



# Utilizing Waste from Palm Oil Mills as a Substrate to Produce Antimicrobial Agents Using Fermentation by *Streptomyces philanthi* RM-1-138: Effectiveness, Stability, and Compound Identification

Sawai Boukaew<sup>1</sup> · Poonsuk Prasertsan<sup>2</sup> · Teera Chookaew<sup>3</sup> · Benjamas Cheirsilp<sup>4</sup>

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## Abstract

Antimicrobial agents were produced through submerged and solid substrate fermentation using palm oil mill effluent (POME) and oil palm decanter cake (OPDC) as substrates, respectively. This study aimed to compare the effectiveness of extracts from *Streptomyces philanthi* RM-1-138 that were obtained from POME and OPDC culture against three strains of bacterial plant pathogens and three strains of oil palm pathogenic fungi. The antimicrobial agents produced in the POME medium could inhibit these pathogens better than those produced in the OPDC medium. The antimicrobial agents exhibited strong antimicrobial activity against *Xanthomonas axonopodis* pv. *glycines* and *X. campestris* pv. *campestris* (MIC 16 µg ml<sup>-1</sup>) and *Curvularia oryzae* and *Ganoderma boninense* (MIC 128 µg ml<sup>-1</sup>). The effect of temperature (–20 to 121 °C for 15 min) and pH (4.0–8.0) on the stability of the antimicrobial agents against *C. oryzae* was investigated. The antimicrobial agents had excellent stability at the highest temperature and a wide range of pH tests. Identification of these antimicrobial agents produced in POME and OPDC substrate using LC-Q-TOF MS/MS revealed 8 and 6 compounds, respectively. These compounds could be functioned as fungicides (oxadixyl), herbicides (chloreturon), insecticides (isoprocarb and xylylcarb), and antibiotics (anisomycin). Therefore, the POME and OPDC mediums has shown promising potential for being utilized as substitute substrates in the production of antimicrobial agents from *S. philanthi* RM-1-138.

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✉ Sawai Boukaew  
sawai.bo@skru.ac.th

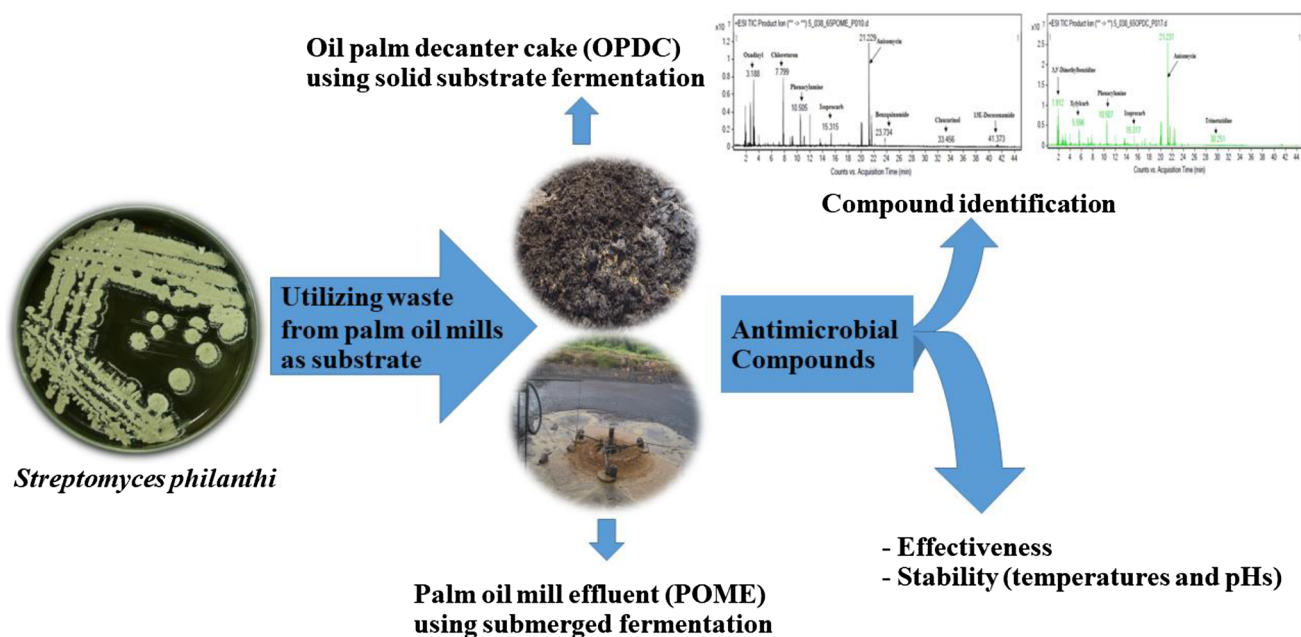
<sup>1</sup> College of Innovation and Management, Songkhla Rajabhat University, Songkhla 90000, Thailand

<sup>2</sup> Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Faculty of Agro-Industry, Prince of Songkla University, Hatyai 90110, Thailand

<sup>3</sup> Surat Thani Oil Palm Research Center, Field and Renewable Energy Crops Research Institute, Department of Agriculture, Ministry of Agriculture, Surat Thani 84340, Thailand

<sup>4</sup> International Program in Biotechnology, Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Faculty of Agro-Industry, Prince of Songkla University, Hatyai 90110, Thailand

## Graphical Abstract



**Keywords** Antimicrobial agents · Palm oil mill wastes · Waste utilization · *Streptomyces philanthi* · Identification

### Statement of Novelty

This study presents a novel approach to producing antimicrobial agents using POME and OPDC substrates for submerged and solid substrate fermentation, respectively. The effectiveness of extracts from *Streptomyces philanthi* RM-1-138 obtained from these cultures was compared against three strains of bacterial plant pathogens and three strains of oil palm pathogenic fungi. Antimicrobial agents produced in POME medium exhibited stronger inhibitory activity against tested pathogens compared to those produced in OPDC medium, while also showing excellent stability over a wide range of pH and high temperatures. These findings suggest that POME and OPDC substrates have great potential for use as antimicrobial agents and natural fungicides in agriculture and other fields.

### Introduction

The palm oil milling process can be classified into two categories: the wet process, which produces palm oil mill effluent (POME), and the dry process, which produces oil palm decanter cake (OPDC) [1]. The extraction of palm oil through the wet process is the most prevalent method, particularly in Thailand. The POME is characterized by high

levels of chemical oxygen demand (COD) (ranging from 45,200 to 56,300 mg COD<sup>-1</sup>) and biochemical oxygen demand (BOD) (ranging from 12,000 to 24,300 mg BOD<sup>-1</sup>) [2]. Bioconversion involves the use of microorganisms to metabolize the nutrient-rich organic content in POME and generate biomass and specific end-products, such as bioenergy and biochemicals. Previous studies have reported the application of POME as a substrate for bioenergy [3–8]. OPDC is a solid waste generated after passing the sludge through a decanter (or three-phase separator) that separates solid particles, oil, and liquid (decanter effluent) [9]. It is important to note that POME still contains a significant percentage (30–40%) of oil and is often improperly disposed of in landfills, which can lead to serious water and air pollution problems [10]. It has been reported that OPDC could be used as a growth media for the production of biosurfactants [11]. There have been two previous reports about using POME and OPDC as a substrate for producing bioactive compounds using *Streptomyces* spp. [12, 13]. Thus, the utilization of waste as an alternative nutrient source for *Streptomyces* spp. was seen as a viable solution to address the serious problems associated with water and air pollution, rendering it an ideal substrate for the production of bioactive agents.

Actinomycetes are among the most effective producers of secondary metabolites, and are highly significant from an industrial perspective. *Streptomyces* is a diverse genus of bacteria well-known for its ability to produce a wide range of

commercially valuable bioactive compounds [14–17]. *Streptomyces* species are frequently present in natural surroundings and can be harnessed as biocontrol agents owing to their ability to synthesize secondary metabolites like enzyme inhibitors and antibiotics, which demonstrate a wide range of biological properties. These activities encompass inhibition of diverse oil palm pathogenic fungi, including *Ganoderma boninense* [18–20], *Pyricularia oryzae* [21], and *Thielaviopsis punctulata* [22], as well as several bacteria such as *Acidovorax avenae*, *Clavibacter michiganensis* ssp. *michiganensis*, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas vasicatoria*, *Xanthomonas campestris*, and *Xanthomonas phaseolicoli* var. *fuscus* [23, 24]. Additionally, they have shown inhibitory activity against *Ralstonia solanacearum* [24–26] and *Pectobacterium carotovorum* [24]. Prior studies conducted on *Streptomyces philanthi* RM-1-138 have indicated that it can synthesize antifungal and antibacterial substances when grown on synthetic medium (glucose yeast-malt extract, GYM), which demonstrated antifungal activity against diverse plant pathogenic fungi, such as *Sclerotium rolfsii* [27], *Rhizoctonia solani*, *Pyricularia grisea*, *Bipolaris oryzae*, *Fusarium fujikuroi*, *Colletotrichum gloeosporioides*, *C. capsici* [28, 29] and *Aspergillus* spp. [30], and bacteria like *Ralstonia solanacearum* [27].

The tropical and sub-tropical climate of Thailand, characterized by extended periods of high rainfall and humidity, creates a conducive environment for the proliferation of different types of bacteria and fungi that can result in plant diseases [31]. Among the bacteria that cause significant damage to crop plants worldwide are gram-negative bacteria such as *Xanthomonas campestris* pv. *campestris*, *X. oryzae* pv. *oryzae*, and *X. axonopodis* pv. *glycines* [32–34], which are also prevalent in Thailand [35, 36]. For example, black rot disease, caused by *Xanthomonas campestris* pv. *campestris*, is a seed-borne pathogen that affects several members of the Brassicaceae family, such as Brassica and Arabidopsis [32]. Likewise, bacterial leaf blight disease, caused by *Xanthomonas oryzae* pv. *oryzae*, is a major problem for rice cultivation in Asian countries, including Thailand [33]. Additionally, *X. axonopodis* pv. *glycines* is a foliar pathogen responsible for causing bacterial pustule disease, one of the most serious diseases of soybean [37].

*Curvularia oryzae*, *Ganoderma boninense*, and *Ceratocystis paradoxa* are fungal species that are considered significant menaces to oil palm plantations in Southeast Asia, particularly in nations such as Malaysia, Indonesia, and Thailand [31, 38], as they can inflict substantial harm to the agricultural yield. *C. oryzae* causes a severe leaf spot disease that affects seedling stocks in private nurseries [31]. *G. boninense* and *C. paradoxa*, both soil-borne pathogens, cause basal stem rot disease, which is a major concern for oil palm growers.

The current study has the following objectives: (i) to investigate the potential of using POME and OPDC as alternative substrates for producing antimicrobial agents by *S. philanthi* RM-1-138 and evaluate their effectiveness in combating microorganisms, (ii) to examine the stability of the antimicrobial agents produced by the strain RM-1-138 under different pH and temperature conditions and evaluate their effectiveness against *Curvularia oryzae*, and (iii) to characterize the antimicrobial agents produced by the strain RM-1-138 using liquid chromatography-quadrupole time of flight mass spectrometry (LC-Q-TOF MS/MS).

## Materials and Methods

### Microorganisms, Media, and Cultivation Conditions

Following its isolation and characterization by Boukaew et al. [27], *Streptomyces philanthi* RM-1-138 was incubated on glucose yeast-malt extract agar (GYM) at room temperature ( $28 \pm 2$  °C) for 10 days before being utilized.

*Curvularia oryzae* and *Ganoderma boninense*, which are fungi that cause disease in oil palm, were acquired from the Suratthani Oil Palm Research Center, which operates under the Department of Agriculture, Ministry of Agriculture and Cooperative in Thailand. A fungus called *Ceratocystis paradoxa*, which is recognized for its ability to induce black seed rot disease in sprouted oil palm seeds [39], was obtained from the fermentation of the oil palm trunk [40]. The potato dextrose agar (PDA) slants were employed to cultivate the fungal strains, which were then kept at  $28 \pm 2$  °C for 3–5 days before preservation at 4 °C for future use. A fresh subculture was prepared for each experiment. The spore inoculum for *C. oryzae* and *C. paradoxa* was prepared by suspending them in sterile 0.1% Tween 80. After calculating the spore count with a hemacytometer, the inoculum was diluted to a concentration of  $10^6$  spores ml<sup>-1</sup> using potato dextrose broth (PDB). *G. boninense* inoculum was created according to the National Committee for Clinical Laboratory Standards [41].

Three plants pathogenic bacteria, specifically *Xanthomonas axonopodis* pv. *glycines*, *Xanthomonas oryzae* pv. *oryzae*, and *Xanthomonas campestris* pv. *campestris*, were sourced from Kasetsart University in Thailand. The bacteria were cultured on nutrient agar (NA) slants at  $28 \pm 2$  °C for 24–48 h and stored at 4 °C. The bacterial inoculum was prepared by growing them in nutrient broth (NB) for 24 h on a rotary shaker (150 rpm). Before use, the inoculum concentration was adjusted to  $10^6$  CFU ml<sup>-1</sup>.

## Palm Oil Mill Effluent and Decanter cake

Palm oil mill effluent (POME) and oil palm decanter cake (OPDC) were sourced from a palm oil mill (Larp Tavee Industries Co., Ltd.) situated in La-ngu city, Satun province, Southern Thailand. The OPDC was dehydrated in an oven at 95 °C until its moisture content dropped below 5%, after which it was kept in a freezer at –20 °C until required [2]. The characteristics of POME were as follows: acidic pH (4.65), chemical oxygen demand (COD) 95,933 mg l<sup>-1</sup>, total Kjeldahl nitrogen (TKN) 1050 mg l<sup>-1</sup>, and total suspended solids (TSS) 42,666 mg l<sup>-1</sup> [12]. The OPDC was characterized as 126,000 mg l<sup>-1</sup> COD, 1850 mg l<sup>-1</sup> TKN, and 235,000 mg l<sup>-1</sup> TSS, and had a pH of 5.33 [13].

## Impact of POME and OPDC Mediums Without Bacteria on the Mycelial Growth of Plant Pathogenic Fungi

A study was carried out to determine the impact of POME and OPDC media on the mycelial growth of three oil palm pathogens. To prepare the media, 1 ml of sterilized POME (diluted 50% with distilled water) [12] and OPDC (extracted with a 1:1 phosphate buffer) [42, 43] were added to 9 ml of melted sterile PDA. The mixture was then poured onto a 9 cm diameter culture plate. A 5 mm diameter mycelial plug from a 3-day-old colony of each oil palm pathogen was taken and placed in the center of the test PDA plates. The plates were then incubated at 28 ± 2 °C, and the growth of mycelia was observed after 2 days for *C. paradoxa*, 4 days for *C. oryzae*, and 7 days for *G. boninense*. The experiment was replicated three times.

## Extraction of the Strain RM-1-138 Antimicrobial Agents from POME and OPDC Mediums

To prepare the antimicrobial agents of the strain RM-1-138, cultivated in POME and OPDC mediums, we followed the method described by Boukaew et al. [12] and Boukaew et al. [13], respectively. The antimicrobial agents were extracted using equal volumes of ethyl acetate at 37 °C, with shaking at 150 rpm. Next, the organic layer was separated from the liquid media using a separatory funnel and evaporated under reduced pressure at 37 °C, resulting in a crude extract. The crude extract was dispersed in 2 ml of DMSO, filtered through a 0.45 µm Millipore membrane, and then tested for antimicrobial activity. Additionally, we used a quadrupole time-of-flight mass spectrometer/MS (LC-Q-TOF MS/MS) to identify the components of the extract.

## Effects of the Strain RM-1-138 Extracts on Plant Pathogenic Fungi and Bacteria

The antimicrobial activity of the crude extract from the strain RM-1-138 against bacterial and fungal plant pathogens using broth micro-dilution techniques according to the CLSI guidelines (National Committee for Clinical Laboratory Standards) [44]. The assay was performed on a 96-well microtiter plate. Minimum inhibitory (MIC) and minimum fungicidal (MFC), and minimum bactericidal (MBC) concentrations were determined by using the micro-dilution method [45]. The crude extract was DMSO to a final concentration of 10 µg ml<sup>-1</sup>. Twofold serial dilutions of crude extracts in the range of 0.25 to 500 µg ml<sup>-1</sup> were tested.

In the antibacterial assay, 100 µl of bacterial culture in nutrient broth (NB) was added to each well of sterile 96-well microplates. Then, 100 µl of each stock crude extract solution (10 µg ml<sup>-1</sup>) was added to the first well of a 96-well microplate and twofold serially diluted in sterile NB to obtain a final concentration range of 0.25 to 500 µg ml<sup>-1</sup>. One hundred microliters of a bacterial suspension containing 10<sup>6</sup> CFU ml<sup>-1</sup> were dispensed into each well of a sterile 96-well microtiter plate. Positive and negative controls were included in the assay using gentamicin (1.0 µg ml<sup>-1</sup>) and DMSO, respectively. The microtiter plates were placed in plastic bags to prevent contamination and incubated at 28 ± 2 °C overnight. After incubation, 40 µl of 0.2 µg/ml *p*-iodonitrotetrazolium violet (INT) (Sigma, Germany) was added to each well to indicate microbial growth. The MIC was determined to follow the method described by Shai et al. [46]. The experiment was repeated in triplicate.

For the antifungal assay, 100 ml of fungal culture in PDB was added to each well of a sterile 96-well microplate. Then, 100 ml of each stock crude extract solution (10 µg ml<sup>-1</sup>) was added to the first well of a 96-well microplate and serially diluted twofold in sterile PDB to obtain a final concentration range of 0.25 to 500 µg ml<sup>-1</sup>. One hundred microliters of a spore suspension (10<sup>6</sup> spores ml<sup>-1</sup> or 10<sup>6</sup> CFU ml<sup>-1</sup>) was added to each well of a sterile 96-well microplate. Positive and negative controls were established using Prochloraz® (Sportak) (45% (w/v)) and DMSO, respectively. The MIC value was determined as the lowest concentration of the sample that inhibited fungal growth after 24 h of incubation for *C. oryzae* and *Ceratocystis paradoxa* and after 48 h for *G. boninense* [47]. Fungal growth was assessed by measuring the absorbance of the microplate at 600 nm, as previously described by Elof et al. [48]. The experiment was repeated in triplicate.

## Investigating the Impact of Temperature and pH on the Efficiency and Durability of Crude Extracts Against *Curvularia oryzae*

The sensitivity of the antimicrobial agents in the crude extract produced in POME and OPDC mediums from *S. philanthi* RM-1-138 was characterized by assessing their response to variations in temperature and pH.

In the pH stability test, the freeze-dried powder of the sterile antimicrobial agents of crude extract was reconstituted in 50 mM phosphate buffer (pH 7.0) to assess their antifungal activity [49]. The pH of the crude extract was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 using 1 M HCl and 1 M NaOH before evaluating their inhibitory activity against *C. oryzae*. The antifungal activity was determined by assessing the effect of each treatment on the mycelial growth of *C. oryzae* on PDA plates, following the modified method of Droby et al. [50]. Briefly, a sterile PDA medium containing 1 ml of the antimicrobial agent of the crude extract was poured into a 9 cm diameter culture plate after being melted. Controls were established using DMSO and a non-treated treatment mixed with an equivalent amount of melted sterile PDA. A 5 mm diameter mycelial plug was obtained from a 3-day-old *C. oryzae* colony and placed at the center of each test agar plate. The plates were then incubated for 4 days at  $28 \pm 2^\circ\text{C}$ . The size of the colony in each treatment was measured and used to calculate the percentage of hyphal growth inhibition. The experiment was performed in triplicate for each treatment.

The resilience of the antimicrobial agents in the crude extract to temperature changes was evaluated by exposing them to a range of cooling and heating processes. The treatments included exposure to  $-20^\circ\text{C}$  and  $4^\circ\text{C}$  for 30 min, and  $40^\circ\text{C}$ ,  $60^\circ\text{C}$ ,  $80^\circ\text{C}$ , and  $100^\circ\text{C}$  for 30 min, as well as  $121^\circ\text{C}$  for 15 min. Controls were established using DMSO and non-treated samples. The effect of each treatment on the antifungal activity against *C. oryzae* was determined as described above, with three replicates for each treatment.

### Analysis of Antimicrobial Agents in Crude Extract Using Liquid Chromatography Connected to Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS)

The crude extract obtained from POME and OPDC medium was characterized by its antimicrobial agents using a LC-QTOF-MS analysis following the method described by Alara et al. [51]. Separation of compounds was achieved using a UHPLC column (Zorbax Eclipse Plus C18 Rapid Resolution HD 150 mm length  $\times$  2.1 mm inner-diameter, particle size 1.8  $\mu\text{m}$ , Agilent) with a column temperature of  $25^\circ\text{C}$ . The mobile phase, consisting of 0.1% formic acid in  $\text{H}_2\text{O}$  (A) and 0.1% formic acid in acetonitrile (B), was used

at a flow rate of  $0.4 \text{ ml min}^{-1}$ . The gradient program of the mobile phase had the following as described by Boukaew et al. [52]. Five microliters of the sample were injected, and the LC-Q-TOF MS/MS instrument was set to detect ions using positive electrospray ionization ( $^+\text{ESI}$ ) mode. The optimized parameters included and mass-to-charge ( $m/z$ ) of substances were measured as described in detail by Boukaew et al. [52]. The LC-MS chromatogram in  $^+\text{ESI}$  mode was compared using Mass Hunter METLIN metabolite PCD and PCDL version 8, which are databases and libraries used for identifying compounds.

### Statistical Analysis

To ensure statistical significance, the experiments were performed in triplicate ( $n=3$ ), and the data obtained were analyzed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp, Armonk, NY). Tukey's HSD (Honestly Significant Difference) test was used for comparing means, and a significance level of  $p < 0.05$  was considered statistically significant.

## Results

### The Effect of the POME and OPDC Medium Without Bacteria (Control) on Mycelial Growth of Plant Pathogenic Fungi

The effect of POME and OPDC mediums without bacteria (control) on the mycelial growth of *C. oryzae*, *C. paradoxa*, and *G. boninense* on the PDA plates is illustrated in Fig. 1. The results indicated that all three strains of oil palm pathogens were able to grow and colonize the PDA medium, as evidenced by the observed mycelial growth. Therefore, we concluded that the POME and OPDC medium did not have any significant impact on the growth of plant pathogenic fungi.

### Antimicrobial Activity of the Crude Extract

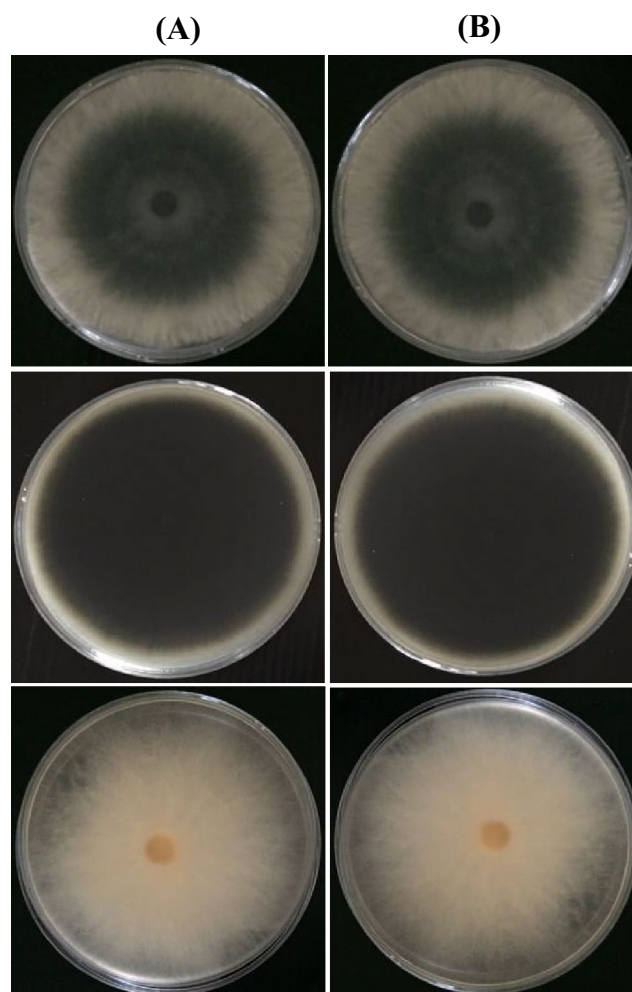
Antimicrobial activity of the crude extract produced in POME and OPDC medium against plant pathogenic bacteria (*X. axonopodis* pv. *glycines*, *X. campestris* pv. *campestris*, and *X. oryzae* pv. *oryzae*) and fungi (*C. oryzae*, *G. boninense*, and *C. paradoxa*) were determined. The antibacterial profiles in terms of MIC/MBC of the crude extract are shown in Table 1. The antimicrobial agents of crude extract obtained in the POME medium exhibited stronger inhibition on three strains of plant pathogenic bacteria (MIC values between 16 and  $128 \mu\text{g ml}^{-1}$ ) than those from using the OPDC medium (MIC values between 64 and  $500 \mu\text{g ml}^{-1}$ ) (Table 1). The highest antibacterial activity was against *X.*

**Fig. 1** The effect of the POME (A) and OPDC (B) medium on mycelial growth of the three oil palm pathogenic fungi after incubation at  $28 \pm 2$  °C for 2 days of *C. Paradoxa*, 4 days of *C. oryzae*, and 7 days of *G. boninense*

*Ceratocystis paradoxa*

*Curvularia oryzae*

*Ganoderma boninense*



**Table 1** Minimal Inhibitory Concentration (MIC) of antimicrobial compounds ( $500\text{--}0.25$   $\mu\text{g mL}^{-1}$ ) from *S. philanthi* RM-1-138 produced in palm oil mill effluent (POME) and on oil palm decanter cake (OPDC) medium against plant pathogenic bacteria

Treatments	Plant pathogen		
	<i>X. axonopodis</i> pv. <i>glycines</i>	<i>X. oryzae</i> pv. <i>oryzae</i>	<i>X. campestris</i> pv. <i>campestris</i>
POME medium	16	128	16
OPDC medium	64	500	64
Gentamicin	0.5	0.25	0.5

*axonopodis* pv. *glycines* and *X. campestris* pv. *campestris* which was 8 times higher than against *X. oryzae* pv. *oryzae* (MIC of 16 and 128  $\mu\text{g mL}^{-1}$ , respectively). The antibiotic gentamicin as a positive control had MIC values ( $0.25$  to  $0.5$   $\mu\text{g mL}^{-1}$ ) much lower than those of the studied antimicrobial agents of crude extract. For MBC, the antimicrobial agents of crude extract produced in POME medium (MBC value range of 120 to 500  $\mu\text{g mL}^{-1}$ ) had higher bactericidal

**Table 2** Minimal Bactericidal Concentration (MBC) of antimicrobial compounds ( $500\text{--}0.25$   $\mu\text{g mL}^{-1}$ ) from *S. philanthi* RM-1-138 produced in palm oil mill effluent (POME) and on oil palm decanter cake (OPDC) medium against plant pathogenic bacteria

Treatments	Plant pathogen		
	<i>X. axonopodis</i> pv. <i>glycines</i>	<i>X. oryzae</i> pv. <i>oryzae</i>	<i>X. campestris</i> pv. <i>campestris</i>
POME medium	128	500	128
OPDC medium	200	> 500	200
Gentamicin	0.5	0.5	1.0

activities against three strains of bacteria pathogen than those produced in OPDC medium ( $200$  to  $> 500$   $\mu\text{g mL}^{-1}$ ) (Table 2).

The antifungal profiles in terms of MIC/MFC of the crude extract are presented in Tables 3 and 4. The antifungal metabolites of crude extract produced in the POME medium (MIC values between 128 and 200  $\mu\text{g mL}^{-1}$ ) showed stronger inhibition against three strains of the oil palm pathogenic

**Table 3** Minimal Inhibitory Concentration (MIC) of antimicrobial compounds ( $500\text{--}0.25\ \mu\text{g mL}^{-1}$ ) from *S. philanthi* RM-1-138 produced in palm oil mill effluent (POME) and on oil palm decanter cake (OPDC) medium against oil palm pathogenic fungi

Treatments	Plant pathogen		
	<i>C. oryzae</i>	<i>G. boninense</i>	<i>C. paradoxa</i>
POME medium	128	128	200
OPDC medium	500	500	500
Prochloraz®	8	0.25	16

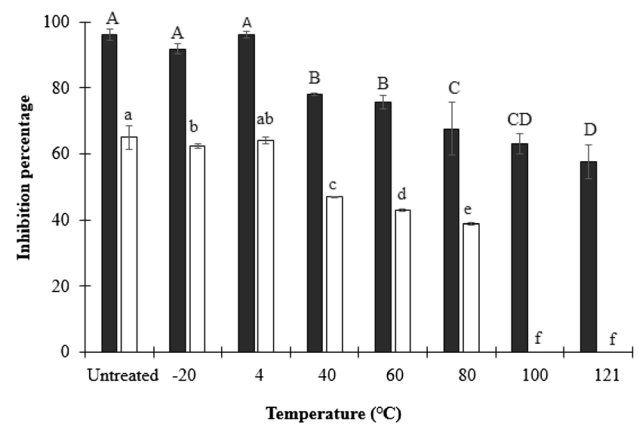
**Table 4** Minimum Fungicidal Concentration (MFC) of antimicrobial compounds ( $500\text{--}0.25\ \mu\text{g mL}^{-1}$ ) from *S. philanthi* RM-1-138 produced in palm oil mill effluent (POME) and on oil palm decanter cake (OPDC) medium against oil palm pathogenic fungi

Treatments	Plant pathogen		
	<i>C. oryzae</i>	<i>G. boninense</i>	<i>C. paradoxa</i>
POME medium	500	500	> 500
OPDC medium	> 500	> 500	> 500
Prochloraz®	32	0.25	> 32

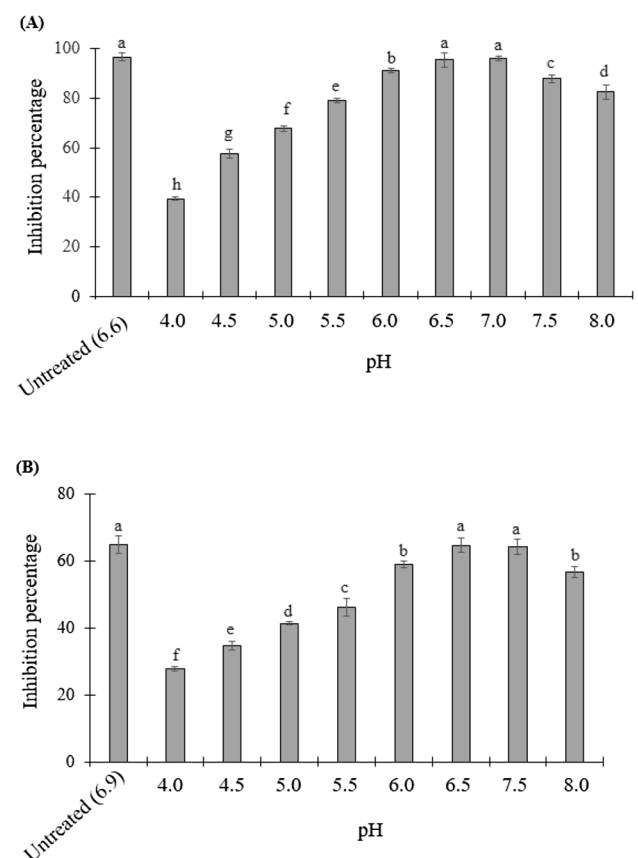
fungi than those produced in the OPDC medium (MIC value  $500\ \mu\text{g mL}^{-1}$ ). The inhibitory effect of antifungal activity of crude extract produced in POME medium inhibited *C. oryzae* and *G. boninense* stronger than *C. paradoxa* (with a MIC at  $128\ \mu\text{g mL}^{-1}$  and  $200\ \mu\text{g mL}^{-1}$ , respectively). The three strains of the fungal pathogen exhibited relatively higher MIC values ( $500\ \mu\text{g mL}^{-1}$ ) against the crude extract produced in OPDC medium, indicating lower antifungal activity. These antifungal activities were compared with the commercial prochloraz® possessing MIC values ranging from 0.25 to  $16\ \mu\text{g mL}^{-1}$  (Table 3). For MFC results, the antifungal activity of crude extract produced in POME (MFC value  $500\ \mu\text{g mL}^{-1}$ ) had strong inhibitory activity against *C. oryzae* and *G. boninense* than those produced in OPDC medium (MFC value  $> 500\ \mu\text{g mL}^{-1}$ ) (Table 4). In addition, *C. paradoxa* exhibited resistance to the antifungal crude extract at the concentrations tested (0.25 to  $500\ \mu\text{g mL}^{-1}$ ).

### Investigating the Impact of Temperature and pH on the Efficiency and Durability of Crude Extracts Against *Curvularia oryzae*

Antimicrobial agents of crude extract produced in the POME medium were tolerant to temperatures higher than  $100^\circ\text{C}$ , while those produced in the OPDC medium could be tolerant to temperatures up to  $80^\circ\text{C}$ . The antimicrobial agents of crude extracts kept at  $-20$  and  $4^\circ\text{C}$  showed no significant differences ( $p > 0.05$ ) in antifungal activity against *C. oryzae* compared to the untreated treatment



**Fig. 2** Effect of temperature on activity and stability of antimicrobial agents produced in palm oil mill effluent (MOPE) (black color) and on oil palm decanter cake medium (OPDC) (white color) of *S. philanthi* RM-1-138 against *C. oryzae*. The data were the mean values of triplicate determination  $\pm$  standard deviation. Values with the same letter are not significantly different (ANOVA,  $p < 0.05$ ; Duncan multiple range test)



**Fig. 3** Effect of pH on activity and stability of antimicrobial agents produced **A** in oil mill effluent (OPDC) and **B** on oil palm decanter cake (OPDC) medium from *S. philanthi* RM-1-138 against *C. oryzae*. The data were the mean values of triplicate determination  $\pm$  standard deviation. Values with the same letter are not significantly different (ANOVA,  $p < 0.05$ ; Duncan multiple range test)

**Table 5** Antimicrobial agents emitted by *S. philanthi* RM-1-138 produced in palm oil mill effluent (POME) medium detected by LC-Q-TOF MS/MS analysis

Number	Predicted compounds	t <sub>R</sub> (min)	Formula	m/z	Mass error (mDa)	Proportion (%)
1	Oxadixyl	3.18	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O <sub>4</sub>	279.13	- 0.67	0.89
2	Chloreturon	7.79	C <sub>11</sub> H <sub>15</sub> ClN <sub>2</sub> O <sub>2</sub>	243.08	- 2.30	2.41
3	Benzquinamide	10.50	C <sub>22</sub> H <sub>32</sub> N <sub>2</sub> O <sub>5</sub>	404.23	- 0.61	0.24
4	Isoproc carb	15.31	C <sub>11</sub> H <sub>15</sub> NO <sub>2</sub>	194.11	- 0.68	1.35
5	Phenacylamine	21.22	C <sub>8</sub> H <sub>9</sub> NO	135.06	- 2.66	3.65
6	Anisomycin	23.73	C <sub>14</sub> H <sub>19</sub> NO <sub>4</sub>	265.13	- 3.95	87.41
7	Clausarinol	33.45	C <sub>24</sub> H <sub>30</sub> O <sub>6</sub>	414.20	2.59	0.96
8	13E-Docosenamide	41.37	C <sub>22</sub> H <sub>43</sub> NO	337.33	- 4	0.43

The antimicrobial agents emitted by *S. philanthi* RM-1-138 produced in the MOPE medium were tentatively identified by mass spectra comparison to those in the Mass Hunter METLIN metabolite PCD and PCDL (probability based match > 90%)

(Fig. 2). The antifungal activity of *S. philanthi* RM-1-138 remained partially active after heat treatment at the sterilization temperature (121 °C for 15 min), showing only 58% inhibition.

The results concerning pH stability indicated that the antimicrobial agents of crude extract were insensitive to pH variation. The antimicrobial activity remained unaffected within the pH range of 6.5–7.0 for the POME medium and 6.5–7.5 for the OPDC medium. However, it was observed to decrease significantly under highly acidic (< 6) and alkaline (> 7.5) pH conditions, as shown in Fig. 3.

### Analysis of Antimicrobial Agents in Crude Extract Using Liquid Chromatography Connected to Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF-MS)

The LC-Q-TOF MS/MS was utilized to identify the antimicrobial agents present in the crude extract. A total of 8 and 6 bioactive compounds were identified in the strain RM-1-138 produced in the POME and OPDC medium, respectively, based on retention time (t<sub>R</sub>), molecular mass, and mass spectra (MS) fragmentation (Tables 5 and 6, and Fig. 4) suggesting the response of compounds reflected their relative proportion in the substrate medium. These compounds could be functioned as fungicides (oxadixyl), herbicides (chloreturon), insecticides (isoproc carb and xylylcarb), and antibiotics (anisomycin). Among them, anisomycin, isoproc carb, and phenylamine were produced in both POME and OPDC mediums. The compound response data revealed the relative abundance of metabolites present in the crude extract. The analysis demonstrated that anisomycin comprised 87.41% of all identified compounds, indicating its dominance as the primary compound in *S. philanthi* RM-138.

## Discussion

Previous studies have demonstrated the efficacy of antimicrobial agents produced by *Streptomyces philanthi* RM-1-138 in the waste media of palm oil mill effluent (POME) and oil palm decanter cake (OPDC) against plant pathogenic bacteria (*X. axonopodis* pv. *glycines*, *X. campestris* pv. *campestris*, and *X. oryzae* pv. *oryzae*) and fungi (*C. oryzae*, *G. boninense*, and *C. paradoxa*) [12, 13]. However, limited information is currently available on the response of these antimicrobial agents to changes in temperature and pH. Additionally, the study also investigated the production of antimicrobial agents in POME and OPDC media.

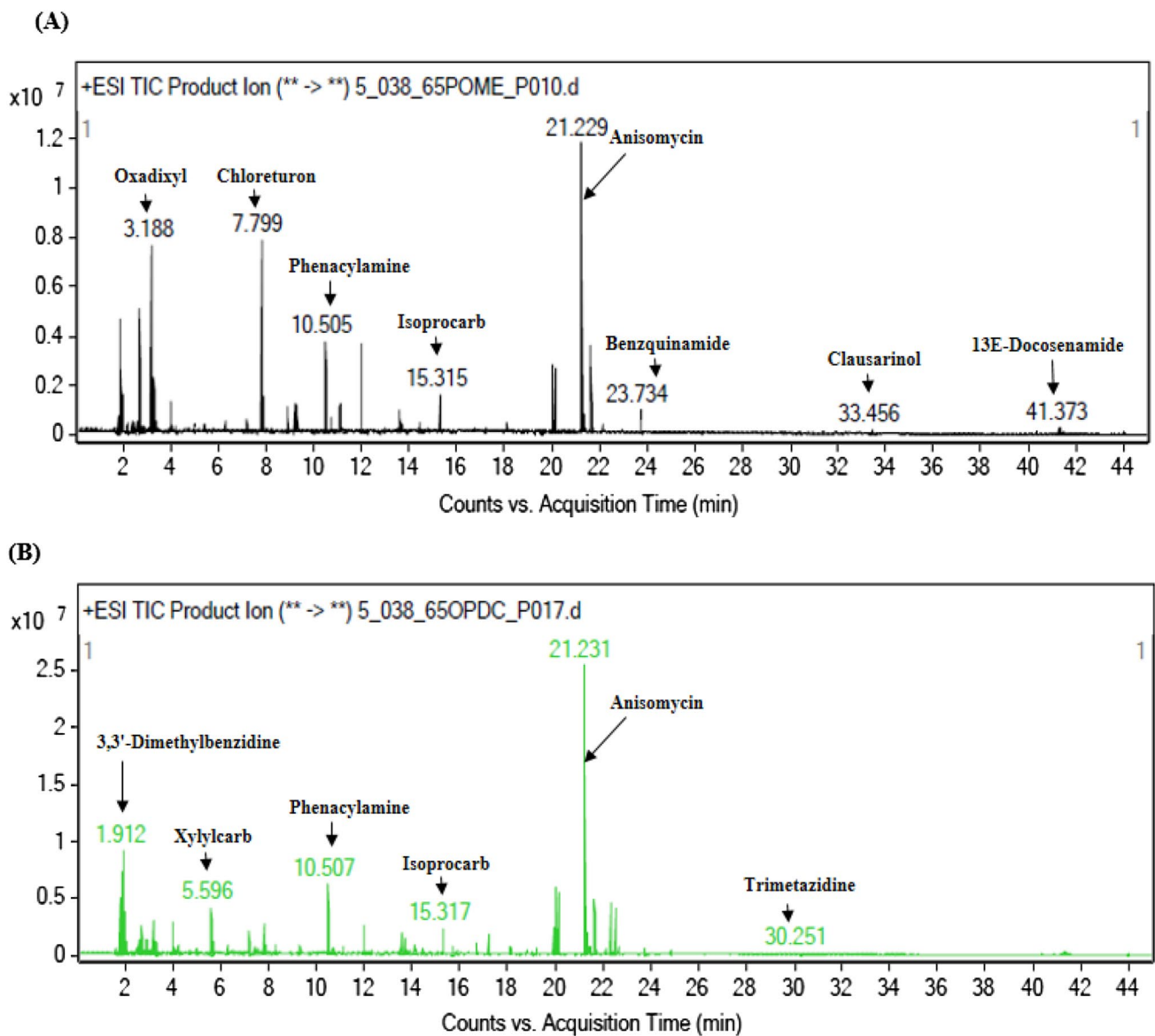
Antimicrobial agents produced by *Streptomyces* species in synthetic media are effective against both bacterial [23–27] and fungal [18–22, 27] plant pathogens. The antimicrobial agents produced by the strain RM-1-138 in a synthetic medium (glucose yeast-malt extract broth (GYM)) are known to have antimicrobial effects against various plant pathogens [27–29]. In the present study, the crude extract obtained from the POME and OPDC waste medium of the strain RM-1-138 were subjected to an antimicrobial activity assay against plant pathogens. This study found that the antimicrobial agents in the crude extract had significant antimicrobial activity against all the test plant pathogens, as determined by the MIC and MBC/MFC assays. This suggests that metabolites are broad-spectrum in nature and that they have potential applications as antimicrobial agents. The MIC and MBC values of their *Streptomyces* species against plant pathogenic bacteria were similar to our study. Girão et al. [53] determined the MIC values of *Streptomyces* sp. against *Staphylococcus aureus* that ranged between < 0.5 and 1000 µg ml<sup>-1</sup>. According to MIC values, the antimicrobial agents in crude extract produced in POME (MIC 128–200 µg ml<sup>-1</sup>) medium were stronger than the antifungal activity of *S. blastmyceticus* strain 12-6 against *Botrytis cinerea* and *Fusarium oxysporum* (MIC value > 500 µg ml<sup>-1</sup>) [54] but lower than that of *S. lilacinus* NRRL B-1968T



**Table 6** Antimicrobial agents emitted by *S. philanthi* RM-1-138 produced on oil palm decanter cake (OPDC) medium detected by LC-Q-TOF MS/MS analysis

Number	Predicted compounds	t <sub>R</sub> (min)	Formula	m/z	Mass error (mDa)	Proportion (%)
1	3,3'-Dimethylbenzidine	1.91	C <sub>14</sub> H <sub>16</sub> N <sub>2</sub>	212.13	- 0.04	0.68
2	Xylylcarb	5.59	C <sub>10</sub> H <sub>13</sub> NO <sub>2</sub>	180.10	- 0.32	0.98
3	Phenacylamine	10.50	C <sub>8</sub> H <sub>9</sub> N O	135.06	- 2.66	3.65
4	Isoprocarb	15.31	C <sub>11</sub> H <sub>15</sub> NO <sub>2</sub>	194.11	- 0.68	1.35
5	Anisomycin	21.23	C <sub>14</sub> H <sub>19</sub> NO <sub>4</sub>	265.13	- 3.95	87.41
6	Trimetazidine	30.25	C <sub>14</sub> H <sub>22</sub> N <sub>2</sub> O <sub>3</sub>	266.16	- 3.5	1.16

The antimicrobial agents emitted by *S. philanthi* RM-1-138 produced on the OPDC medium were tentatively identified by mass spectra comparison to those in the Mass Hunter METLIN metabolite PCD and PCDL (probability based match > 90%)



**Fig. 4** Mass spectra showing antimicrobial agents emitted by *S. philanthi* RM-1-138 produced **A** in palm oil mill effluent (POME) and **B** on oil palm decanter cake (OPDC) medium detected by LC-Q-TOF MS/MS analysis

against *Pyricularia oryzae* and *Rhizoctonia solani* (MIC value  $25 \mu\text{g ml}^{-1}$ ) [55]. Our investigation found that POME and OPDC waste media do not have a positive influence on the growth of plant pathogenic fungi. Therefore, it can be concluded that the crude extract exhibited potent antimicrobial activity against plant pathogens, and its efficacy was not compromised by the presence of waste media. This evidence suggests that the POME and OPDC media can be effective in producing antimicrobial agents with *S. philanthi* RM-1-138, and that this strain of bacteria does produce antimicrobial agents that are effective against plant pathogens.

Understanding the characteristics of antimicrobial metabolites was crucial for developing effective treatments for various diseases caused by pathogens. One such disease was the leaf spot disease caused by the fungus *C. oryzae*, which was a significant problem for oil palm seedlings [56]. To develop an effective treatment, it was essential to study the stability of the antimicrobial agents in different environmental conditions, such as temperature and pH. A recent study investigated the stability of the active crude extract at a range of temperatures and pH values, and it was found that the antimicrobial agents in the extract were highly stable [57, 58]. This stability was an important factor for its potential use in agricultural fields or industrial applications. The study found that the antimicrobial agents in the crude extract remained active even after being subjected to high temperatures of  $121 \text{ }^\circ\text{C}$  for 15 min, indicating excellent thermo-stability. These findings were promising and suggested that the antimicrobial agents in the crude extract could be a potential solution to combat leaf spot disease in oil palm seedlings. In addition to the recent study, there have been other reports of heat-stable antimicrobial agents produced by various microorganisms. For example, *Streptomyces* strain KX852460 [58] and *Bacillus subtilis* NSRS 89-24 [59] have been found to produce antimicrobial agents that remain stable at high temperatures. This demonstrates the potential for utilizing various microorganisms to develop antimicrobial agents with high thermo-stability, which can be used for a range of applications, including agriculture and industrial settings. The findings from these studies highlight the importance of exploring different microorganisms and their metabolites to develop effective solutions for combating diseases caused by pathogens.

The antimicrobial study found several important findings about the crude extract obtained from different media. Specifically, the study found that the antimicrobial agents in the crude extract obtained from the POME medium were heat-stable and could tolerate temperatures over  $100 \text{ }^\circ\text{C}$  without loss of activity, while those obtained from the OPDC medium were only tolerant to temperatures up to  $80 \text{ }^\circ\text{C}$ . This suggested that some antimicrobial agents may be more sensitive to heat than others, and the observed inhibition was likely due to a combination of these agents. Additionally,

the pH values of the crude extract remained stable within a range of 6.0 to 8.0, but were not stable at lower pH values. These findings were consistent with previous studies that investigated the stability of antimicrobial agents at different temperatures and pH values. For instance, Uddin et al. [60] reported on the stability of antimicrobial filtrate at different temperatures and pH values, while Zhao and Wu [61] previously reported on the stability of antimicrobial cultural filtrate from *Streptomyces*. Studies showed that extracellular lytic enzymes and antibiotics from *Streptomyces* strain KX852460 had potent biocontrol properties against fungi [58]. These agents could act alone or in synergy to degrade the fungal cell walls [58]. As a result, the antimicrobial agents in the crude extract obtained from the POME medium, which remained heat-stable and showed tolerance to a wide range of pH values and temperatures, were considered a potential biocontrol agent for fungal diseases. Overall, the high tolerance of antimicrobial agents in the crude extract to temperatures and pH suggested that they could potentially be used as biofungicides for applications in the agricultural field, particularly in the control of leaf spot disease caused by *C. oryzae*.

LC-Q-TOF MS/MS is a novel technique that can be used to identify secondary metabolites in *Streptomyces* fermentation broth. This technique is very effective for accurately identifying the individual compounds present in complex biochemical products. The capability of *Streptomyces* cultures to produce antibiotics is not constant and can fluctuate significantly depending on the nutritional and cultivation conditions [62]. Therefore, the composition of the medium and the metabolic capabilities of the organism greatly impact the biosynthesis of antibiotics. In this study, we further investigated the antimicrobial agents in the crude extract of the strain RM-1-138 produced in POME and OPDC medium and analyzed by LC-Q-TOF MS/MS. *Streptomyces* species have long been recognized as a prominent producers of secondary metabolites, accounting for a significant portion of clinically relevant antibiotics derived from natural sources [63, 64]. In comparison between the among 8 and 6 chemical compounds from the strain RM-1-138 grown in POME and OPDC medium, respectively, only anisomycin, phenacylamine and isoprocarb were found in both media. Among them, anisomycin, representing 87.41% of all identified components, was the dominant compound. In contrast, acetic acid was exclusive to the GYM medium [65]. The LC-Q-TOF MS/MS analysis revealed that strain RM-1-138 produced more antimicrobial agents in the POME medium (8 compounds) compared to the OPDC medium (6 compounds). However, the number of compounds produced in POME and OPDC media was less than the 32 compounds detected in the GYM medium using GC-MS [65]. The differences in both types and concentrations of antimicrobial compounds resulted from the differences in the medium

composition of POME, OPDC, and GYM. The onset of biosynthesis in *Streptomyces* species is influenced by several factors, including the quantity and type of available carbon and nitrogen sources, as well as external environmental conditions such as the presence of trace elements, oxygen availability, temperature, light, and pH [66, 67]. Various precise regulatory mechanisms are involved in the initiation, sustenance, and termination of antibiotic biosynthesis [68, 69]. Carbon source regulation is a crucial factor in controlling secondary metabolism among these mechanisms, with the molecular level revealing that the carbon source can impact the synthesis of secondary metabolites either by blocking transcriptional activation or through repression [70]. This latter mechanism, repression by the carbon source, is commonly referred to as carbon catabolite repression (CCR). Sanchez et al. [67] found that glucose interferes with the synthesis of antibiotics in *Streptomyces*. The effects of glucose on carbon source uptake, central carbon and nitrogen metabolism, nitrogen regulation, development, and the production of undecylprodigiosins (RED), CDA (calcium-dependent antibiotic), and cryptic polyketide (CPK) antibiotics were significant [71]. Additionally, culture conditions like submerged and solid substrate fermentation impact bioactive compound production. For instance, the strain RM-1-138 can produce eight compounds in the POME medium through submerged fermentation, and six compounds in the OPDC medium via solid substrate fermentation. Our findings indicated that the regulation of bioactive agent production in strain RM-1-138 was influenced by both the environmental conditions and the composition of the culture medium. Therefore, we concluded that these factors played a crucial role in the biosynthesis of bioactive compounds in this strain.

The functional properties of some identified antimicrobial agents were reported. Anisomycin is an antibiotic produced by *S. griseolus* and *S. roseochromogenes* [72] that is primarily known for its ability to inhibit protein synthesis in eukaryotic organisms. It works by binding to and inhibiting the peptidyl transferase activity of the 60S ribosomal subunit [73]. Previously reported antimicrobial agents from *S. lydicus* M01 [74], including isoprocarb, xylylcarb, oxadixyl, and chloreturon, have also been found in the strain RM-1-138. The metabolites detected in this study were all small molecules with low molecular weights, and their antimicrobial mechanisms likely differ from those of larger biomacromolecules like lipopeptides [75]. These small molecules may act by inhibiting enzymes critical for cell metabolism, or by interfering with protein synthesis and cell membrane structure [76]. Based on our findings, it is reasonable to conclude that the antimicrobial activity of the crude extract is partly attributed to the presence of bioactive agents, which we identified using LC-Q-TOF MS/MS analysis. This analytical technique enabled us to identify a diverse range of

compounds within the sample. However, the complexity of the sample and the potential co-elution of various compounds pose challenges in definitively attributing specific compounds solely based on the analysis results. Therefore, it is crucial for future studies to focus on isolating and purifying individual compounds of interest to further characterize and verify their presence. While our current findings offer valuable insights into the overall composition and potential compounds present, it is important to interpret these results cautiously. By addressing the need for individual compound purification and subsequent testing, future research can enhance the reliability and accuracy of compound identification in similar studies.

In conclusion, the extracts from strain RM-1-138 displayed a broad spectrum of antimicrobial activity against various plant pathogenic bacteria and fungi. Moreover, the antimicrobial agents in crude extract showed excellent stability to high temperatures (121 °C) and a wide range of pH (4–8) against oil palm pathogenic fungi *Curvularia oryzae*. The utilization of LC-Q-TOF MS/MS enabled the identification of 8 and 6 compounds in the POME and OPDC medium, respectively, through the analysis of secondary metabolite profiles. These compounds were predominantly composed of fungicides (oxadixyl), herbicides (chloreturon), insecticides (isoprocarb and xylylcarb), and antibiotics (anisomycin). Overall, the study suggests that POME and OPDC hold substantial potential as alternative media for the production of antimicrobial agents from the strain RM-1-138.

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## Declarations

**Conflict of interest** The authors declare no competing interests.

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