

# Biocontrol of Orchid-pathogenic Mold, *Phytophthora palmivora*, by Antifungal Proteins from *Pseudomonas aeruginosa* RS1

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## ABSTRACT

Black rot disease in orchids is caused by the water mold *Phytophthora palmivora*. To gain better biocontrol performance, several factors affecting growth and antifungal substance production by *Pseudomonas aeruginosa* RS1 were verified. These factors include type and pH of media, temperature, and time for antifungal production. The results showed that the best conditions for *P. aeruginosa* RS1 to produce the active compounds was cultivating the bacteria in Luria-Bertani medium at pH 7.0 for 21 h at 37 °C. The culture filtrate was subjected to stepwise ammonium sulfate precipitation. The precipitated proteins from the 40% to 80% fraction showed antifungal activity and were further purified by column chromatography. The eluted proteins from fractions 9–10 and 33–34 had the highest antifungal activity at about 75% and 82% inhibition, respectively. SDS-PAGE revealed that the 9–10 fraction contained mixed proteins with molecular weights of 54 kDa, 32 kDa, and 20 kDa, while the 33–34 fraction contained mixed proteins with molecular weights of 40 kDa, 32 kDa, and 29 kDa. Each band of the proteins was analyzed by LC/MS to identify the protein. The result from Spectrum Modeler indicated that these proteins were closed similarly to three groups of the following proteins; catalase, chitin binding protein, and protease. Morphological study under scanning electron microscopy demonstrated that the partially purified proteins from *P. aeruginosa* RS1 caused abnormal growth and hypha elongation in *P. palmivora*. The bacteria and/or these proteins may be useful for controlling black rot disease caused by *P. palmivora* in orchid orchards.

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## 1. Introduction

Synthetic chemical fungicide application is the most popular method used to reduce the incidence of plant diseases caused by fungi and molds. However, using chemical agents has several negative impacts such as the chemical residues that remain in environment, the reduction of beneficial soil microorganisms, the physiological adaptation of pathogens to resist the chemical fungicide, and the direct exposure to chemical mixtures on human health [1,2]. Owing to recent concerns regarding the impact of chemical fungicides on the environment and human health, natural bioactive compounds as well as biological control agents are being used more often instead of agrochemicals [3]. In addition, the biological control method was reported to be an eco-friendly and safe approach to control plant pathogens, thus is now used as an alternative method to prevent and/or protect plants from diseases [4]. Various beneficial microorganisms have been used as biocontrol agents against fungal and mold plant pathogens [5]. Good examples of microorganisms that are able to antagonize the growth of

phytopathogenic fungi and mold include many species of bacteria in the genera *Streptomyces*, *Bacillus*, and *Pseudomonas* [6–8].

Numerous bacterial biocontrol agents have been manufactured using *Pseudomonas* spp., some of which have become commercially available [9,10]. *Pseudomonas* spp. can produce many kinds of extracellular biofungicides. For example, *Pseudomonas fluorescens* produces volatile 1-undecene to inhibit *Phytophthora infestans* [11,12], *Pseudomonas aeruginosa* SS14 produces biosurfactant against *Fusarium verticillioides* [13], *P. fluorescens* produces a cyclic lipopeptide that suppresses *Gaeumannomyces graminis* var. *tritici* and *Rhizoctonia solani* [14], and *P. aeruginosa* produces pafungin to inhibit *Fusarium oxysporum* [15]. Several *Pseudomonas* spp. are also reported to produce antifungal proteins as biocontrol substances. For example, two bifunctional chitinases/lysozymes from *P. aeruginosa* K-187 have shown antifungal activity against 36 strains of fungi [9], and protease from *P. aeruginosa* has demonstrated antifungal activity by inhibiting spore germination and hyphal elongation of *Fusarium solani* [16].

Orchidaceae is not only the largest flowering plant family but also produces the greatest floral diversity of all Angiosperms [17]. Orchids are found in several countries worldwide such as Netherlands, Thailand, Taiwan, Singapore, and New Zealand [18]. In Thailand, the orchid export industry generated around US\$50 million in 2001 [19]. However, the orchid industry suffers financially from poor crop yields due to the problem of plant diseases. Most orchid diseases are acquired through infection from bacteria [20], viruses [21], and fungi [22]. Thus, controlling disease is an important practice for maintaining the quality of orchids.

Fungal and mold pathogens cause several diseases in orchids, such as *Curvularia lunata*, causes rust spot disease on the flowers [23], *C. eragrostidis* causes rusty flower disease [24], *Fusarium moniliforme* causes column blight disease, *Fusarium solani* causes leaf yellowing and root and collar rot diseases [25], *Fusarium oxysporum* causes wilt disease, *Sclerotium rolfsii* causes collar rot disease, *Phoma exigua* causes flower spot disease, *Colletotrichum gloeosporioides* causes anthracnose disease [26], and *Phytophthora palmivora* causes black rot disease [23]. *P. palmivora* is considered a water mold that causes black rot disease in wide variety of orchids including *Aerides*, *Ascocenda*, *Brassavola*, *Dendrobium*, *Gongora*, *Maxillaria*, *Miltonia*, *Oncidium*, *Paphiopedilum*, *Phalaenopsis*, *Rhynchostylis*, and *Schomburgkia* [27]. After infection, orchids initially display small black lesions on the root or basal portion of the pseudobulbs, afterwards the black lesions expand to completely cover the pseudobulbs and leaves [27]. The mold spreads and infects other parts of the orchid until the orchid dies.

In this research, the natural antagonistic bacteria that inhibit the growth of *P. palmivora* were selected. The antifungal proteins were purified and then identified by LC-mass spectrometry.

## 2. Materials and methods

### 2.1. Microorganisms and culture media

*P. aeruginosa* RS1 (GenBank accession number MF344551) from the culture collection of Department of Microbiology, Faculty of Science, Chulalongkorn University under Thailand Bioresource Research Center TBRC as MSCU 0860 which was found to inhibit the growth of *P. palmivora*, was maintained on a nutrient agar (NA) plate (0.5% peptone, 0.3% beef extract and 1.5% agar) at 37 °C.

*Phytophthora palmivora* (DOAC2057) which was obtained from the Department of Agriculture (Bangkok, Thailand) was cultured on a V8 agar plate

(5% V8 vegetable juice (original) and 1.5% agar) at 26 °C.

Inhibition of *P. palmivora* by *P. aeruginosa* RS1 was performed by inoculating an agar plug of *P. palmivora* at the center of the V8 agar plate, and dropping the suspended bacteria next to the fungal plug. The plate was incubated at 26 °C for 2 days. The vegetative growth of *P. palmivora* was measured by comparing it with the growth of the mold growing without bacteria. The percentage inhibition was calculated using formula  $IP = (C - T/C) * 100$ ; where IP = inhibitory percentage, C = average diameter of *P. palmivora* from the control, and T = average diameter from the test plate [28]. Mycelia at the periphery of the colony from both conditions were observed under light microscope.

### 2.2. Optimization conditions for producing antifungal substances from *P. aeruginosa* RS1

*P. aeruginosa* RS1 was cultured in nutrient broth (NB) at 37 °C until the optical density (OD) at 660 nm reached about 0.5. A 1% inoculum of the culture was added to nutrient broth (NB), Luria-Bertani (LB) broth, and tryptic soy broth (TSB); all at pH 6. The culture was incubated at 37 °C for 18 h with shaking at 200 rpm. The culture was then centrifuged at 4 °C, 5870 × g for 15 min to obtain a supernatant. The supernatant was tested for antifungal activity as described below. The medium that showed the highest antifungal activity was selected for pH optimization, which included pH 6, 7, 8, and 9. The bacteria were cultured in the optimum medium and pH at different temperatures; 30 °C, 37 °C, and 40 °C. After the optimum temperature was obtained, the optimum cultivation time was determined.

### 2.3. Purification of the antifungal proteins

*P. aeruginosa* RS1 was cultured under optimum conditions and the supernatant was obtained. The antifungal proteins were then precipitated by ammonium sulfate precipitation followed by anion exchange chromatography. Briefly, the proteins in the *P. aeruginosa* RS1 supernatant were precipitated by the ammonium sulfate concentration in two steps; 0–40% and 40–80%. The protein pellet was dissolved with the minimum volume of 50 mM Tris-HCl buffer at pH 7.5 and dialyzed three times (5 h each) using a dialysis bag with the cutoff at 3500 (SnakeSkin Dialysis Tubing, Thermo Fisher Scientific, Rockford, IL) against 50 mM Tris-HCl buffer at pH 7.5. The final step of dialysis was performed in the same buffer which contained 30% of glycerol. The crude protein from the ammonium sulfate precipitation step was applied to a DEAE-

cellulose column that pre-equilibrated with 50 mM Tris-HCl buffer at pH 7.5. The unbound protein fractions were collected and the bound protein fractions were eluted with a linear gradient of 0.0–1.0 M NaCl in the same buffer. All fractions were monitored at 280 nm for the presence of proteins. Two eluted fractions were combined to decrease the sample numbers during the antifungal activity assay. Antifungal activity was calculated as an Arbitrary Unit (AU) defined as the highest dilution of the protein that yielded a clear inhibition zone against *P. palmivora*.

#### 2.4. Electrophoresis and LC mass spectrometry

The protein from ammonium sulfate precipitation and the protein fractions from the DEAE-cellulose column were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli and Favre [29]. A page ruler prestained protein ladder (10–170 kDa) (Thermo Fisher Scientific, Rockford, IL) was used as molecular weight marker. Identification of purified proteins was accomplished by liquid chromatography equipped with Q Exactive™ Plus hybrid quadrupole-orbitrap mass spectrometry (LC/MS) (Thermo Fisher Scientific, Rockford, IL) at the Proteomics Core Facility, Faculty of Medicine, Chulalongkorn University (Bangkok, Thailand). Data analysis was performed by using an X! TANDEM Spectrum Modeler (<http://www.thegpm.org>) against the Global Proteome Machine Database. Searches were run with trypsin as the cleaving enzyme with a maximum of one missed cleavage, carbamidomethyl cysteine as a fixed modification, and oxidation of methionine as the potential variable modification.

#### 2.5. Antifungal activity assay

The supernatant which was collected from each condition was filtered through a 0.45 µm membrane filter to remove cells. The antifungal activity was inspected by pour plate method [30] using a 1:9 ratio of supernatant to V8 media for all experiments, except the protein fractions from ammonium sulfate precipitation, which used a 1:1 ratio. The agar plug

with a 6-mm diameter of *P. palmivora* was placed at the center of each plate, and the plate was incubated at 26 °C for 48 h. The control plate contained the fungus growing on the media with only the V8 agar. The vegetative growth of the fungus was measured, and the percentage of inhibition was calculated by using formula provided above. The experiments were replicated four times.

#### 2.6. Microscopy

The effect of purified protein to *P. palmivora* was examined under optical microscope and scanning electron microscope (SEM). The result was compared with the control *P. palmivora* growing without bacterial protein.

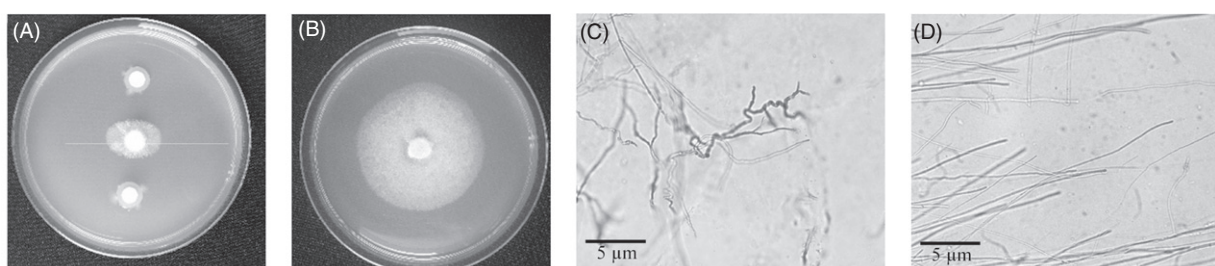
### 3. Results

#### 3.1. Antagonistic activity of *P. aeruginosa* RS1 against *P. Palmivora*

*Pseudomonas aeruginosa* RS1 was shown to inhibit the growth of *P. palmivora* on a V8 agar plate as demonstrated in Figure 1(A) comparing to the control in Figure 1(B). The inhibition efficiency was around 50%. Observation under microscope revealed malformed hyphae of *P. palmivora* growing with *P. aeruginosa* RS1, while a normal appearance with smooth elongate hyphae was observed in *P. palmivora* growing alone (Figure 1(C) and 1(D), respectively).

#### 3.2. Optimization conditions for *P. aeruginosa* RS1 to produce antifungal substances

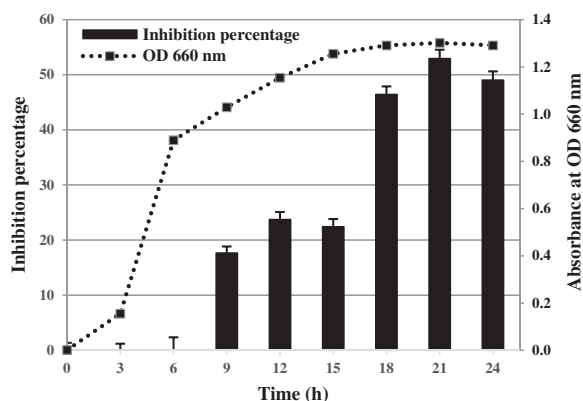
*Pseudomonas aeruginosa* RS1 was cultured in different media (LB, NB and TSB) and the culture filtrate to medium ratio of 1:9 was used to determine the antifungal activity on *P. palmivora*. The result showed that the highest inhibition activity was obtained from cultivation in LB, follow by NB and TSB with the percentage inhibition at  $53.33 \pm 2.72\%$ ,  $29.03 \pm 2.63\%$ , and  $13.33 \pm 3.85\%$ , respectively (Table 1). Bacteria were cultured in LB medium with pH levels at 6, 7, 8, and 9. The result revealed that LB medium at pH 7 demonstrated



**Figure 1.** Inhibition of *P. palmivora* by *P. aeruginosa* RS1 on a V8 agar plate (A) and control growth of the mold without the bacteria (B). Microscopic observation (400×) of the hyphae of *P. palmivora* growing with (C) and without (D) *P. aeruginosa* RS1.

**Table 1.** Percentage of inhibition by culture filtrate from *P. aeruginosa* RS1 at different variable factors.

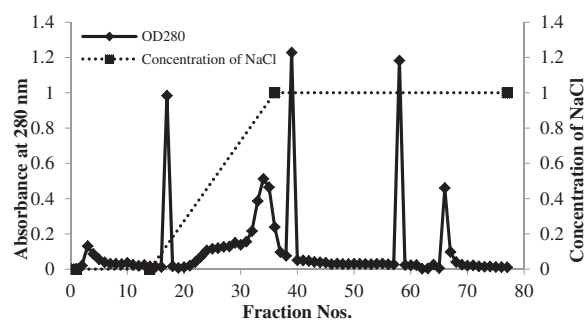
Variable factors	Conditions	% Inhibition
Media	NB, pH7	29.03 ± 2.63
	LB, pH7	53.33 ± 2.72
	TSB, pH7	13.33 ± 3.85
pH	LB, pH6	48.57 ± 2.33
	LB, pH7	52.63 ± 2.15
	LB, pH8	32.35 ± 2.40
	LB, pH9	22.58 ± 2.63
Temperature	LB, pH7, 30 °C	41.94 ± 2.63
	LB, pH7, 37 °C	54.84 ± 3.72
	LB, pH7, 40 °C	35.48 ± 2.63
Ammonium sulfate	0–40%	0.00 ± 2.55
	40–80%	100.00 ± 0.00
	80–100%	0.00 ± 2.55

**Figure 2.** Inhibition percentage against *P. palmivora* and absorbance at OD<sub>660 nm</sub> of *P. aeruginosa* RS1 in LB medium (pH 7) at 37 °C, at time points measured every 3 h, 24 h.

52.63 ± 2.15% inhibition, which was better than at pH 6, pH 8, and pH 9 (48.57 ± 2.33%, 32.35 ± 2.40%, and 22.58 ± 2.63% inhibition, respectively) (Table 1). After bacteria were cultured in LB medium at pH 7 at different temperatures; 30 °C, 37 °C, or 40 °C; it was found that the best inhibition activity was observed at 37 °C with the inhibition percentage at 54.84 ± 3.72% (Table 1). A time course experiment was performed at this optimum condition, which demonstrated that the growth of *P. aeruginosa* RS1 entered a logarithmic phase at 3–6 h, and a late logarithmic phase at 6–15 h, after which the growth gradually reduced and entered a stationary phase at about 18 h (Figure 2). The culture filtrate started to show antifungal activity from 9 h of growth, and reached the highest activity at 21 h, which yielded an inhibition percentage of about 52.94 ± 1.60% (Figure 2).

### 3.3. Purification and electrophoresis of the antifungal proteins

The antifungal protein from *P. aeruginosa* RS1 was purified by ammonium sulfate precipitation and DEAE-cellulose chromatography. The crude protein from ammonium sulfate precipitation at 40–80% inhibited the growth of *P. palmivora* up to 100% (Table 1). The protein from this fraction was further

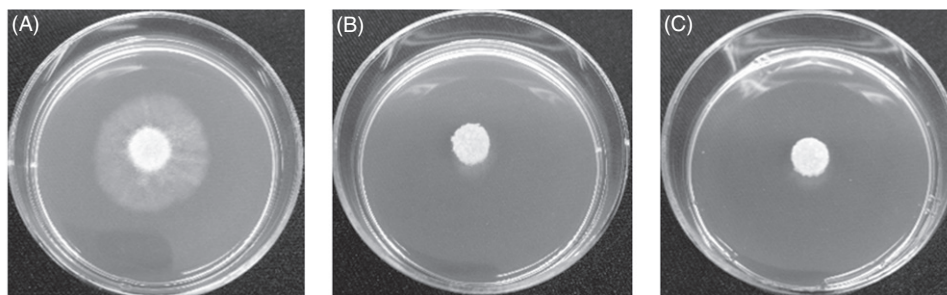
**Figure 3.** Chromatogram from DEAE-cellulose column chromatography indicates the absorbance at OD<sub>280 nm</sub> of the proteins eluting from the column using gradient concentrations of NaCl.

purified by DEAE-cellulose column chromatography, and the chromatogram is shown in Figure 3. Several protein peaks were obtained. Every protein fraction peak showed antifungal activity ranging from 27.94–82.35% (data not shown). The protein from fractions 9–10 and 33–34 exhibited the highest antifungal activity at about 75 ± 2.08% and 82.35 ± 2.08% inhibition, respectively (Figure 4). The 9–10 fraction was eluted before adding the gradient of NaCl, while the 33–34 fraction was eluted during about 0.7–0.9 M NaCl (Figure 3).

As shown in Table 2, the culture filtrate, 40–80% ammonium sulfate fraction, DEAE fraction 9–10, and DEAE fraction 33–34 yielded total activity of 8000, 384, 8.4, and 16 AU, respectively. The ion exchange chromatography resulted in 11.25 and 1.67 fold increases in purification of the proteins from the 9–10 and 33–34 fractions, respectively. These fractions revealed the specific activity of 250 AU/mg and 37.04 AU/mg, respectively. SDS-PAGE confirmed the presence of proteins from these fractions. Fewer protein bands appeared in the DEAE-cellulose column chromatography step compared with those in 40–80% ammonium sulfate precipitation step (Figure 5). Some bands were concentrated after the DEAE-cellulose column chromatography step because those bands did not appear in the ammonium sulfate fraction. The results also demonstrated that the proteins in DEAE fraction 9–10 and DEAE fraction 33–34 were partially purified because several polypeptide bands were observed. At least three bands appeared in DEAE fraction 9–10 with molecular weights of about 54 kDa, 32 kDa, and 20 kDa, respectively (Figure 5, #1–3); whereas at least three bands appeared in DEAE fraction 33–34 with molecular weights of about 40 kDa, 32 kDa, and 29 kDa, respectively (Figure 5, #4–6).

### 3.4. Identification of antifungal proteins with LC mass spectrometry analysis

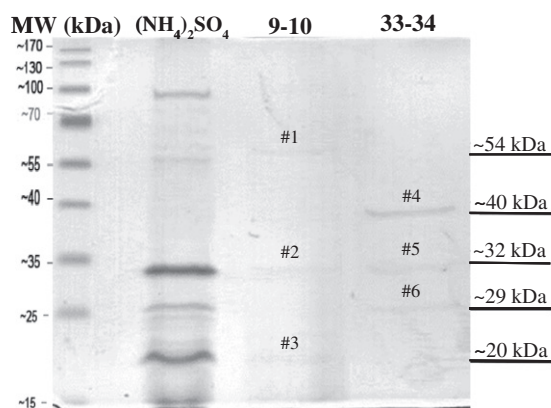
All six protein bands (#1–6) were excised from the gel and subjected to protein identification by



**Figure 4.** Inhibition of *P. palmivora* by eluted proteins from DEAE-cellulose column chromatography. *P. palmivora* grew on a V8 agar plate: control without protein (A), with protein fraction 9–10 (B), and with protein fraction 33–34.

**Table 2.** Summary for purification of the antifungal protein from *P. aeruginosa* RS1.

Sample	Volume (ml)	Protein (ng/ml)	Total protein (mg)	Activity (AU/ml)	Specific activity (AU/mg protein)	Total activity (AU)	Purification (fold)
Culture filtrate	5000.00	72.00	360.00	1.60	22.22	8000.00	1.00
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 40–80%	24.00	718.00	17.232	16.00	22.28	384.00	1.00
DEAE #9–10	4.20	8.00	0.0336	2.00	250.00	8.40	11.25
DEAE #33–34	4.00	108.00	0.432	4.00	37.04	16.00	1.67



**Figure 5.** SDS-PAGE of partial purification of the antifungal proteins from the ammonium sulfate precipitation and anion exchange chromatography steps. The labels 9–10 and 33–34 refer to the proteins from DEAE fractions 9–10 and 33–34, respectively.

LC/MS. The data obtained were compared with the Global Proteome Machine Database, and the results of each protein band are reported in Table 3. Protein band #1–3 which was obtained from fraction 9–10 showed high similarity to catalase, protease, and protease IV, with a protein coverage of 65%, 53%, and 40%, respectively. Protein band #4–6 from fraction 33–34 showed high similarity to chitin binding domain, protease, and protease with protein coverage of 86%, 53%, and 33%, respectively. These matched proteins belonged to the proteins from *Pseudomonas aeruginosa* LESB58.

### 3.5. Microscopy

Morphology of *P. palmivora* after incubation with purified antifungal protein for 2 days was observed under SEM. The result revealed that the hyphae of the control *P. palmivora* without the purified protein had straight and smooth hyphae (Figure 6(A)).

However, *P. palmivora* grown with both the purified protein from fractions 9–10 and 33–34 had abnormal growth showing short branched, rugous, and swollen hyphal tips (Figure 6(B) and 6(C), respectively).

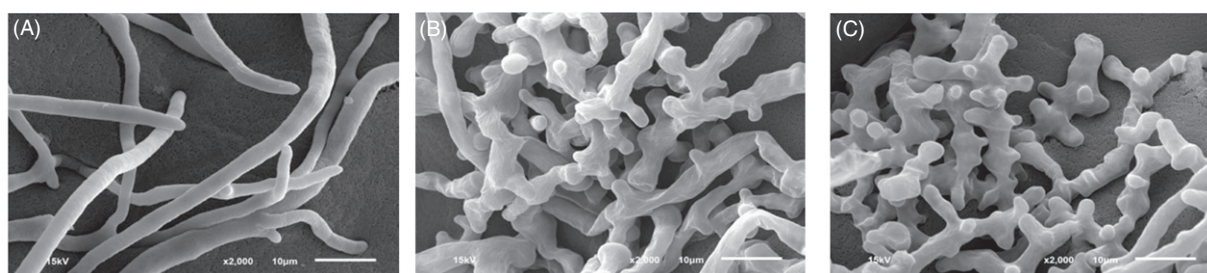
## 4. Discussion

In this research, to obtain high antifungal activity against *P. palmivora* by *P. aeruginosa* RS1, cultivation conditions for the bacteria including type of medium, pH, temperature, and time-course were optimized. The antifungal proteins were purified from supernatant of the bacteria by ammonium sulfate precipitation, followed by DEAE-column chromatography, and identified by LC/MS. This work demonstrates that *P. aeruginosa* RS1 is capable of producing several anti-*P. palmivora* proteins that may be useful for controlling black rot disease caused by *P. palmivora* in orchid orchards.

The results revealed that varying the type of culture medium was highly influential to the antifungal activity, while the variation of pH and temperature showed less affect. In our study, LB was found to be the best medium for producing the antifungal substance as compared with TSB and NB at 4 and 1.8 fold, respectively. The difference in inhibition efficiency also indicated that media composition has an effect on anti-*P. palmivora* metabolite production. Although generally, TSB is the richest nutrient composition medium when compared with NB and LB media, and can well support the growth of several bacteria, TSB and NB may interfere with antifungal production as previously observed [31,32]. The original conditions, pH 7 and 37 °C, seemed to be optimal and demonstrated the highest percentage of inhibition when compared with other tested conditions. Thus, the optimal condition for producing an antifungal agent by *P. aeruginosa* RS1 in

**Table 3.** Identification of each protein band from anion exchange chromatography fractions 9–10 and 33–34.

Sample	Log (e)	Protein coverage	No. of unique peptide found	Molecular weight (kDa)	Accession No.	Protein	Organism
Fraction 9–10, #1	–737.5	65%	64	55.6	gi   218889435	Catalase	<i>Pseudomonas aeruginosa</i> LESB58
Fraction 9–10, #2	–1881.8	53%	145	53.7	gi   218890000	Protease	<i>Pseudomonas aeruginosa</i> LESB58
Fraction 9–10, #3	–1349.0	40%	99	48.2	gi   218889496	Piv gene product (protease IV)	<i>Pseudomonas aeruginosa</i> LESB58
Fraction 33–34, #4	–1648.1	86%	128	41.9	gi   218893181	Chitin binding protein	<i>Pseudomonas aeruginosa</i> LESB58
Fraction 33–34, #5	–1881.8	53%	145	53.7	gi   218890000	Protease	<i>Pseudomonas aeruginosa</i> LESB58
Fraction 33–34, #6	–864.7	33%	68	45.5	gi   218892171	Protease	<i>Pseudomonas aeruginosa</i> LESB58

**Figure 6.** Morphology of *P. palmivora* under scanning electron microscope (2000×) after incubation for 2 days without (A) and with the purified antifungal protein from DEAE-cellulose column chromatography at fractions 9–10 (B) and 33–34 (C).

this study was to culture the bacteria in LB medium at pH 7 and at 37°C for 21 h. The bioactive antifungal compounds were non-growth associated products since the activity was appeared in the late logarithmic phase of *P. aeruginosa* RS1, which are similar to most metabolites produced by bacterial biocontrol agents.

The purification of antifungal proteins in this research consisted of two steps including ammonium sulfate precipitation and DEAE ion exchange chromatography. The 40–80% ammonium sulfate saturation contained antifungal proteins. The proteins in this fraction were purified further by DEAE-cellulose column chromatography. Several pools of eluted proteins retained antifungal activity, which indicated that there were several types of antifungal proteins produced by *P. aeruginosa* RS1. The highest activity was obtained from fractions 9–10 and 33–34 with the inhibition of  $75.00 \pm 2.08\%$  and  $82.35 \pm 2.08\%$ , respectively. The proteins in fraction 9–10 were eluted before starting the gradient of NaCl, while the proteins in fraction 33–34 were eluted at about 0.7–0.9 M gradient of NaCl. These results indicated that the proteins in fraction 9–10 have no negative charge with which to bind a positive ion onto DEAE, whereas the proteins in fraction 33–34 have a strong negative charge [33–35]. SDS-PAGE indicated that there were three apparent protein bands from each pooled fraction. Bands #1–3 were from fraction 9–10 and bands #4–6 were from fraction 33–34. LC/MS revealed that these proteins were closed similarly to catalase, protease and chitin binding proteins.

Catalase is an enzyme that is produced by variety of organisms exposed to oxygen. It catalyzes the

decomposition of hydrogen peroxide to water and oxygen [36]. Catalase was reported as antifungal substance, for example, the *Bacillus subtilis* strain LEV-006 showed antifungal activity against *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Alternaria brassicae*, and *Leptosphaeria maculans*. One antifungal protein was found to be similar to vegetative catalase 1; however, expression of catalase in *Escherichia coli* showed catalase activity but no antifungal activity [37]. Thus, the antifungal mechanism of catalase is still unknown and needs to be clarified.

Proteases can be found in animals, plants, fungi, bacteria, archaea, and viruses. They are also called peptidases or proteinases, and are any enzyme that breaks down proteins into smaller polypeptides or amino acids by hydrolysis of peptide bonds. The different classes of protease illustrate the same reaction by completely different catalytic mechanisms [38]. In antifungal applications, alkaline antifungal protease was discovered from *B. subtilis* strain N7. The enzyme completely inhibited spore and hyphae growth of *Fusarium oxysporum* f. sp. *cucumerinum* by hydrolysis and loss of integrity of the fungal cell wall [39]. Based on SEM, it appeared that the action of the partial purified proteins occurred during hypha development or cell wall synthesis. In case of *Phytophthora*, cell wall compositions were composed of 80–90% glucan, 5–10% protein, and 1–2% lipids [40]. In addition, cell-wall-associated proteins were also identified in this oomycete mold, and these proteins were proposed to be important for pathogenicity [41]. The antifungal proteins of the protein bands #2, #3, #5, and #6 may individually or in

combination act on the proteins on the cell wall of *P. palmivora*.

The secretion of several chitinolytic enzymes and chitin-binding proteins has been found in the extracellular environment of several species of *Serratia*, *Bacillus*, *Pseudomonas*, and *Vibrio* [42]. The functions of chitinolytic enzymes and chitin-binding proteins are to degrade chitin on the cell wall of fungi. It was reported that proteins that consisted of only chitin-binding domains revealed fungal growth inhibition [43]. In addition, the chitin-binding protein of *P. aeruginosa* strain PAO25 has been reported to secrete chitin-binding proteins and have antifungal activity against *R. solani* and *F. oxysporum* [43]. Although it was reported that the cell wall of oomycete molds does not contain chitin, recent research has found that these water molds could temporarily produce chitin at the hyphal tips before converting it to cellulose [44]. Thus, the chitin binding domain of protein band #4 could act on chitin at this stage. It is also possible that the chitinolytic activity alone may not directly affect the growth of *P. palmivora* and requires a synergistic action of other bioactive compounds to be effective [45,46].

In this study, it was demonstrated that *P. aeruginosa* RS1 produced several proteins that can inhibit the growth of *P. palmivora*. Although the mode of action of each purified protein needs to be verified, these mixed proteins can also be used to obtain synergistic antifungal actions. Recent study reported that *P. aeruginosa* strain JO and strain JO7 produced  $\beta$ -1,3-glucanase, cellulase, protease, and chitinase. These production of several wall lytic enzymes attributed to high antifungal efficiency against *F. oxysporum* and *Alternaria solani* [47].

Several *P. aeruginosa* strains were found to produce antifungal substances against both human and plant pathogenic fungi. For instance, several derivatives of phenazine were produced by *P. aeruginosa*, which had antifungal activity towards human pathogenic fungi such as *Candida albicans* [48]. *Pseudomonas aeruginosa* K-187 produced pafungin that affected *F. oxysporum*, causing swelling and lysis of the fungal hyphae, which subsequently inhibited damping-off disease in alfalfa [15]. Generally, due to public concern regarding human health and environment, *P. aeruginosa* has not been widely accepted as a safe biocontrol agent in agriculture. However, non-pathogenic *P. aeruginosa* strains ATCC 15442 with noninvasive and noncytotoxic properties has been reported [49]. Therefore, genome sequencing as well as these properties of *P. aeruginosa* RS1 would suggest further for its public concern issue. Nevertheless, its cell-free metabolites would be an alternative approach in agriculture to inhibit the growth of *P. palmivora*, the causal pathogen of orchids.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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