Research Article

Partial Purification and Characterization of Antifungal Peptides Produced by *Bacillus amyloliquefaciens* PEP3 Against *Phytophthora capsici*

Hairul A. Roslan*, Ahmad Husaini and Samuel Lihan
Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia

Mohd F. Kota
Sarawak Biodiversity Centre, KM20, Jalan Borneo Heights, Semengoh, Kuching Sarawak, Malaysia

* Corresponding author. E-mail: hairulroslan@hotmail.com DOI: 10.14416/j.asep.2020.01.004
Received: 10 October 2019; Revised: 2 December 2019; Accepted: 23 December 2019; Published online: 22 January 2020

Abstract
The prevalence of antibiotic-resistant microorganisms has triggered the exploration for novel antimicrobial compounds. Peptides are biological molecules that are found in all living organisms. They play key roles in many biological processes including organism's defense. We have previously isolated *Bacillus* species (*Bacillus amyloliquefaciens* PEP3) that showed antagonistic properties against fungi. Metabolites precipitates obtained from *B. amyloliquefaciens* PEP3 showed antifungal activity against plant pathogenic fungus, *Phytophthora capsici*. Further analysis showed that these metabolites were stable in the pH range of 6 to 8 and temperatures of between 25°C to 85°C after which it began to lose its activity. Polyacrylamide gel analysis indicated that the molecular weight of the metabolites to be approximately 5 kDa. The presence of biosynthetic lipopeptides producing genes namely *ituD*, *srfA*, *fenD* and *Ipa-14* genes were detected via the use of specific primers. The metabolites were then isolated via methanol extraction, however none of the major lipopetides family were detected using liquid chromatogrpahy-mass spectrometry (LC-MS). Nevertheless, the results derived from this work suggested that *B. amyloliquefaciens* PEP3 to have biocontrol research value and warrants further analysis.

Keywords: Bacillus amyloliquefaciens, ituD, srfA, *fenD*, Ipa-14, Antifungal peptides

1 Introduction

Bacillus species are known to produce antifungal compounds and lipopeptides. However, not all *Bacillus* species are capable of producing all antifungal lipopeptides and utilize them in defense mechanism. Antifungal lipopeptides from the family of iturin, fengycin and surfactin, are reported to be produced by only two types of *Bacillus* species, *B. subtilis* and *B. amyloliquefaciens*. Antifungal lipopeptides from the family of iturin, fengycin and surfactin are synthesized through a system call nonribosomal peptide synthesis (NRPS) [1]. NRPS is a large, multi-domain enzymes which produces peptide molecules containing unusual amino acids including D-amino acids, β-amino acids, and hydroxy- or N-methylated amino acids. As opposed to the traditional protein synthesis, beneficial molecules such as antifungal lipopeptides are produced without an mRNA template.

Researchers opted to combine both molecular and biochemical approaches to determine the presence of antifungal lipopeptides. From a biochemical approach, acid precipitation of cell free liquid supernatant of a specific producer is normally used. By lowering the acidity of the cell free liquid supernatant, antifungal lipopeptides will precipitates as results of chemical

Please cite this article as: H. A. Roslan, M. F. Kota, A. Husaini, and S. Lihan, "Partial purification and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* PEP3 against *Phytophthora capsici*," *Applied Science and Engineering Progress*, vol. 13, no. 1, pp. 56–66, Jan.–Mar. 2020.

interactions between the compounds and changes of pH. The partially purified antifungal lipopeptides can be detected and identified using liquid chromatography system. Molecular approaches can further support the findings from biochemical approaches through detection of NRPS biosynthetic genes and their expression. This kind of information will provide insight on the ability of the producer to produce antifungal lipopeptides. Recent findings suggested that utilization of *Ipa-14* gene, which encodes for 4-phosphopantetheinyl transferases, is a quick and efficient method to determine the ability of a bacteria to produce antifungal lipopeptide [2], [3]. This is because the absence of the gene lead to deactivation of the carrier protein domains of polyketide synthases (PKS), non ribosomal peptide synthases (NRPS) and fatty acid synthases (FAS) [3], [4].

We have previously isolated *B. amyloliquefaciens* PEP3 and showed its antagonistic activity towards *Phytophthora capsici* identified as through production of hydrolytic enzymes and volatile organic compounds (VOCs) [5]. Here we report the isolation and identification of lipopeptides and their corresponding genes from *B. amyloliquefaciens* PEP3. This work provided further insights on what kind of antifungal lipopeptides being produced and the prospects of their further application.

2 Materials and Methods

2.1 *Strains and culture conditions*

B. amyloliquefaciens PEP3 used in this study was isolated and characterized as reported in Kota and co-workers [5]. The *B. amyloliquefaciens* PEP3 was maintained on Luria–Bertani agar (LBA) (peptone, 10 g; yeast extracts, 5 g; NaCl, 10 g; agar, 18 g; and distilled water, 1 L). Liquid Luria-Bertani media (LB), was used for production of lipopeptides. Indicator strain, *Phytophthora capsici* was donated by Malaysia Pepper Board (MPB) and maintained on malt extract agar (MEA) (malt, 12.75 g; dextrin, 2.75 g; peptone, 0.78 g, agar, 18 g; and distilled water, 1 L).

2.2 *Production and extraction of lipopeptides*

B. amyloliquefaciens PEP3 maintained on LBA was inoculated into 50 mL of LB broth and cultivated at 37°C for 24 h. The seed culture of *B. amyloliquefaciens* PEP3 was transferred to four 1 L Erlenmeyer flask that contained for 500 mL of LB broth. The culture was incubated on a rotary shaker (140 rpm) at 37°C for 72 h. The 72 h fermentation broth of *B. amyloliquefaciens* PEP3 was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected at filter-sterilized using 0.45 μm filter membrane. Precipitation of antifungal peptide was achieved by adjusting the pH of the filter-sterilized fermentation broth to pH 2 using 5 M HCl and incubated at 4°C for overnight. The acid precipitates were recovered by centrifugation at 10,000 rpm for 10 min with the temperature of 4°C. The pellet obtained was solubilized methanol. The methanolic antifungal peptide extract was further concentrated using rotary evaporator and resuspended in Tris buffer (50 mm, pH 7.5) [6], [7].

2.3 *Antifungal peptide activity assay*

Antifungal peptide activity assay was carried out based on disc plate diffusion assay as described by Moyne and c-workers [8]. The centre of the malt extract agar (MEA) was inoculated with 1 cm^2 of mycelial plugs from the edges of actively growing *P. capsici*. Four sterile filter paper discs was placed 2 cm from the edge of fungal culture on the MEA surface. Thirty microliters of samples was applied to the three sterile filter paper discs. The remaining disc was applied with 30 μL of Tris buffer (50 mm, pH 7.5) as control. The plates were incubated at room temperature. After 7 days, the diameter of inhibition zone was measured and the mean was calculated.

2.4 *Effects of temperature and pH on antifungal activities*

Sensitivity of the antifungal peptide to temperature was determined at six different temperatures. Samples were incubated for 15 min at 55°C, 65°C, 75°C, 85°C, 95°C and 121°C. After incubation, each sample was assayed for activity in triplicate. Meanwhile, sensitivity towards pH was determined according to Zhang and co-workers in 2012 [9]. Samples were adjusted to pH 2, 4, 6, 8, 10 and 12 using 0.5 M of NaOH or HCl, and incubated for 24 h at room temperature. Each sample then were restored to pH 7.5 and subjected to disc plate diffusion assay.

2.5 *Molecular weight determination*

2.5.1 Gel preparation and running conditions

Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was undertaken using Mini-PROTEAN Tetra cell slab (Biorad, USA) to determine the molecular weight of the sample containg peptide with antifungal activity [10]. One millimeter spacer was used to set the thickness of the gels. Each gel consisted of; 1 cm stacking gel (4% T, 3% C), 1.5 cm spacer gel (10% T, 3% C), and a 3.0 cm separating gel (16.5% T, 3% C). Where T denotes the total percentage concentration of acrylamide and bisacrylamide, while C denotes the percentage concentration of crosslinker relative to T. Each portion of the gel was made according to the Table 1.

Table 1: Composition of separating gel, spacer gel, and stacking gel

	Separating Gel	Spacer Gel	Stacking Gel
30% (w/v) acrylamide stock	2.8 mL	1.65 mL	195 µL
0.5 M Tris, pH 6.8	/	/	375 µL
10% (w/v) SDS	/	/	15 µL
Gel Buffer*	1.65 mL	1.65 mL	/
Glycerol	0.17 g	/	/
10% (w/v) ammonium persulphate	15 µL	15 µL	7.5 µL
TEMED	2 µL	2 µL	2 µL
Water to a final volume of	5 mL	5 mL	1.5 mL

Gel buffer*: 3M Tris, pH8.45, + 0.3 % (w/v) SDS

Prior to loading onto the gel, the samples were dissolved in loading buffer and heated to 95°C for 5 min. Electrophoresis was conducted at 75 V for 3 h or until the tracking dye reached the end of the gel. The components for the loading buffer, anode buffer, and cathode buffer are listed in Table 2.

Table 2: Composition of loading buffer, anode buffer, and cathode buffer

	Loading buffer	Anode buffer	Cathode buffer
0.5 M Tris, pH 6.8	1.0 mL	/	/
Glycerol	0.8 mL	/	/
10 % (w/v) SDS	1.6 mL	/	5.0 mL
2-β mercaptoethanol	0.4 mL	/	/
0.05 % (w/v) bromophenol blue	0.2 mL	/	/
Tris	/	12.11 g	6.06 g
Tricince	/	/	8.96 g
Adjust pH to	/	8.8	8.25
Water to a final volume of	8.0 mL	500 mL	500 mL

2.5.2 Gel staining techniques

The gel was visualized by silver staining according to Gromova and Celis in 2006 [11]. The gel was fixed for 2 h with 50% methanol, 12% acetic acid, and 0.05% formalin, and then washed with 20% ethanol for 20 min prior to the addition of sensitizing solution [0.02% (w/v) sodium thiosulfate ($Na_2S_2O_3$)]. The gel was immersed in sensitizing solution for 2 min with gentle rotation. Sensitizing solution was discarded and the fixed gel was washed with deionized water twice, 1 min each, and then soaked in staining solution [0.2% (w/v) silver nitrate ($AgNO_3$) and 0.076% formalin] for 20 min with gentle shaking. The staining solution was then poured off and the gel was rinsed with a large volume of deionized water for 20–60 s to remove excess unbound silver ions. Finally, the gel was developed with developing solution [6% (w/v) sodium carbonate (Na_2CO_3), 0.0004% (w/v) sodium thiosulfate ($Na_2S_20_3$), 0.05% formalin] and the reaction was stopped with 12% acetic acid.

2.6 *Antifungal peptide identification*

Antifungal peptide identification was conducted using liquid chromatography-mass spectrometry (LC-MS) by Proteomics International (Australia). Single band from Tricine-SDS-PAGE gel containing the desired antifungal peptide was excised and analyzed. The sample was digested with trypsin and resulting peptides were extracted according to standard techniques [12]. The peptides were analyzed by electrospray ionization mass spectrometry using the Prominence nano HPLC system (Shimadzu, Japan) coupled to a 5600 TripleTOF mass spectrometer (SCIEX, USA). Tryptic peptides analyzed using Agilent Zorbax 300SB-C18, 3.5 µm (Agilent Technologies, USA) and separated with a linear gradient water/acetornitrile/0.1% formic acid (v/v). Spectra were analyzed to identify protein of interest using Mascot sequence matching software (Matrix Science, USA) with MSPnr100 database.

2.7 *Detection of antifungal peptide producing genes*

B. amyloliquefaciens PEP3 genomic DNA was extracted from overnight culture using Wizard® genomic DNA purification kit (Promega, USA). Four primers were used to detect the presence of biosynthetic genes in *B.*

amyloliquefaciens PEP3 based on the genes of *fenD* (fengycin synthetase), *ituD* (iturin A synthetase D), *srfAA* (surfactin synthetase subunit 1) and *lpa-14* (4'-phosphopantetheinyl transferase biosynthesis protein) (Table 3). PCR was carried out using *Swift MiniPro* thermal cycler (Esco, USA), and in a final volume of 25 µL containing 1x PCR buffer, 1.5 mm $MgCl_2$, 0.2 mm dNTP (Invitrogen, USA), 0.2 µm of each primer, 2.0 U of Taq DNA polymerase (Fermentas, Lithuania), and 3 µL of genomic DNA. The PCR cycling program was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing temperature for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were analyzed on 1.0% agarose gel. The amplified DNA fragments were isolated and sequenced by First BASE Laboratories (Malaysia).

2.8 *Expression study on antifungal peptide producing genes*

2.8.1 Total RNA extraction

Total RNA extraction and cDNA synthesis to detect expression of biosynthetic genes were undertaken as described by Leães and co-workers [13]. Total RNA was isolated from overnight cell cultures of *B. amyloliquefaciens* PEP3 using 1 mL of TRIzol reagent (Invitrogen, USA) based on manufactor's instructions, and and analyzed on 1% agarose gel at 90 V for 45 min. Prior to application in further reactions, total RNA extracted were subjected to DNAse (New England Biolabs, USA) treatment.

2.8.2 cDNA synthesis and reverse transcription polymerase chain reaction (RT-PCR)

The total RNA isolated was used to generate cDNA using M-MLV reverse transcriptase (Promega, USA) following the protocol provided by the manufacturer. First strand cDNA was generated using oligo-dT primer. Subsequent PCR to detect the expression of the genes were performed using the same set of primers and PCR conditions as mentioned in section 2.7. The PCR products were visualized on 1% agarose gel at 90 V for 45 min, and subsequently isolated and sequenced by First BASE Laboratories (Malaysia).

3 Results

3.1 *Production and partial purification of antifungal peptide*

Production of antifungal peptide was carried out from LB medium for 72 h. The antifungal peptide was present in the medium as an extracellular product from *B. amyloliquefaciens* PEP3. The precipitate obtained via acidification of cell free supernatant was treated with methanol to extract the antifungal peptide. Approximately 0.07±0.01 g of precipitate containing antifungal peptide was obtained from 100 mL of cell free supernantant. Upscaling production was carried by increasing the volume of LB medium to 2 L resulting in a total of 0.8±0.01 g of precipitate containing antifungal peptide. The precipitate was resuspended in Tris buffer (50 mm, pH 7.5) and stored at –20°C prior to usage.

Table 3: Oligonucleotide primers used to detect the presence of biosynthetic genes in *B. amyloqliquefaciens* PEP3

Expression Product	Primer Name	Primer Sequence (5' to 3')	Product Length (bp)	Annealing Temperature (°C)	Reference
Fengycin	*FEND*-F *FEND*-R	ggcccgttctctaaatccat gctgtccgttctgctttttc	269	60.0	Mora *et al*., 2012
Iturin	*ITUD*-F *ITUD*-R	atgaacaatcttgccttttta ttattttaaaatccgcaatt	1203	50.0	Ali *et al*., 2014
Surfactin	*SRFA*-F *SRFA*-R	tcgggacaggaagacatcat ccactcaaacggataatcctga	201	60.0	Mora *et al*., 2012
4'-Phosphopantetheinyl transferase	SFP-F SFP-R	atgaagatttacggaattta ttataaaagctcttcgtacg	675	46.0	Stanković *et al*., 2012

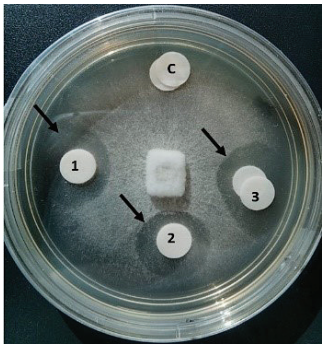

Figure 1: Halo zones from antifungal activity of antifungal precipitate against *P. capsici*.

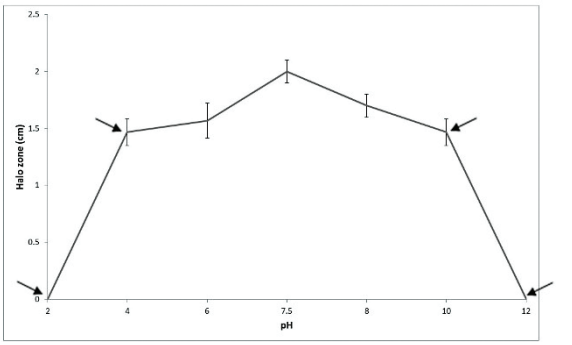

Figure 2: Effect of pH on the activity of the resuspended precipitate containing antifungal peptide. Points marked with an arrowhead are significantly different from control, where pH 7.5 served as control, ($p < 0.05$ for a paired t test). All samples were assayed for halo zone. Error bars represent standard deviation.

3.2 *Antifungal peptide activity assay*

Detection of antifungal peptide activity was carried out using disc diffusion assay. *P. capsici* was used as fungal indicator and the assay was conducted for 7 days. Diameter of inhibition zone was recorded at 2.00 ± 0.10 cm (Figure 1). Discs labelled 1, 2 and 3 were inoculated with 30 µL of resuspended precipitates suspected to contain antifungal peptide, meanwhile disc labelled C (negative control) was inoculated with 30 µL sterile resuspension buffer.

3.3 *Effects of temperature and pH on antifungal activities*

The antifungal activity of resuspended precipitate was tested against a wide range of temperatures and pH. Figure 2 shows the effect of pH on the stability of precipitate, which was determined by measuring the halo zone formed. All samples were adjusted to pH 7.5 with Tris buffer after treatment of different pH prior to assay. For samples treated to pH 6 and pH 8 showed a slight decreased in halo zone formation as compared to the control. Halo zone formation for pH 4 and pH 10 were recorded to be decreased significantly ($p < 0.05$ for a paired t-test). Antifungal activity of the samples were lost when the samples treated with pH 2 and pH 12.

The effect of temperature was determined at six different temperatures. The pH of samples and control were adjusted to pH 7.5 and incubated for 15 minutes before observing the halo zone formation (Figure 3). At the temperatures of 95°C and 121°C, a significant decrease in halo zone formation was observed as compared to the control at 25°C. At pH 7.5, antifungal peptide activity began to lose its activity for temperature beyond 85°C, and a complete loss of activity was detected at 121°C.

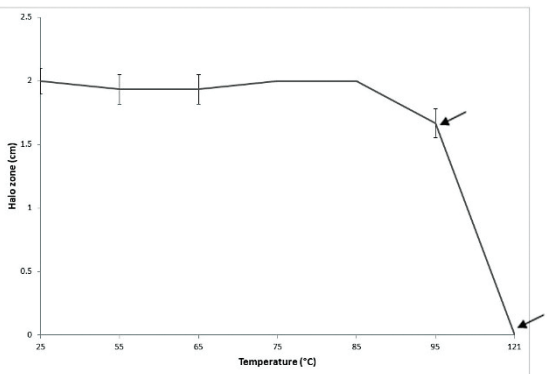

Figure 3: Effect of temperature on the activity of the resuspended precipitate containing antifungal peptide. Points marked with an arrowhead are significantly different from control, where 25°C served as control, ($p < 0.05$ for a paired t test). All samples were assayed for halo zone. Error bars represent standard deviation.

3.4 *Molecular weight determination*

The SDS polyacrylamide gel in Figure 4 indicated an approximation of the molecular weight of the sample. The sample which was suspected to contain antifungal lipopeptides was loaded into the lane labeled as S. The

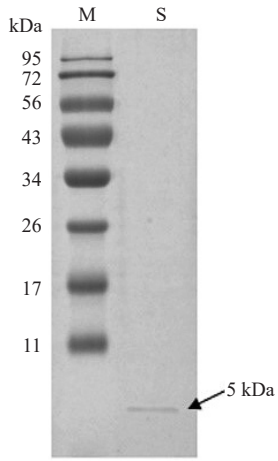

Figure 4: Tricine-SDS-polyacrylamide gel silver-stained. Lane M: BLUeye Prestained Protein Ladder molecular weight marker (kDA); lane S: sample fraction with antifungal activity was separated on the gel.

band was significantly smaller than smallest molecular weight marker (lane M) of 11 kDa. Based on the molecular weight markers, the sample was determined to be approximately 5 kDa.

3.5 *Antifungal peptides identification*

The peptides were analyzed by electrospray ionization mass spectrometry and after trypic digestion, a total of 322 fragments were obtained. Out of these fragments, 37 fragments were found to be identical with other

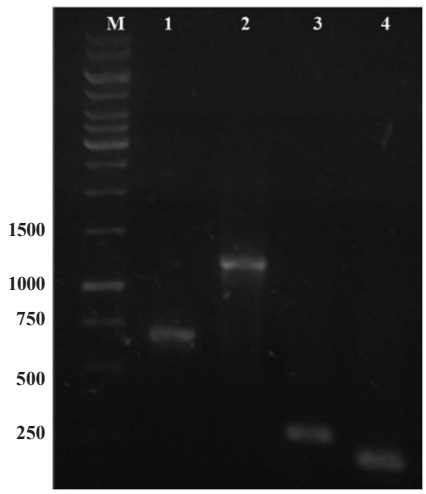

Figure 5: PCR products obtained using genomic DNA with PCR specific primers designed to detect lipopeptides-producing biosynthetic genes in *B. amyloliquefaciens* PEP3. Lane M: 1 kbp DNA Ladder; Lane 1- SFP; Lane 2- *ITUD*; Lane 3- *SRFA*; Lane 4- *FEND*.

fragments of protein from taxonomy of *B. subtilis* by using MSPnr100 database. A total of 31 fragments were found to be significantly identical with protein score higher than 34 ($p > 0.05$) and are derived from ions scores as a non-probabilistic basis to rank protein hits. Although no antifungal peptides were identified, five fragments were found to be associated with antifungal peptide synthesis (Table 4).

Table 4: Summary on peptides identified from the sample

Residue		Peptide(s)	Score	Protein Matched	Accession Number
Start	End				
11	24	IIVDRLGVDEADVK	297	Acyl carrier protein	A0A080UJX1
63	77	IATVGDAVNYIQNQQ			
506	514	FAPLVKEIK	82	Iturin A synthetase	B3TLA1
1297	1307	TVISNASGIR			
1922	1928	DTVEDLR			
84	109	ITSPMVGTFYASSSPEAGPYVNQGSK	75	Acetyl-CoA carboxylase	A0A0A0TYX1
19	41	VKEEEIDAESEMR	59	Polyketide synthase	WP_032863666.1
100	108	TEPSIPS			
901	908	ELGAIETK	54	Polyketide synthase	WP_038463900.1
2233	2241	QKDMLETVR			
3941	3947	LQELLAK			

3.6 *Detection of antifungal peptide producing genes*

Genomic DNA from *B. amyloliquefaciens* PEP3 was subjected to PCR screening using specific primers for the presence of lipopeptide biosynthetic genes *fenD*, *ituD*, *srfAA* and lpa-14 (Figure 5). This indicates that *B. amyloliquefaciens* PEP3 harboring these biosynthesis peptide genes in its genome. BLASTn analysis of contigs from the PCR products revealed that: contig using SFP primers was identified as a part of *Ipa-14* gene, the *FEND* and *SRFA* primers matched with condensation domain of *fenD* and *srfAA* genes, respectively, while *ITUD* primers matched with *ituD* gene.

3.7 *Expression of antifungal peptide producing biosynthetic genes*

Standard conditions for the detection of lipopeptides producing biosynthetic genes were performed by growing *B. amyloliquefaciens* PEP3 for 24 h in LB broth. From the RT-PCR analysis, expression of *fenD* (fengycin D), *ituD* (iturin A), *srfAA* (surfactin) and *Ipa-14* (4'-phosphopantetheinyl transferase) genes was detected. The RT-PCR products were subsequently analyzed via BLASTx program and the targeted genes were confirmed to be present (Table 5).

Table 5: The BLASTx summary of PCR products derived from cDNA

Expression Product	Primer Name	Gene	Overlap-Differences	Accession Number
Fengycin synthetase (partial)	FEND-F FEND-R	*fenD*	88-2	AJZ72674.1
Malonyl CoA-acyl carrier protein transacylase	ITUD-F ITUD-R	*ituD*	348-2	WP_014418049.1
Surfactin A synthetase	SRFA-F SRFA-R	*srfAA*	43-1	WP_007409350.1
4'-phospho-pantetheinyl transferase	SFP-F SFP-R	lpa-14	221-3	WP_014416880.1

4 Discussion

Bacillus subtilis, commonly reported as the superior species that exhibits a broad spectrum of antagonism against different plant pathogens. This ability was contributed by its capability to produce different types of antimicrobial compounds. Closely related species, *B. amyloliquefaciens*, has been reported in numerous publications to have the same ability [14]. Antifungal lipopeptides was reported to be the dominant compounds contributing to antagonism activity on fungi [15], [16]. Among the antifungal compounds produced by *Bacillus* species includes: iturins, surfactins, fengycins, mycobacillins, bacillomycins, mycosubtilins and subsporins. In addition, *Bacillus* species are also known to produce siderophore and volatile organic compounds (VOCs). VOCs are low molecular weight compounds that easily evaporates at normal temperature and pressure [17]. The effective components of VOCs varies between different strains. For example, Gao and co-workers (2017) found four antifungal VOCs, pyrzine017, benzothiazole, 4-chloro-3methyl, and phenol-2,4-bis (1,1-dimethylethyl), released by *B. velezensis* ZSY-1 [18]. Meanwhile, Yuan *et al*. (2012) reported that from a total of 36 VOCs detected from *B. amyloliquefaciens* NJN-6, only eleven compounds were reported to completely inhibit fungal growth [19].

Antifungal lipopeptides produced by *Bacillus* species are known to be lipopeptides with small molecular weight and generally in cyclic form. Three major families of antibiotics namely, iturin, fengycin and surfactin are known to be produced by *Bacillus* species [15], [16]. Antifungal activities have been previously reported for iturin and fengycins [20]. Intriguingly, less reports have been reported on fungi toxic effects for surfactin. Kong and co-workers in 2010, reported that *B. lichneformis* N1 was capable of producing two types of lipopeptides, iturin and surfactin, and the capability of surfactin to inhibit tomato gray mold, tomato late blight and pepper anthracnose [21]. In a report by Tendulkar and co-workers, showed that purified surfactin from *B. licheniformis* effectively inhibit the growth of *Magnaporthe grisea*, a rice fungal pathogen [22].

The cell free supernatant of *Bacillus amyloliquefaciens* PEP 3 served as the starting point for the purification of antifungal lipopeptides. Bulk purification of the antifungal lipopeptides was achieved through acidification to pH 2.0. By altering the pH of the cell free supernatant, several compounds were precipated out of the solution as a rusty-brown precipitate, which was collected via centrifugation. Although quantification and application of antifungal lipopeptides can be proceeded by resuspending the

precipitate in appropriate buffer, further purification using organic solvents to extract antifungal lipopeptides were reported to ease the identification and enhanced the antifungal activity of antifungal lipopeptides [23]. According to Yokota and co-workers, 0.089 mg mL^{-1} of iturin was recovered from *B. subtilis* ATCC 21556 grown for 2 days in No.3S medium (1% polypepton S, 1% glucose, 0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$) after further extraction using organic solvents [23].

Antifungal activity of the precipitates suspected to contain antifungal lipopeptides was demonstrated by disc diffusion assay. The antifungal lipopeptides extracted from *B. amyloliquefaciens* PEP3 was observed to have a 2.0 ± 0.1 cm inhibition zone when tested against *P. capsici*. Ji and co-workers (2013) reported purified Iturin A produced by *Bacillus amyloliquefaciens* CNU114001 isolated from mud flat in Korea recorded a 2.67 cm inhibition zone when tested against *P. capsici* [24]. Meanwhile Cho *et al*. (2007) reported a lower level of inhibition was observed from *Paneibacillus polymyxa* GS01 and *Bacillus* sp. GS06 when tested against *P. capsici*, with both of bacteria only recorded 1.32 cm and 0.84 cm, respectively [25].

Antifungal activity was observed for precipitates which were extracted further with methanol. However, direct application of the precipitate resuspended in Tris buffer (50 mm, pH 7.5) did not show any antifungal activity. This could be due to inhibition by other compounds present in the precipitate [23]. Grover and co-workers, conducted a series of additional extractions using different polar and non-polar organic solvents to precipitates recovered from *B. subtilis* after 96 h of incubation, and reported only managed to detect the presence of surfactin and fengycin besides iturin A after further extraction using methanol [26]. The presence of hydrophobic moiety in these compounds enable them to be dissolve in methanol.

To further characterize the antifungal activity, the precipitates were tested for thermal and pH stability. The results showed that antifungal activity was observed and have a small range of pH stability, in the range of pH 6 to 8. Although inhibition were still detected for pH 4 and pH 10, the diameter of inhibition zone were significantly reduced as compared to the control (pH 7.5). Cui and co-workers reported that antifungal lipopeptides produced by *B. licheniformis* to be stable in neutral and alkaline conditions with optimal pH for antifungal activity at pH 8 [27]. The stability test of the antifungal lipopeptides were conducted by treating the lipopeptide to extreme pH range and temperatures. When the antifungal lipopeptide was treated to pH 4 and 10, significant decrease was observed in the diameter of the halo zone. Meanwhile total loss of activity was observed when the lipopeptide was treated to pH 2 and pH 12. Compared with untreated control, the treatment with extreme pH (pH 2 and 12) also led to a large precipitation with a complete loss of the antifungal activity in the soluble phase [28].

For thermal stability, a significant drop in inhibition zone was recorded when the precipitates were heated to 95°C and total loss of activity was observed at 121°C. Decreasing halo zone diameter is contributed to the denaturation of the peptide bonds located at the lipopeptide tail [28]. This is in agreement with most reports where the thermal stability of antifungal lipopeptides starts to decrease from 80–95°C [29]. This is expected as antifungal lipopeptides consisted of fatty acid attached to peptide ring. Bonds between amino acids are susceptible to high temperature, resulting in denaturation. However, some researchers managed to isolate antifungal lipopeptides which could withstand exposure at 100°C [9], [30].

In this work, the molecular weight of the partially purified precipitates with antifungal activity from *B. amyloliquefaciens* PEP3 was approximately 5 kDa. However, the peptide size in this work did not match with the molecular mass of reported antifungal lipopeptide (iturin, surfactin and fengycin), which were around 1.1–1.5 kDa [31]. This phenomenon could be because of lipopeptides aggregation due to the presence of hydrophobic tail on the antifungal lipopeptides that forms a micelle and appear to have a higher molecular mass when visualized on SDS-PAGE [32]. Although this phenomenon causes differences in size, these aggregates are thought to enhance antimicrobial activity and could contribute to their resistance to degradation [29].

Liquid chromatography with electrospray ionization mass spectrometry (LC-MS) was used to detect and identify the antifungal lipopeptides. However, none of the proposed antifungal lipopeptides were detected. Despite no antifungal lipopeptides were identified, LC-MS analysis revealed that several peptide fragments were found to be identical to proteins associated with antifungal lipopeptide synthesis in non-ribosomal peptide synthesis (NRPS). The identified fragments,

among others, matched with acyl carier protein (ACP) and iturin A synthetase. The ACP in the form of 4'-phosphopantetheinyl transferase, is responsible for the activation of non-ribosomal peptide synthetases, including α-aminoadipate reductase (AAR) for lysine biosynthesis and polyketide synthases, that biosynthesize peptide and polyketide secondary metabolites [4].

PCR-based genome mining for biosynthetic gene clusters showed the presence of surfactin, iturin, and fengycin biosynthetic genes in the isolate *B. amyloliquefaciens* PEP3. On top of the presence of these biosynthetis genes, expression study revealed that these genes are activated and translated into functional proteins. These biosynthesis genes, *Ipa-14*, *srf*, *fenD*, and *ituD* encodes for 4-phosphopantetheinyl transferase, surfactin synthetase, fengycin synthetase D and malonyl CoA transacylase, were also found in other species of *Bacillus* [16], [33]. The gene, *Ipa-14* was also detected to be present and expressed by *B. amyloliquefaciens* PEP3 which indicates that *B. amyloliquefaciens* PEP3 is capable of producing antifungal lipopeptides. The absence or inactivation of the gene lead to deactivation of the carrier protein domains of the polyketide synthases, non ribosomal peptide synthases and fatty acid synthases [3], [4].

5 Conclusions

Acid precipitation of cell free liquid supernatant results in brown color precipitates. The precipitates show antifungal activity towards *P. capsici* and to have pH stability in the range of pH 6–8. Loss of activity was observed when the precipitates were exposed to temperature higher than 95°C. The molecular size of the precipitates were approximately 5 kDa. Despite no antifungal lipopeptides were detected via LC-MS, the detection of expression products of the biosynthetic genes indicated that *B. amyloliquefaciens* PEP3 habors the vital biosynthetic genes and capable of producing antifungal lipopeptides. The results suggested that *B. amyloliquefaciens* PEP3 to have the potential to be further developed in biocontrol research.

Acknowledgements

This research project is funded by Malaysia Ministry of Education (MoE) under the Exploratory Research Grant (ERGS/01(02)/852/2012(4)), UNIMAS PhD studentship grant (F07(DPP09)/1161/2014(09)) and in-part by Malaysia Fundamental Research Grant (FRGS/ST03(01)/1069/2013(15)). The authors would also like to thank the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, for providing the research facilities and Malaysia Pepper Board for the fungal stocks.

References

[1] J. E. E. U. Hellberg, M. A. Matilla, and G. P. C. Salmond, "The broad-spectrum antibiotic, zeamine, kills the nematode worm *Caenorhabditis elegans*," *Frontiers in Microbiology*, vol. 6, p. 137, Feb. 2015.

[2] I. A. Abd El Daim, P. Häggblom, M. Karlsson, E. Stenström, and S. Timmusk, "*Paenibacillus polymyxa* A26 Sfp-type PPTase inactivation limits bacterial antagonism against *Fusarium graminearum* but not of *F. culmorum* in kernel assay," *Frontiers in Plant Science*, vol. 6, p. 368, May 2015.

[3] R. Bunet, R. Riclea, L. Laureti, L. Hôtel, C. Paris, J. M. Girardet, D. Spiteller, J. S. Dickschat, P. Leblond, and B. Aigle, "A single Sfp-type phosphopantetheinyl transferase plays a major role in the biosynthesis of PKS and NRPS derived metabolites in *Streptomyces ambofaciens* ATCC23877," *PLoS One*, vol. 9, no. 1, p. e87607, 2014.

[4] N. A. Zainudin, B. Condon, L. De Bruyne, C. Van Poucke, Q. Bi, W. Li, M. Höfte, and B. G. Turgeon, "Virulence, host-selective toxin production, and development of three *Cochliobolus* phytopathogens lacking the Sfp-type 4'-phosphopantetheinyl transferase Ppt1," *Molecular Plant-Microbe Interactions*, vol. 28, no. 10, pp. 1130–1141,Oct. 2015.

[5] M. F. Kota, A. A. S. A. Husaini, S. Lihan, M. H. M. Hussain, and H. A. Roslan, "*In vitro* antagonism of *Phytophthora capsici* and *Fusarium solani* by bacterial isolates from Sarawak," *Malaysian Journal of Microbiology*, vol. 11, no. Special Issue 2, pp. 137–143, 2015.

[6] A. F. de Faria, D. Stéfani, B. G. Vaz, Í. S. Silva, J. S. Garcia, M. N. Eberlin, M. J. Grossman, O. L. Alves, and L. R. Durrant, "Purification and structural characterization of fengycin homologues produced

by *Bacillus subtilis* LSFM-05 grown on raw glycerol," *Journal of Industrial Microbiology & Biotechnology*, vol. 38, no. 7, pp. 863–871, Jul. 2011.

[7] P. Il Kim, J. Ryu, Y. H. Kim, and Y.-T. Chi, "Production of biosurfactant lipopeptides Iturin A, fengycin and surfactin A from *Bacillus subtilis* CMB32 for control of *Colletotrichum gloeosporioides*," *Journal of Microbiology and Biotechnology*, vol. 20, no. 1, pp. 138–145, Jan. 2010.

[8] A. L. Moyne, R. Shelby, T. E. Cleveland, and S. Tuzun, "Bacillomycin D: An iturin with antifungal activity against *Aspergillus flavus*," *Journal of Applied Microbiology*, vol. 90, no. 4, pp. 622–629, Apr. 2001.

[9] S. Zhang, Y.-X. Wang, L.-qiang Meng, J. Li, X.-yu Zhao, X. Cao, X.-Ling Chen, A.-xue Wang, and J.-Fu Li, "Isolation and characterization of antifungal lipopeptides produced by endophytic *Bacillus amyloliquefaciens* TF28," *African Journal of Microbiology Research*, vol. 6, no. 8, pp. 1747–1755, 2012.

[10] H. Schagger, "Tricine-SDS-PAGE," *Nature Protocols*, vol. 1, no. 1, pp. 16–22, 2006.

[11] I. Gromova and J. Celis, "Protein detection in gels by silver staining: A procedure compatible with massspectrometry," in *Cell Biology. 3rd ed.*, Massachusetts: Academic Press, 2006, pp. 219–223.

[12] S. Bringans, S. Eriksen, T. Kendrick, P. Gopalakrishnakone, A. Livk, R. Lock, and R. Lipscombe, "Proteomic analysis of the venom of *Heterometrus longimanus* (Asian black scorpion)," *Proteomics*, vol. 8, no. 5, pp. 1081–1096, Mar. 2008.

[13] F. Leães, R. Velho, D. Caldas, J. Pinto, S. Tsai, and A. Brandelli, "Influence of pH and temperature on the expression of *sboA* and *ituD* genes in *Bacillus* sp. P11," *Antonie Van Leeuwenhoek*, vol. 104, July 2013.

[14] P. Fickers, "Antibiotic compounds from *Bacillus* : Why are they so amazing?," *American Journal of Biochemistry and Biotechnology*, vol. 8, no. 1, pp. 38–43, 2012.

[15] H. Cawoy, D. Debois, L. Franzil, E. De Pauw, P. Thonart, and M. Ongena, "Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*," *Microbial Biotechnology*, vol. 8, no. 2, pp. 281–295, Mar. 2015.

[16] I. Mora, J. Cabrefiga, and E. Montesinos, "Cyclic lipopeptide biosynthetic genes and products, and inhibitory activity of plant-associated *Bacillus against* phytopathogenic bacteria," *PLoS One*, vol. 10, no. 5, p. e0127738, 2015.

[17] A. Gotor-Vila, J. Usall, R. Torres, C. Solsona, and N. Teixidó, "Enhanced shelf-life of the formulated biocontrol agent *Bacillus amyloliquefaciens* CPA-8 combining diverse packaging strategies and storage conditions," *International Journal of Food Microbiology*, vol. 290, pp. 205–213, 2019.

[18] Z. Gao, B. Zhang, H. Liu, J. Han, and Y. Zhang, "Identification of endophytic *Bacillus velezensis* ZSY-1 strain and antifungal activity of its volatile compounds against *Alternaria solani* and *Botrytis cinerea*," *Biological Control*, vol. 105, pp. 27–39, 2017.

[19] J. Yuan, W. Raza, Q. Shen, and Q. Huang, "Antifungal Activity of *Bacillus amyloliquefaciens* NJN-6 Volatile Compounds against *Fusarium oxysporum* f. sp. *cubense*," *Applied and Environmental Microbiology*, vol. 78, no. 16, pp. 5942– 5944, Aug. 2012.

[20] S. P. Chowdhury, J. Uhl, R. Grosch, S. Alquéres, S. Pittroff, K. Dietel, P. Schmitt-Kopplin, R. Borriss, and A. Hartmann, "Cyclic lipopeptides of *Bacillus amyloliquefaciens* subsp. *plantarum* colonizing the lettuce rhizosphere enhance plant defense responses toward the bottom rot pathogen *Rhizoctonia solani*," *Molecular Plant-Microbe Interactions*, vol. 28, no. 9, pp. 984–995, Sep. 2015.

[21] K. Hyun-Gi, K. Jin-Cheol, C. Gyoung-Ja, L. Kwang-Youll, K. Hyun-Ju, H. Eul-Chul, M. Byung-Ju, and L. Seon-Woo, "Production of surfactin and iturin by *bacillus licheniformis* N1 responsible for plant disease control activity," *The Plant Pathology Journal*, vol. 26, no. 2, pp. 170–177, Jun. 2010.

[22] S. R. Tendulkar, Y. K. Saikumari, V. Patel, S. Raghotama, T. K. Munshi, P. Balaram, and B. B. Chattoo, "Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC98, and its effect on phytopathogen *Magnaporthe grisea*," *Journal of Applied Microbiology*, vol. 103, no. 6, pp. 2331–2339, Dec. 2007.

[23] K. Yokota, M. Yatsuda, E. Miwa, and K. Higuchi, "Comparative study on sample preparation methods for the HPLC quantification of iturin from culture supernatant of an antagonistic *Bacillus strain*," *Journal of the International Society for Southeast Asian Agricultural Sciences*, vol. 18, pp. 70–75, Jan. 2012.

[24] S. H. Ji, N. C. Paul, J. X. Deng, Y. S. Kim, B.-S. Yun, and S. H. Yu, "Biocontrol activity of *Bacillus amyloliquefaciens* CNU114001 against fungal plant diseases," *Mycobiology*, vol. 41, no. 4, pp. 234–242, Dec. 2013.

[25] K. M. Cho, S. Y. Hong, S. M. Lee, Y. H. Kim, G. G. Kahng, Y. P. Lim, H. Kim, and H. D. Yun, "Endophytic bacterial communities in ginseng and their antifungal activity against pathogens," *Microbial Ecology*, vol. 54, no. 2, pp. 341–351, Aug. 2007.

[26] M. Grover, L. Nain, S. B. Singh, and A. K. Saxena, "Molecular and biochemical approaches for characterization of antifungal trait of a potent biocontrol agent *Bacillus subtilis* RP24," *Current Microbiology*, vol. 60, no. 2, pp. 99–106, Feb. 2010.

[27] T.-B. Cui, H.-Y. Chai, and L.-X. Jiang, "Isolation and partial characterization of an antifungal protein produced by *Bacillus licheniformis* BS-3," *Molecules*, vol. 17, no. 6, pp. 7336–7347, Jun. 2012.

[28] M. Gong, J. D. Wang, J. Zhang, H. Yang, X. F. Lu, Y. Pei, and J. Q. Cheng, "Study of the antifungal ability of *Bacillus subtilis* strain PY-1 *in vitro* and identification of its antifungal substance (iturin A)," *Acta Biochimica et Biophysica Sinica (Shanghai)*, vol. 38, no. 4, pp. 233–240, Apr. 2006.

[29] D. Lin and A. Grossfield, "Thermodynamics of micelle formation and membrane fusion modulate antimicrobial lipopeptide activity," *Biophysical Journal*, vol. 109, no. 4, pp. 750–759, Aug. 2015.

[30] H. B. Ayed, N. Hmidet, M. Béchet, M. Chollet, G. Chataigné, V. Leclère, P. Jacques, and M. Nasri, "Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21," *Process Biochemistry*, vol. 49, no. 10, pp. 1699–1707, 2014.

[31] S. Kaewklom, S. Lumlert, W. Kraikul, and R. Aunpad, "Control of *Listeria monocytogenes* on sliced bologna sausage using a novel bacteriocin, amysin, produced by *Bacillus amyloliquefaciens* isolated from Thai shrimp paste (Kapi)," *Food Control*, vol. 32, no. 2, pp. 552–557, 2013.

[32] S. Sur and A. Grossfield, "Understanding the function of the cyclic antifungal lipopeptide fengycin using all-atom Md simulation," *Biophysical Journal*, vol. 108, no. 2, p. 84a, 2015.

[33] M. K. Solanki, R. K. Singh, S. Srivastava, S. Kumar, P. L. Kashyap, and A. K. Srivastava, "Characterization of antagonistic-potential of two *Bacillus strains* and their biocontrol activity against *Rhizoctonia solani* in tomato," *Journal of Basic Microbiology*, vol. 55, no. 1, pp. 82–90, Jan. 2015.