | 13.434 ± 0.7550 |
| Phenylalanine*** | 0.907 ± 0.0451 | 11.090 ± 0.7780 |
| Proline** | 3.267 ± 0.0586 | 9.437 ± 0.4970 |
| Serine | 1.890 ± 0.0755 | 7.074 ± 0.5280 |
| Threonine* | 1.800 ± 0.0500 | 7.582 ± 0.4030 |
| Tyrosine** | 2.567 ± 0.0764 | 10.652 ± 0.4400 |
| Valine*** | 1.180 ± 0.0755 | 12.227 ± 0.5880 |
| Total AAs | 39.480 ± 0.3503[a] | 155.442 ± 0.2538[a] |
| Essential AAs | 12.040 ± 0.1559[a] | 81.527 ± 0.4629[b] |
| Hydrophobic AAs | 14.753 ± 0.2411[b] | 77.080 ± 1.1033[a] |

[a]Fermented *B. thoracites* protein (FBTP) used to estimate the proximate composition was obtained under optimal lactic acid fermentation conditions; Essential A.A.*; Hydrophobic A.A.**; Amino acid analysis data were expressed as mean ± standard deviation (n=3), Different superscripts within each row indicate significant differences ($p$<0.05).

The total amino acid content, essential amino acids, and hydrophobic amino acids are significantly increased ($p$<0.05) to a range of 39.48-155.44, 12.04-81.52 and 14.75-77.08 g/100 g after fermentation compared to the respective control, *B. thoracites* crude extract at 39.48, 12.04 and 14.75 g/100 g. The final total amino acid concentration is a net balance of consumption and production by LAB, depending on their proteolytic activities and autolysis (release of amino acids; Christensen *et al*., 1999). The generation or consumption of amino acids during LAB metabolism varies with LAB strains and media used (Park *et al*., 2016). The significant production of amino acids by *L. casei* indicates its strong proteolytic activities.

The varied increase of essential amino acids during fermentation might be due to the inoculum itself (Shankman, 1943; Christias *et al*., 1975; Watson, 1976). The increased microbial growth during fermentation increased its own amino acid content, which might have been reflected in the fermented samples. Microorganisms utilise carbohydrates as a source of energy and the bio-conversion of such carbohydrates into microbial protein by intermediary metabolism might also be responsible for the increase observed in the amino acid profiles (Shi *et al*., 2016).

## CONCLUSION

To summarise, this study revealed the potential of antimicrobial protein hydrolysates from *B. thoracites*. The fermentation of *B. thoracites* protein using *L. casei* resulted in bioactive peptides with high antimicrobial activity against rice pathogens with appropriate protein and amino acid contents. However, utilising the generated protein hydrolysates in agricultural industries would require more research to optimise the lactic acid fermentation conditions to achieve the highest level of antimicrobial activity. Additionally, further study is necessary, especially in emphasising the fractionation and potential applications of protein hydrolysates.

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