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CHARACTERIZATION OF BACTERIA FROM NEST PRODUCTS OF STINGLESS BEE, *Heterotrigona itama* (COCKERELL, 1918)

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CHARACTERIZATION OF BACTERIA FROM NEST PRODUCTS OF STINGLESS BEE, *Heterotrigona itama* (COCKERELL, 1918)

By

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CHARACTERIZATION OF BACTERIA FROM NEST PRODUCTS OF STINGLESS BEE, *Heterotrigona itama* (COCKERELL, 1918)

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April 2019

Chairman : Suriana binti Sabri, PhD
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Stingless bee, *Heterotrigona itama* nest products which are propolis, honey, and bee bread are rich in antimicrobial activities which particularly limit the development of microorganisms. Regardless of the antimicrobial activities, some bacteria still can survive in nest products. To date, little is known about the bacterial species (other than lactic acid bacteria) in the nest products of *H. itama*. Therefore, the aim of this study was to isolate and characterise bacterial species (other than lactic acid bacteria) in the *H. itama* propolis, bee bread, and honey. The nest products were aseptically collected from four geographical localities of Malaysia. Total plate count (TPC), bacterial identification, phenotypic profile, enzymatic, and antimicrobial activities were studied.

In this study, low levels of TPC were detected in the propolis, honey, and bee bread due to the reported antimicrobial activities of the nest products. The TPC originated from propolis ranges from 6.3×10^3 to 1.8×10^4 cfu/g, honey (0 to 8.0×10^3 cfu/g), and bee bread (0 to 1.1×10^4 cfu/g). Here, 41 isolates were obtained: propolis (18 isolates), bee bread (14 isolates), and honey (9 isolates) using spread plate method on nutrient agar. The 16S rDNA identification and further confirmation by phylogenetic tree analysis have found that the isolates belonged to the phylum Firmicutes, Proteobacteria, and Actinobacteria.

Fifteen isolates with different identified species namely *Bacillus oleronius* PD3, *Bacillus nealsonii* PD4, *Bacillus stratosphericus* PD6, *Bacillus amyloliquefaciens* PD9, *Bacillus toyonensis* PD13, *Bacillus subtilis* BD3, *Bacillus altitudinis* BD4, *Bacillus aryabhattai* BD8, *Bacillus safensis* BD9, *Bacillus cereus* HD1, *Bacillus pseudomycoides* HM2, *Enterobacter asburiae* PD12, *Enterobacter cloacae* PM4, *Pantoea dispersa* PG1, and

Streptomyces kunmingensis BG1 were used for phenotypic profile, enzymatic assays, and antimicrobial activity determination. The GENIII MicroPlate™ system revealed that the isolates were capable to utilise various carbohydrates, amino acids, and carboxylic acids. Proteolytic, lipolytic, and cellulolytic activities were detected from *B. amyloliquefaciens* PD9, *B. stratosphericus* PD6, *B. subtilis* BD3, and *B. safensis* BD9. Broad spectrum of antimicrobial activity was found from *B. amyloliquefaciens* PD9 that can inhibit Gram-positive and Gram-negative bacteria.

This is the first study on the isolation and characterisation of bacterial species (other than lactic acid bacteria) from the Malaysian stingless bee, *H. itama* nest products. The characteristics of bacterial species associated with *H. itama* were investigated. The results suggested that the isolates might contribute to the formation of bee products by the enzymes and antimicrobial metabolites production.

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia sebagai memenuhi keperluan untuk Master Sains

PENCIRIAN BAKTERIA DARIPADA HASIL SARANG LEBAH KELULUT, *Heterotrigona itama* (COCKERELL, 1918)

Oleh

MOHAMAD SYAZWAN BIN NGALIMAT

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Hasil sarang lebah kelulut, *Heterotrigona itama* seperti propolis, madu, dan roti lebah kaya dengan aktiviti antimikrob yang sangat membatasi perkembangan mikroorganisma. Tanpa menghiraukan aktiviti antimikrob, segelintir bakteria masih mampu mendiri dalam hasil sarang lebah. Sehingga kini, sedikit yang diketahui mengenai spesies bakteria (selain bakteria asid laktik) dalam hasil sarang *H. itama*. Oleh itu, tujuan kajian ini adalah untuk mengasingkan dan mencirikan spesies bakteria (selain bakteria asid laktik) di *H. itama* propolis, roti lebah, dan madu. Hasil sarang telah dikumpulkan secara aseptik daripada empat lokasi geografi di Malaysia. Jumlah kiraan plat (TPC), pengenalan bakteria, profil fenotip, aktiviti enzimatik, dan antiviti antimikrob telah dikaji.

Dalam kajian ini, tahap TPC yang rendah telah dikesan pada propolis, madu, dan roti lebah kerana aktiviti antimikrob yang telah dilaporkan dalam hasil sarang lebah. TPC berasal dari propolis berkisar antara 6.3×10^3 sehingga 1.8×10^4 cfu/g, madu (0 sehingga 8.0×10^3 cfu/g), dan roti lebah (0 sehingga 1.1×10^4 cfu/g). Di sini, 41 isolat telah diperolehi: propolis (18 isolat), roti lebah (14 isolat), dan madu (9 isolat) menggunakan kaedah plat penyebaran pada agar nutrien. Pengenalpastian 16S rDNA dan pengesahan selanjutnya oleh analisis pokok filogenetik mendapati bahawa isolat-isolat dimilik oleh filum Firmicutes, Proteobacteria, dan Actinobacteria.

Lima belas isolat berbeza yang telah dikenalpasti iaitu *B. oleronius* PD3, *B. nealsonii* PD4, *B. stratosphericus* PD6, *B. amyloliquefaciens* PD9, *B. toyonensis* PD13, *B. subtilis* BD3, *B. altitudinis* BD4, *B. aryabhattai* BD8, *B. safensis* BD9, *B. cereus* HD1, *B. pseudomycoides* HM2, *E. asburiae* PD12, *E. cloacae* PM4, *P. dispersa* PG1, dan *S. kunmingensis* BG1 telah digunakan untuk penentuan profil fenotip, asai-asai enzimatik, dan aktiviti

antimikrob. Sistem MicroPlate™ GENIII mendedahkan bahawa isolat-isolat mampu menggunakan pelbagai jenis karbohidrat, asid amino, dan asid karboksilik. Aktiviti proteolisis, lipolisis, dan selulolisis telah dikesan pada *B. amyloliquefaciens* PD9, *B. stratosphericus* PD6, *B. subtilis* BD3, dan *B. safensis* BD9. Spektrum aktiviti antimikrob yang luas telah ditemui dari *B. amyloliquefaciens* PD9 yang boleh merencat bakteria Gram-positif dan Gram-negatif.

Ini adalah kajian pertama mengenai pengasingan dan pencirian spesies bakteria (selain bakteria asid laktik) daripada hasil sarang lebah kelulut Malaysia, *H. itama*. Ciri-ciri spesies bakteria yang dikaitkan dengan *H. itama* telah dijelaskan. Hasilnya menunjukkan bahawa isolat-isolat mungkin menyumbang kepada pembentukan produk-produk lebah melalui pengeluaran enzim-enzim dan metabolit-metabolit antimikrob.

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I certify that a Thesis Examination Committee has met on 17 April 2019 to conduct the final examination of Mohamad Syazwan bin Ngalimat on his thesis entitled "Characterization of Bacteria from Nest Products of Stingless Bee, *Heterotrigona itama* (Cockerell, 1918)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Declaration by graduate student

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the the writing of this thesis was under our supervision;
- supervision responsibilities as slated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

α	Alpha
β	Beta
ρ	Para
°C	Degree celsius
%	Percentage
A_{600nm}	Optical density at wavelength 600 nanometer
µL	Microliter
mm	Milimeter
µmol	Micromoles
ATCC	American Type Culture Collection
DSM	German Collection of Microorganisms and Cell Cultures
JCM	Japan Collection of Microorganisms
NBRC	National Institute of Technology and Evaluation
bp	Base pair
DNA	Deoxyribonucleic acid
g	Gram
kb	Kilobase
L	Litre
M	Molar
T	Transmission
V	Volt

CHAPTER 1

INTRODUCTION

1.1 Background of study

Stingless bees (Hymenoptera: Apidae: Meliponi) or Kelulut is well distributed in Malaysia with approximately 45 stingless bees' species were found nationwide (Jaapar & Jajuli, 2016). Although mostly considered as pollinating agent (Camargo et al., 2013; Michener, 2013), the continuous research on stingless bees have increased the awareness on the usefulness of stingless bees to the honey industry (Jaapar & Jajuli, 2016). Stingless bee honey contains high antioxidants and many pharmaceutical benefits, causing the demand for honey continues to increase (Roowi et al., 2016; Kelly et al., 2014). Realising the tremendous demand of stingless bee honey, the Malaysian Standard on Kelulut (Stingless Bee) Honey-Specification has been developed in Malaysia to ensure the production of high quality of Kelulut honey (Roowi et al., 2017). Thus, these, without doubt have enabled the development of meliponiculture (stingless bee beekeeping) in Malaysia.

Stingless bees can produce nest products such as honey, bee bread, and propolis. These products have high market value and industrial applications (Kelly et al., 2014). Of various industries, the three most important are cosmeceutical, pharmaceutical, and food industries (Jalil et al., 2017; Mostoles et al., 2014). Nest products generated from plant based materials are rich in macromolecules and possess various biological and pharmacological properties (Rao et al., 2016; Komosinska-Vassev et al., 2015). It has antimicrobial properties which can suppress the development of several types of microorganisms (Sinacori et al., 2014). Regardless of numerous growth inhibiting factors such as concentrated sugar, acidity, hydrogen peroxide (H_2O_2), and phytochemical compounds (Gilliam et al., 1990), some microbes such as bacteria still can survive in nest products. However, details information on their characteristics are yet poorly understood.

Association of bacteria with stingless bees are well described (Leonhardt & Kaltenpoth, 2014; Promnuan et al., 2009), but their roles in the biology of the bees and nest products are still unclear. Bacteria have been hypothesised to involve in the formation of nest products (Gilliam et al., 1990) and inhabitation of spoilage microorganisms in the storage pots (Promnuan et al., 2009) where some study considered the microorganism found were as biological contaminants (Olaitan et al., 2007). DNA evidence of bacteria from the genus *Bacillus* was found in the abdominal contents of

dead stingless bee, *Proplebeia dominicana* preserved for 25 to 40 million years in the Dominican amber (Camargo, 2013). Some studies have found that most bacteria isolated from honey were from the genus *Bacillus*, *Brevibacterium*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, *Neisseria*, *Pseudomonas*, *Xanthomonas*, *Streptomyces*, *Lactobacillus*, and *Fructobacillus* (Yaacob et al., 2018; Pucciarelli et al., 2014; Promnuan et al., 2009).

Investigations on the association of bacteria in stingless bee nest products were done mostly focusing on the lactic acid bacteria isolation and identification (Yaacob et al., 2018; Tamarit et al., 2015; Leonhardt & Kaltenpoth, 2014). The association of bacteria in stingless bee has also been confirmed using metabarcoding analysis (Díaz et al., 2016). To date, little is known about the bacteria (other than lactic acid bacteria) that can be found in the honey, bee bread, and propolis of Malaysian stingless bee, *Heterotrigona itama*. Hence, concerted efforts into the identification and characterisation of bacteria in nest products are necessary and merit scientific attention. The study on the characterisation of bacterial species from bee products can be the starting point to understand their metabolic and physiological ability which might lead to the contribution of nest products formation.

1.2 Problem statement

Stingless bees produced beneficial nest products such as honey, bee bread, and propolis. These products possessed antimicrobial properties that limit the growth of bacteria (Amin et al., 2018). However, certain bacteria could survive in the nest products. There are many studies merely focusing on isolation and identification of the lactic acid bacteria from Malaysian stingless bee, *H. itama*. However, studies on other types of bacteria from *H. itama* nest products and the details information on their characteristics is scarce.

1.3 Research objectives

This work aimed to identify and establish the characteristics of bacterial species in the *H. itama* propolis, bee bread, and honey. The specific objectives of this research are:

1. To quantify, isolate, and identify bacteria from *H. itama* nest products;
2. To analyse the phenotypic profile of the bacterial isolates; and
3. To determine the enzymatic and antimicrobial activities of the bacterial isolates

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