INVESTIGATION OF *Bactronophorus thoracites* PROTEIN BREAKDOWN PRODUCTS AS ANTIMICROBIALS AGAINST *Pantoea* spp.

By

SITI NORAZURA BINTI JAMAL

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

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In addressing the challenge posed by *Pantoea* spp. pathogens in paddy cultivation, this study explores the potential of *Bactronophorus thoracites* protein breakdown products as innovative antimicrobial peptides. The research aimed to investigate these products' efficacy against *Pantoea* spp. The initial phase involved optimizing enzymatic hydrolysis and lactic acid fermentation of *B. thoracites* crude protein (BTCP) using Response Surface Methodology (RSM). The resulting *B. thoracites* fermented protein (BTFP) exhibited superior antimicrobial activity and was further analyzed for its chemical and amino acid composition. Advanced techniques such as ^{1}H-Nuclear Magnetic Resonance (NMR) spectroscopy and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) were employed to assess the fermentation's impact and to profile the peptides. Optimal fermentation conditions were determined to be 4 days of fermentation with a 3% (w/v) glucose concentration and a 0.92% (w/v) substrate-to-water ratio.

Under these conditions, the antimicrobial efficacy against *Pantoea ananatis* increased to 63.95% and against *Pantoea stewartii* to 53.53%, compared to the results from enzymatic hydrolysis (41.16% and 37.55%, respectively). Further, the most effective BTFP concentrations were identified as 125 µg/mL Minimum Inhibitory Concentration (MIC) and 500 µg/mL Minimum Bactericidal Concentration (MBC) against *P. ananatis* and *P. stewartii*. Nutritional composition analysis indicated a significant increase ($p < 0.05$) in protein and ash content to 12-15%, and a reduction in fat and carbohydrates to 7-19%. Lactic acid fermentation significantly enhanced ($p < 0.05$) the amino acid profile of BTFP, with leucine registering the highest increase (12.54 g/100g) and lysine the lowest (4.93 g/100g). Using ^{1}H-NMR spectroscopy, 27 metabolites with notable differences ($p < 0.05$) were identified between BTCP and BTFP. Notably, indole-3-lactate was the most concentrated metabolite in BTFP (351.131 mmol/L), while arabinose was predominant in BTCP (69.117 mmol/L). The principal component analysis (PCA) highlighted metabolites such as 1,3-diaminopropane, acetoacetate, alanine, and others as key discriminators. LC-MS/MS analysis revealed 18 cationic peptides with molecular weights ranging from 393 to 14,500 Da, originating from various protein sources including mollusc, fish, and invertebrate proteins. In summary, the lactic acid fermentation of BTCP emerges as a promising source of bioactive compounds, exhibiting potent antimicrobial properties that could be harnessed to address plant pathogen challenges in agriculture.

Keywords: Antimicrobial activity, *Bactronophorus thoracites*, lactic acid fermentation, enzymatic hydrolysis, metabolomic analysis, peptide profiling

SDG: Zero Hunger

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENYIASATAN PRODUK PECAHAN PROTEIN *Bactronophorus thoracites* SEBAGAI ANTIBAKTERIA TERHADAP *Pantoea* spp.

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Dalam menghadapi cabaran yang ditimbulkan oleh patogen *Pantoea* spp. dalam penanaman padi, kajian ini meneroka potensi produk pemecahan protein *Bactronophorus thoracites* sebagai peptida antimikrob inovatif. Penyelidikan ini bertujuan untuk menyiasat keberkesanan produk ini terhadap *Pantoea* spp. Fasa awal melibatkan pengoptimuman hidrolisis enzimatik dan fermentasi asid laktik protein mentah *B. thoracites* (BTCP) menggunakan Metodologi Permukaan Respons (RSM). Protein *B. thoracites* yang difermentasi (BTFP) yang dihasilkan menunjukkan aktiviti antimikrob yang lebih unggul dan dianalisis lebih lanjut untuk komposisi kimia dan asid amino. Teknik canggih seperti spektroskopi Resonans Magnet Nuklear ^{1}H (NMR) dan Kromatografi Cecair-Spektrometri Jisim Beriringan (LC-MS/MS) digunakan untuk menilai kesan fermentasi dan profil peptida. Keadaan fermentasi yang optimum ditentukan sebagai 4 hari fermentasi dengan kepekatan glukosa 3% (w/v) dan nisbah substrat-ke-air 0.92% (w/v). Di bawah keadaan ini, keberkesanan antimikrob terhadap *Pantoea ananatis* meningkat kepada 63.95% dan terhadap *Pantoea stewartii* kepada 53.53%, berbanding dengan hasil dari hidrolisis enzimatik (41.16% dan 37.55%, masing-masing). Selanjutnya, kepekatan BTFP yang paling berkesan dikenal pasti sebagai 125 µg/mL Kepekatan Rencatan Minimum (MIC) dan 500 µg/mL Kepekatan Bakterisidal Minimum (MBC) terhadap *P. ananatis* dan *P. stewartii*. Analisis komposisi nutrisi menunjukkan peningkatan yang signifikan ($p < 0.05$) dalam kandungan protein dan abu kepada 12-15%, dan pengurangan dalam lemak dan karbohidrat kepada 7-19%. Fermentasi asid laktik secara signifikan meningkatkan ($p < 0.05$) profil asid amino BTFP, dengan leusin mencatatkan peningkatan tertinggi (12.54 g/100g) dan lisin yang terendah (4.93 g/100g). Melalui spektroskopi ^{1}H-NMR, 27 metabolit dengan perbezaan ketara ($p < 0.05$) dikenal pasti antara BTCP dan BTFP. Secara khusus, indole-3-laktat adalah metabolit yang paling pekat dalam BTFP (351.131 mmol/L), sementara arabinosa adalah yang paling dominan dalam BTCP (69.117 mmol/L). Analisis komponen utama (PCA) menonjolkan metabolit seperti 1,3-diaminopropana, asetoasetat, alanina, dan lain-lain sebagai pembeza utama. Analisis LC-MS/MS mendedahkan 18 peptida kationik dengan berat molekul yang berkisar dari 393 hingga 14,500 Da, yang berasal dari pelbagai sumber protein termasuk protein moluska, ikan, dan invertebrata. Kesimpulannya, fermentasi asid laktik BTCP muncul sebagai sumber sebatian bioaktif yang menjanjikan, menunjukkan sifat antimikrob yang kuat yang boleh digunakan untuk mengatasi cabaran patogen tumbuhan dalam pertanian.

Kata Kunci: Aktiviti Antimikrob, *Bactronophorus thoracites*, fermentasi asid laktik, hidrolisis enzimatik, analisis metabolomik, profil peptide

SDG: Kelaparan Sifar

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Date: 13 June 2024

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LIST OF ABBREVIATIONS

%	Percentage
°C	Celcius
µg	Microgram
^{1}H-NMR	Proton Nuclear Magnetic Resonance
2D NMR	Two-Dimensional Nuclear Magnetic Resonance Spectroscopy
Adj.	Adjusted
AFTA	ASEAN Free Trade Area
AMPs	Antimicrobial Peptides
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
BBD	Box-Behnken Design
BLB	Bacterial Leaf Blight
C	Cytosine
Ca2+	Calcium Ion
CCD	Central Composite Design
CD_3OD	Methanol-*d4*
cfu/ml	Colony-Forming Unit Per Millilitre
Cm	Centimetre
CRIM	Centre For Research and Instrumentation Management
D_2O	Deuterium Oxide
D.H	Degree of Hydrolysis
DMSO-d6	Dimethyl Sulfoxide-*d6*

DoE	Design of Experiments
E/S	Enzyme-To-Substrate Ratio
FBTP	Fermented *B.Thoracites* Protein
FFD	Full Factorial Design
G	Guanine
G	Gram
GABA	Gamma-Aminobutyric Acid
GRAS	Generally Recognized As Safe
H	Hour
HCl	Hydrochloric Acid
HMDB	Human Metabolome Database
HPLC	High-Performance Liquid Chromatography
KDa	Kilodalton
Kg	Kilogram
KH_2PO_4	Potassium Dihydrogen Phosphate
L	Liter
LAB	Lactic Acid Bacteria
LB	Luria Broth
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LPS	Lipopolysaccharides
M	Molar
MBC	Minimum Bactericidal Concentration
Mg	Milligram
Mg2+	Magnesium Ion
MHz	Megahertz
MIC	Minimum Inhibitory Concentration

Min	Minutes
Ml	Millilitre
Mm	Millimetre
MRS	De Man, Rogosa, And Sharpe
MS	Mass Spectrometry
MVDA	Multivariate Data Analysis
N	Normality
NA	Nutrient Agar
NaOD	Sodium Deuterium Oxide
NaOH	Sodium Hydroxide
NFBTP	Non-Fermented *B.Thoracites* Protein
Nm	Nanometre
NMR	Nuclear Magnetic Resonance
OPA	O-Phtaldialdehyde
OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
P/L	Peptide-To-Lipid Ratio
PCA	Principal Component Analysis
pH	Potential Of Hydrogen
PLS-DA	Partial Least Squares-Discriminant Analysis
Q-TOF	Quadrupole Time-Of-Flight
R^2	R-Square
rpm	Rotation Per Minute
RSE	Residual Standard Error
RSM	Response Surface Methodology
S/W	Substrate/Water Ratio
S.D	Standard Deviation

SEM	Standard Error
SRI	System Of Rice Intensification
TCA	Tricarboxylic Acid
TMS	Tetramethylsilane
TSP	Trimethylsilyl propionic acid
UKM	Universiti Kebangsaan Malaysia
UPM	Universiti Putra Malaysia
USA	United State Of America
UV-VIS	Ultraviolet-Visible
v/v	Volume Per Volume
v/v/v	Volume Per Volume Per Volume
W.P	Wilayah Persekutuan
w/v	Weight Per Volume
WTO	World Trade Organization
α	Alpha
β	Beta
δ	Delta
μL	Microliter
μmole	Micromole

CHAPTER 1

INTRODUCTION

1.1 Research Background

In Malaysia, paddy fields constitute the third-largest agricultural sector, spanning approximately 526,000 hectares, with a significant 86% of the national rice yield coming from Peninsular Malaysia's 400,000 hectares of paddy fields (Awang et al., 2021; Sabran & Abas, 2021). However, the agricultural economy faces challenges from bacterial leaf blight (BLB) disease, which severely impacts crop yield and quality, potentially leading to yield reductions as high as 70% in some areas (Savary et al., 2019; Chukwu et al., 2019; IRRI, 2019; Yasmin et al., 2017).

The *Pantoea* genus is recognized worldwide as a source of BLB disease, with instances like *Pantoea agglomerans* causing BLB in Venezuela since 2002 (González et al., 2015). Further, *Pantoea* spp. were implicated in BLB outbreaks in India and Korea (Mondal et al., 2011; Lee et al., 2010). In Malaysia, molecular studies have identified *P. ananatis* and *P. stewartii* as the agents behind BLB in rice-producing regions (Azizi, Ismail et al., 2019; Azizi, Zulperi et al., 2019), with typical symptoms including water-soaked leaf marks and brownish streaks, as observed in Sekinchan, Selangor (Toh et al., 2019).

Managing BLB involves various strategies, including cultural practices, resistant varieties, and chemical controls. However, controlling *Pantoea* spp. remains challenging due to the evolving pathogenic strains and resistance to control measures (Khan et al., 2012; Sharma et al., 2019; Sundin & Wang, 2018; Valarmathi, 2020). This highlights the need for novel bioactive molecules against emerging plant pathogens (Hamed et al., 2018).

Significant research on molluscs has uncovered potential antimicrobial peptides (AMPs) with broad-spectrum activity against various microbes (Avila, 2006; Benkendorff, 2010, 2014; Sable et al., 2017; Tassanakajon et al., 2015). These small, cationic, and amphipathic AMPs disrupt microbial membranes, offering an alternative to conventional antibiotics in the era of increasing antibiotic resistance (De Zoysa, 2013; Mitta et al., 2000; Wang et al., 2019; Seo et al., 2021; Gueguen et al., 2006).

The emergence of antimicrobial resistance and antibiotic residues in agriculture necessitates alternative antimicrobial agents. The World Trade Organisation and ASEAN Free Trade Area emphasize the importance of producing safe, high-quality agricultural products. AMPs, with their effective antimicrobial properties, low propensity for inducing resistance, and biotechnological production potential, are increasingly regarded as viable alternatives in agriculture (Wu et al., 2022; Erdem Büyükkiraz & Kesmen, 2022).

1.2 Problem Statement

Antimicrobial peptides (AMPs) are short amino acids with strong abilities to kill microbes. They are seen as potential tools for controlling plant diseases like leaf blight in rice, often caused by antibiotic-resistant *Pantoea* spp. Examples of these AMPs include defensins, thionins, and cecropins, known for their effectiveness against plant diseases (Sathoff et al., 2019). While there's much research on AMPs in molluscs (Hou et al., 2011; Kim & Wijesekara, 2010), the use of *B. thoracites* protein products to fight rice diseases hasn't been much explored but could be a promising approach.

To find and study AMPs, scientists use methods like enzymatic hydrolysis, microbial fermentation (Paul et al., 2021; Ulug et al., 2021; Mohamad Asri et al., 2020), and advanced techniques like NMR spectroscopy and LC-MS/MS (Muhialdin et al., 2021). However, these methods sometimes overlook important factors that affect the breakdown of *B. thoracites* proteins, which is key to getting the best results and understanding the process better.

The effectiveness of protein breakdown products depends on their size, structure, and the types of amino acids they contain. Thorough hydrolysis creates smaller peptides with strong biological activity. Yet, the changes in their properties during extensive hydrolysis using enzymes and lactic acid fermentation aren't well documented, so more research is needed on how this affects their antimicrobial capabilities.

These protein products contain mixtures of peptides and antimicrobial substances, which can be analyzed using NMR or LC-MS/MS. NMR gives a broad overview but needs larger amounts of samples, while LC-MS/MS is more sensitive and detailed, useful for identifying antimicrobial agents in sea creatures like snails and mussels (Zhong et al., 2013; Liao et al., 2013b; Zhang et al., 2015; Bowden et al., 2020). LC-MS/MS has its own challenges, like complex preparation steps and data analysis, but remains a powerful tool in this field of study (Nilsson et al., 2010).

1.3 Significance of Research

This study focuses on the protein breakdown product of *B. thoracites*, a marine organism with a unique ecological niche, hypothesized to possess distinct antimicrobial properties in its protein composition. Given the organism's specific environmental adaptations, the proteins breakdown product of *B. thoracites* are expected to offer novel peptide sequences and structures, potentially leading to unique antimicrobial mechanisms not observed in terrestrial organisms or other marine species. Moreover, the protein breakdown of *B. thoracites* is anticipated to yield peptides with antimicrobial activities that differ from those in other animals. These variations, possibly due to differences in amino acid composition, sequence, and peptide structure, could result in a diverse spectrum of antimicrobial actions. Investigating protein breakdown of *B. thoracites* is thus a promising avenue for discovering new antimicrobial agents, with potential efficacy against emerging forms of microbial resistance, thereby contributing significantly to the field of antimicrobial research. The overview of the study is displayed in Figure 1.1.

1.4 Research Objective

This study aims to investigate *Bactronophorus thoracites* protein breakdown products as antimicrobials against *Pantoea* spp.

The specific objectives were:

1. To optimise the parameters for enzymatic hydrolysis and lactic acid fermentation of BTCP using RSM.
2. To characterise the BTFP, emphasising antimicrobial activity, chemical, and amino acid composition.
3. To evaluate the optimisation effect on the BTFP metabolites using ^{1}H-NMR-based metabolomics.
4. To profile the peptide constituents in the BTFP with antimicrobial properties using LC-MS/MS.

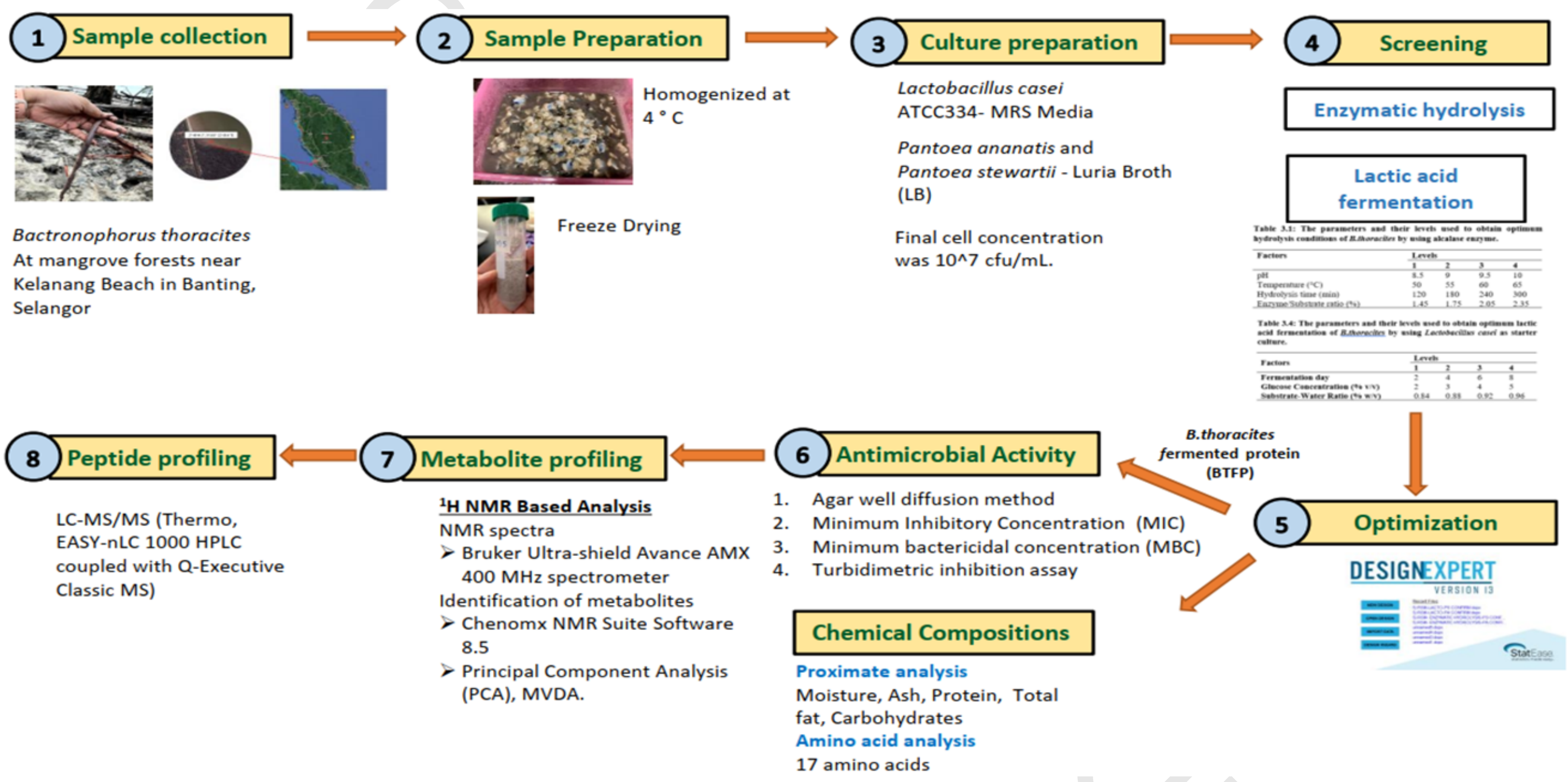

Figure 1.1 : Process flowchart of the experimental works

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