
**UNIVERSITI PUTRA MALAYSIA**

***ANTIMICROBIAL ACTIVITY OF Bacillus velezensis STRAIN PD9 SECONDARY METABOLITE AGAINST METHICILLIN RESISTANT Staphylococcus aureus***

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By

**MOHAMAD MALIK AL-ADIL BIN BAHARUDIN**

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**February 2021**

**Chairman : Suriana binti Sabri, PhD**
**Faculty : Biotechnology and Biomolecular Science**

Methicillin resistant *Staphylococcus aureus* are opportunistic pathogen that are resistant towards many antibiotics particularly methicillin and penicillin class of antibiotic. Infections caused by MRSA have reached epidemic proportion globally. The rapid onset of resistance acquired by this bacterium reduces the efficacy of most commonly known antimicrobial drug; making it more difficult to treat. Therefore, there is an urgent need for an alternative therapeutic strategy and a continuous supply of antibiotic to combat the problem. Natural sources such as bacteria in the genus of *Bacillus* provide a promising potential for the exploration of anti-MRSA metabolites. *Bacillus velezensis* has been widely reported as bio-control agent in agriculture industry due to the vast spectrum of antimicrobial metabolites produced from this bacterium. However, there is lack of information available on the antimicrobial activity of this bacterium against MRSA. Thus, the aims of the study were to characterize antimicrobial activity and physicochemical feature of CFS against MRSA as well as to purify anti-MRSA metabolite(s) from *B. velezensis* strain PD9.

In this study, bacteria initially identified as species belonged to the *Bacillus amyloliquefaciens* operational group were re-classified into specific species within the group based on housekeeping gene, *gyrB*. Cell-free supernatant (CFS) from *B. velezensis* strains were used for antimicrobial test using agar well diffusion assay against MRSA strains and various types of pathogenic bacteria. Antimicrobial activity of CFS from *B. velezensis* PD9 was characterised against MRSA ATCC 33742. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and MRSA growth inhibition effect were analysed. Physicochemical analyses of the strain PD9 CFS against MRSA ATCC 33742 were also determined. In this study, an antimicrobial peptide was purified from CFS of *B. velezensis* PD9 using solvent extraction method and

silica gel column chromatography. The purified product was visualized using SDS-PAGE and zymogram. The purity of silica gel purified antimicrobial peptide was determined by using HPLC.

Based on *gyrB* sequence analysis, 5 strains (PD9, B7, PU1, BP1 and L9) were classified as *B. velezensis*. CFS of all *B. velezensis* strains showed broad inhibitory activity against MRSA strains and several pathogenic Gram-negative and Gram-positive bacteria tested. Inhibitory activity of *B. velezensis* PD9 against MRSA ATCC 33742 were chosen for further analysis as it showed the biggest zone of inhibition (21.0 ± 0.4 mm). MIC and MBC values of *B. velezensis* PD9 CFS against MRSA ATCC 33742 obtained were 125 µl/ml. CFS of *B. velezensis* PD9 showed bactericidal activity against MRSA ATCC 33742 and were stable at various temperature (40 - 80 °C), pH (4-12), surfactant (Tween 20, Tween 80, SDS and Triton X-100) and metal ions (MgCl2, NaCl2, ZnNO3 and CuSO4) tested, but not in proteinase K; these properties resembled the characteristics of a peptide. Anti-MRSA metabolite was successfully concentrated using 1-butanol. Active fractions showing antimicrobial activity against MRSA ATCC 33742 were collected from silica gel column chromatography. The silica gel purified product was visualised on SDS-PAGE and zymogram. The size of the antimicrobial peptide obtained was approximately 5 kDa. The band with halo zone against MRSA ATCC 33742 was observed on zymogram. Single peak observed on HPLC chromagram indicated the purity of silica gel purified product.

The present characterization reveals interesting properties of the antimicrobial peptide from *B. velezensis* PD9 which justifies its promising potential in controlling pathogenic MRSA strains. The antimicrobial peptide produced from *B. velezensis* PD9 might provide an alternative to combat the spread of MRSA infection in the future.

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

**AKTIVITI ANTIMIKROB METABOLIT SEKUNDER *Bacillus velezensis* STRAIN PD9 TERHADAP RINTANG METISILIN *Staphylococcus aureus***

Oleh

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*Staphylococcus aureus* rintang-metisilin (MRSA) merupakan opportunis patogen yang tahan terhadap pelbagai antibiotic terutamanya methisilin dan antibiotik kelas penisilin. Jankitan disebabkan oleh MRSA telah mencapai tahap epidemik diperingkat global. Keupayaan bakteria ini untuk tahan terhadap keberkesanan antibiotik telah menyebabkan penurunan keupayaan antibitik untuk menghentikan jangkitan; seterusnya menyebabkan jangkitan ini sukar untuk ditangani. Oleh itu, alternatif segera perlu dikenalpasti di samping bekalan antibiotik yang efektif perlu berterusan untuk mengekang masalah ini. Sumber semulajadi seperti bakteria dalam genus Bacillus dilihat sebagai potensi yang boleh diterokai bagi mengenalpasti metabolit anti-MRSA. *Bacillus velezensis* terkenal sebagai ejen kawalan biologi dalam industri pertanian disebabkan keupayaan bakteria ini untuk menghasilkan pelbagai metabolit anti-mikrob. Walaubagaimanapun, maklumat mengenai aktiviti antimikrob bakteria ini terhadap MRSA masih terhad. Oleh itu, tujuan kajian ini adalah untuk mencirikan aktiviti antimikrob dan fizikomia supernatan bebas sel (SBS) terhadap MRSA di samping untuk menulenkan metabolit anti-MRSA daripada *B. velezensis* PD9.

Dalam kajian ini, bakteria yang pada awalnya dikenali sebagai sebahagian spesies yang terdapat dalam kumpulan "*B. amyloliqueafaciens* operational group" telah diklasifikasikan semula kepada spesies spesifik dalam kumpulan berdasarkan gen pengemasan, *gyrB*. Supernatan bebas-sel (SBS) daripada strain ini digunakan untuk ujian antimikrob menggunakan kaedah penjerapan perigi agar sebaran terhadap RMSA dan pelbagai jenis bakteria patogen yang lain. Aktiviti antimikrob SBS daripada *B. velezensis* PD9 dicirikan terhadap MRSA ATCC 33742. Kepekatan perencatan minimum (MIC), kepekatan bakterisidal minimum (MBC) dan analisis fizikokimia SBS terhadap MRSA dikenalpasti. Kesan perencatan pertumbuhan RMSA ATCC 33742 oleh strain PD9 juga dianalisis. Dalam kajian ini, peptida antimikrob berfungsi terhadap

MRSA ditulenkan menggunakan kaedah ekstrak pelarut dan kromatografi turus gel silika. Produk hasil penulenan telah dianalisis menggunakan SDS-PAGE dan zimogram. Ketulenan produk tulen kromatografi silica dianalisis menggunakan HPLC.

Berdasarkan analisis jujukan *gyrB*, 5 strain ini dikenali sebagai *B. velezensis*. Supernatan bebas sel daripada semua strain *B. velezensis* menunjukkan aktiviti perencatan yang luas terhadap pelbagai MRSA strain dan bakteria patogen Gram-positif dan Gram-negatif yang diuji. Aktiviti perencatan strain PD9 ke atas MRSA ATCC33742 dipilih untuk analisis yang selanjutnya disebabkan strain ini menunjukkan zon perencatan yang terbesar (21.0 ± 0.4 mm). Nilai MIC dan MBC yang diperoleh ialah 125 µl/ml. Ekstrak kompaun kasar antimikrob menunjukkan aktiviti bakterisidal dan stabil dalam pelbagai suhu (40 – 80 °C), pH (4-12), surfaktan (Tween 20, Tween 80, SDS and Triton X-100) dan ion logam (MgCl2, NaCl2, ZnNO3 dan CuSO4) yang diuji, tetapi tidak terhadap proteinase K; sifat ini memaparkan ciri-ciri suatu peptida. Metabolit antimikrob telah diekstrak menggunakan 1-butanol sebagai pelarut. Fraksi aktif yang menunjukkan antimikrob aktiviti ke atas MRSA ATCC 33742 yang dikumpulkan daripada kromatografi turus gel silika. Produk tulen dari kromatografi gel silika dianalisis menggunakan SDS-PAGE dan zimogram. Saiz antimikrob yang diperoleh adalah sekitar 5 kDa. Jalur dengan zon halo terhadap MRSA ATCC 33742 dapat dilihat melalui zimogram.

Pencirian semasa menujukkann sifat-sifat menarik yang terdapat pada metabolit antimikrob ini, sekaligus memaparkan potensi antimikrob ini untuk digunakan bagi mengawal jangkitan MRSA patogenik. Metabolit antimikrob yang dihasilkan oleh *B. velezensis* strain PD9 dilihat dapat dijadikan sebagai salah satu alternatif untuk mengawal penularan jangkitan MRSA pada masa hadapan.

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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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## LIST OF ABBREVIATIONS

| | |
|---|---|
| UPM | Universiti Putra Malaysia |
| CFS | Cell free supernatant |
| MRSA | Methicillin resistant *Staphylococcus aureus* |
| HA-MRSA | Hospital-associated MRSA |
| CA-MRSA | Community-associated MRSA |
| LA-MRSA | Livestock-associated MRSA |
| µL | Microliter |
| Mm | Milimeter |
| °C | Degree Celsius |
| APS | Ammonium persulfate |
| ATCC | American Type Culture Collection |
| MTCC | Microbial Type Culture Collection |
| $A_{600nm}$ | Optical density at wavelength 600 nanometer |
| bp | Base pair |
| DNA | Deoxyribonucleic acid |
| MSA | Mannitol salt agar |
| MIC | Minimun inhibitory concentration |
| MBC | Minimun bactericidal concentration |
| AMP | Antimicrobial peptide |
| DCM | Dichloromethane |
| MeOH | Methanol |
| WHO | World Health Organization |
| SBS | Supernatan bebas-sel |
| HPLC | High Performance Liquid Chromatography |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

The emergence of antibiotic resistance among the most common bacterial pathogens is considered as a major public health threat affecting human globally. In 2019, World Health Organization (WHO) has listed antimicrobial resistance (AMR) as top 10 threats to global health due to its rapid and ongoing spread (Friedrich, 2019). It is estimated about nearly one million people died every year due to the bacterial infection that cannot be treated with common antibiotic (Neill, 2016). If the rising of antimicrobial resistance threat is not addressed, annual death toll could reach 10 million by the year 2050 (Neill, 2016).

The most common bacterial pathogens associated with antibiotic resistance and virulence are term as “ESKAPE” pathogen which comprised of *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp. (Mulani et al., 2019). Recently, WHO has categorised “ESKAPE” pathogen based on the urgency of new antibiotic needed to combat them; Methicillin-resistant *Staphylococcus aureus* (MRSA) has been listed in high priority group (Mulani et al., 2019). MRSA is an opportunistic pathogen that is resistant against various antibiotics particularly methicillin and other classes of ß-lactam antibiotics (Stapleton & Taylor, 2007). MRSA is reported to be responsible for the most *S. aureus* bacteraemia cases which cause high mortality rates. In Malaysia, 21% cases of bacteraemia were reported to be caused by MRSA (Ahmad et al., 2009).

Many efforts have been made to search for new antimicrobial subtances to kill MRSA strains. Natural sources such as plants (Chew et al., 2018; Khameneh et al., 2019), arthropods (Li et al., 2017) fungus (Gebreyohannes et al., 2019; Phongpaichit et al., 2006) and bacteria (Chalasani et al., 2015; Gowrishankar et al., 2015; Jeyanthi & Velusamy, 2016; Ramachandran et al., 2014) have been widely employed to discover new novel antimicrobial subtances. Among all the natural sources, bacteria in the genus of *Bacillus* have been viewed as the most promising group to identify new antimicrobial metabolites. *Bacillus* spp. have been reported to encode more than 4 % of their genome for antimicrobial metabolites production (Caulier et al., 2019; Rabbee et al., 2019; Sumi et al., 2015). *Bacillus amyloliqueafaciens* operational group which comprised of *B. amyloliquefaciens, B. velezensis* and *B. siamensis* have the capability to produce large number of antimicrobial metabolites such as bacteriocins, lipopeptide, polyketides, volatile compounds and others (Caulier et al., 2019; Rabbee et al., 2019; Schallmey et al., 2004; Sumi et al., 2015).

There are few studies reported on anti-MRSA metabolite produced from *B. amyloliquefaciens* operational group such as aminoglycoside antibacterial substance from *B. velezensis* RP147 (Pournejati et al., 2019) bacillysin A from *B. amyloliqueafaciens* AP183 (Ravu et al., 2015) phenolic compounds from *B. amyloliquefaciens* MHB1 (Jeyanthi & Velusamy, 2016), cyclic dipeptide such as cyclo(L-leucyl-L-prolyl) from *B. amyloliqueafaciens* MMS-50 (Gowrishankar et al., 2015) cyclic lipopeptides such as surfactin, iturin and fengycin A from *B. amyloliquefaciens* JN68 (Chen et al., 2016), amysin from *B. amyloliquefaciens* SP-1-13LM (Kaewklom et al., 2019), antimicrobial peptide (CSpK14) from *B. amyloliqueafaciens* K14 (Regmi et al., 2017a) as well as 4-O-isopropyl genistein, and genistein from *B. amyloliquefaciens* KCTC13588 (Choi et al., 2018).

## 1.2 Problem Statement

In the previous study, cell free supernatant (CFS) from *B. amyloliquefaciens* PD9 (currently re-classified as *B. velezensis* PD9) isolated from stingless bee product showed antimicrobial activity against Gram positive (*B. cereus, S. aureus, Micrococcus luteus*) and Gram negative bacteria (*Entrobacter aerogenes, Echerichia coli, Alcaligene faecalis, Aeromonas hydrophilla, Salmonella typhimurium*) (Ngalimat et al., 2019). However, antimicrobial activity against MRSA has not been tested. To be noted, there are five strains of *Bacillus* spp. isolated from stingless bee were belonged to *B. amyloliquefaciens* operational group which are strain PD9, B7, PU1, L9 and BP1 (currently all strains were re-classified as *B. velezensis*).

*Bacillus velezensis* were reported to encode about 4-5% of its genome for antimicrobial metabolites production (Caulier et al., 2019; Rabbee et al., 2019). This could provide a potential platform for the exploration and discovery of anti-MRSA subtances from their metabolites. Although there are reports on the anti-MRSA activity of *B. velezensis* antimicrobial compounds, however, the types of the antimicrobial compounds produced from this bacteria are strain dependent (Dimitrovski et al., 2014; Giani et al., 2019). In this study, the inhibitory potential of antimicrobial metabolite(s) from *B. velezensis* PD9 against MRSA was explored.

Infections caused by multi-drug resistance strain of *Staphylococcus aureus* (MRSA) has reached an epidemic proportion globally (Deurenberg et al., 2006; Foster, 2017; Grema et al., 2015; Hassoun et al., 2017). The rapid onset of resistance gained by this bacterium reduces the efficacy of most commonly known antimicrobial drug making it more difficult to treat. Thus, there is an urgent need for continuos discovery of new anti-MRSA agents to combat the infection. However, the progress in developing them has been slow (Fischbach & Walsh, 2010). Based on the Infectious Disease Society of America (IDSA), the antibiotic pipeline status report showed a continual drop in the development of new antibiotic since last few decades (Boucher et al., 2013). ne of the strategies to avoid the shortage of effective antibiotics is by continuing the searching of the

new potential anti-MRSA agents. In logical fashion, more novel antimicrobial agent discovered more chances for the new novel agents to be approved for clinical used.

Recently, extracellularly produced antimicrobial metabolite from *B. velezensis* PD9 (previously known as *B. amyloliquefaciens* PD9) showed broad spectrum inhibition against various Gram positive and Gram negative bacteria (Ngalimat et al., 2019). However, the information on the antimicrobial potential of the metabolites against MRSA has not been studied.

## 1.3 Objectives

The main aim of this study was to determine the antimicrobial potential of *B. velezensis* PD9 against MRSA. The specific objectives of the study were:

1) To evaluate antimicrobial activity of *B. velezensis* cell free supernatant against MRSA;
2) To determine the physicochemical characteristics of antimicrobial metabolite(s) produced by *B. velezensis* PD9; and
3) To purify anti-MRSA metabolite produced by *B. velezensis* PD9.

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