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Endophytic bacteria isolated from higher plant in Aceh, Indonesia, and their chemical compounds activity against *Fusarium oxysporum* f. sp. *lycopersici*

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Abstract

Background: Endophytic bacteria are an association between bacteria and plant tissue that could play a role as a biocontrol agent.

Main body: Endophytic bacteria were isolated from several high root plants in Aceh, Indonesia. This study aimed to detect the chemical compounds of the potential endophytic bacteria as a biocontrol agent against *Fusarium oxysporum* f. sp. *lycopersici* (FOL). There were 198 endophytic bacterial isolates detected in roots of 9 higher plant. The hypersensitive reaction showed that 193 isolated endophytic bacteria were non-pathogenic. There were 13 isolated endophytic bacteria that worked to inhibit FOL between 50.0 and 89.2%; such endophytic bacteria were isolated from *Solanum lycopersicum* L., *Psidium guajava* L., *Dendrocalamus asper* (Schult with f.) Backer ex Heyne, *Pinus merkusii* L., *Theobroma cacao* L., and *Albizia chinensis* L. Molecular identification using 16S rRNA gene sequence confirmed that the endophytic bacteria were derived from species *Pseudomonas aeruginosa*, *P. mosselii*, *Arthrobacter* sp., *Bacillus cereus*, *B. thuringiensis*, and *Serratia marcescens*. *P. aeruginosa* that showed the highest inhibition was analyzed using GC-MS analysis. The analysis identified that antibiotics as Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- was produced by *P. aeruginosa* succeeded in suppressing FOL.

Conclusion: The study recommends the species *P. aeruginosa*, as effective endophytic bacteria for the control of FOL pathogen.

Keywords: Endophytic bacteria, Dual culture, *Fusarium oxysporum* f. sp. *lycopersici*, Antibiotics, Molecular identification, GC-MS analysis

Background

Fusarium oxysporum f. sp. *lycopersici* (FOL) is a plant pathogenic fungus causing *Fusarium* wilt of tomato. This pathogen causes losses of 50–100% in crop productions without any effective treatment (Lecomte et al. 2016). Synthetic fungicides have been used for decades to control the plant pathogens as they are affordable and effective. However, the negative impact of the chemical fungicides on environment is also quite serious. Therefore, use of eco-friendly biological control method is a priority task for sustainable agriculture in many countries, protects and increases the antagonistic microorganisms, and reduces damage from pesticides and pathogens. One of the biological methods is by utilizing endophytic bacteria (Abd-Elgawad and Askary 2020).

Endophytic bacteria are capable to colonize inside plant tissues without causing disturbance or harm to host plants. Some of these are known to produce secondary metabolites, which have beneficial roles to the host plants, such as promoting growth, inducing

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protection against infection, and environmental stresses (Eljounaidi et al. 2016). As it has such a positive nature, endophytic bacteria are studied vigorously as potential agents of eco-friendly biological control.

Endophytic bacterium genus *Bacillus* isolated from *Nicotiana glauca* plant produced extracellular metabolites which successfully inhibited FOL growth by 87–100% in vitro. Moreover, these metabolites also protected tomato plant against *Fusarium* wilt disease and enhanced tomato growth by 38–80% (Abdallah et al. 2016). Thirty-five isolated bacteria, screened for antagonistic activity in dual culture against fungi *Fusarium oxysporum*, *P. aeruginosa* showed a high antagonistic activity (58.33%) (Islam et al. 2018). Twenty-nine bioactive chemical constituents have been identified from methanolic extract of the *P. aeruginosa* by gas chromatogram mass spectrometry (GC-MS). *P. aeruginosa* produces many important secondary metabolites with high biological activities such as Oxime-,methoxy-phenyl, Edulan II, Methyl-4[nitromethyl]-4- piperidinol, Acetamide, N-methyl-N-[4-[2-fluoromethyl-1-pyrrolidyl-2-buty, Octahydrochromen-2-one (Altaee et al. 2017).

Based on the previous description, it is necessary to explore and isolate endophytic bacteria derived from some higher plants and performed in vitro testing against wilt *Fusarium* disease and their chemical compounds to play rules as biocontrol agent. In this study, endophytic bacteria, isolated from 9 higher plants roots, were selected as they were abundant in Aceh, Indonesia, and have great potentials to develop of endophytic bacteria as a biological control agent.

Materials and methods

Isolation of endophytic bacteria

Endophytic bacteria were isolated from roots of healthy plants, tomato (*Solanum lycopersicum* L.), guava (*Psidium guajava* L.), soybean (*Glycine max* Merr. L.), bamboo (*Dendrocalamus asper* (Schult with f.) Backer ex Heyne), sugar cane (*Saccharum officinarum* L.), pine (*Pinus merkusii* L), cacao (*Theobroma cacao* L.), sengon (*Albizia chinensis* L), and gamal (*Gliricidia sepium* (Jacq.) Kunth ex Walp.). Samples were collected from several areas of Aceh province, i.e., Banda Aceh, Aceh Besar, and Pidie, Indonesia, using purposive sampling and random sampling. Isolation of endophytic bacteria was performed individually with each plant roots; endophytic bacteria were isolated basically, following the method by Lodewyckx et al. (2002); root samples were cut (ca 0.5 cm), washed with sterilized distilled water (DW), and then soaked in running water for 2 h. After washing, root samples were soaked in 5.25% sodium hypochlorite for 5 min, then rinsed with sterilized DW for 1 min 3 times. The sterilized root samples were grinded in the sterilized mortar and pestle, added to 10 ml of sterilized DW, then serially diluted from 10^{-1} to 10^{-3} with sterilized DW. One milliliter of each 10^{-3} diluted samples was transferred and spread onto the Nutrient Agar (NA) medium and incubated at 28°C for 72 h. A total of 198 colonies was isolated and established as candidate strains for further analysis.

Pathogenicity test on tobacco plant

Bacterial strains streaked on NA medium for 72 h at 28 °C to isolate a single colony. Colonies were picked up and suspended into the 100 ml of sterilized water. Bacterial suspensions were serially diluted from 10^{-1} to 10^{-3} (0.281 in OD 600) by sterilized DW, a volume of 1.5 ml of each suspension (10^{-3} dilution) as infiltrated into the lamina on abaxial/adaxial side of *Nicotiana tabaccum* leaves, using a disposable syringe. Pathogenic test to each bacterium was carried out in 3 replicates/plant and repeated 3 times with plant pots. Inoculated tobacco plants were incubated for 72 h (Nawangsih et al. 2011).

Antagonistic activity of endophytic bacteria

In vitro antagonistic activity of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) test was performed by double culture technique (dual culture) with reference to Suryanto et al. (2011). FOL used in this test was a collection of laboratory plant disease. Endophytic bacteria that incubated for 48 h scratched straight in the middle of PDA (Potato Dextrose Agar) medium against 168 h FOL was placed adjacent to the bacteria on a Petri dish. Observations were carried out of the inhibition zones (clear zones) produced by endophytic bacteria.

Molecular identification of endophytic bacteria

A single colony of candidate bacteria was picked up, transferred into the 4 ml of LB broth in 15-ml tube, and cultured for overnight at 28 °C. After culturing, 700 μl of each bacterial medium was transferred into a cryotube, mixed with 300 μl of 50% glycerol, and then stored in −80 °C freezer. Rests of the bacterial medium were centrifuged at 15,000*g* (14,000 rpm) for 5 min and supernatants were discarded. Genomic DNA from each bacterial palette was extracted, using DNeasy Blood and Tissue Kit (Qiagen, USA), following the instruction manual. Genomic DNA was dissolved in 50 μl of TE buffer pH 8.0. Almost full length of the 16S ribosomal RNA gene (ca 1.5 kbp) was amplified from genomic DNA with the universal primers, 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′), and 1492R (I) (5′-GGT TAC CTT GTT ACG ACT T-3′) (Turner et al. 1999). PCR fragments were purified from agarose gel with NucleoSpin® Gel and PCR Clean-up (Machery-Nagel, Germany). Samples were submitted to Hokkaido System Science Co. (Sapporo, Japan) for sequencing from both strands using primers, 8F, 1492R (I), 519R (5′-GWA

TTA CCG CGG CKG CTG-3′), 533F (5′-GTG CCA GCA GCC GCG GTA-3′), 895F (5′CRC GTC GGG AGT RCR G-3′), 907R (5′-CCG TCA ATT CMT TTR AGT TT-3′), and 1237F (5′-GGG CTA CAC ACG YGC WAC-3′) (Lane 1991; Weisburg et al. 1991 and Hodkinson and Lutzoni 2009). Sequence results were deposited in NCBI GenBank (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB7518780). The MEGA 7.0 program was used to construct phylogenetic trees by maximum likelihood (ML) methods with 1000 iterations (Felsenstein 1985).

GC-MS analysis

The extract obtained from the mentioned procedure was then sent for analysis by gas chromatography-mass spectroscopy (GC-MS) in LIPI, Bogor, Indonesia. The gas chromatography- mass spectroscopy had been carried out on TRACE 1300 GC, TSQ 8000 TRIPLE QUADRUPOLE MS fitted with TG 5MS (30m × 0.25mm, 0.25μm) column and S/SL Injector. The injector temperature had been kept at 250 °C and MS transfer line temperature had been kept at 250 °C along with ion source temperature, also 250 °C. The column temperature had been programmed between 60 and 250 °C at 10°C/min using helium as carrier gas at a carrier flow rate of 1 ml min^{-1}. Injection volume had 1.0 μl prepared in DMSO having split flow 1 ml min^{-1}. The mass spectra had been taken at 75 eV with mass scan range from m/z 40-500 amu. The individual constituents had been identified by comparing their mass spectra with those of standard using NIST (National Institute of Standards and Technology, US Department of Commerce) compounds (Sparkman et al. 2011).

Results and discussion

Isolation of endophytic bacteria

The results were obtained from the stages of the exploration, isolation, and purification of bacteria. There were 26 endophytic bacteria isolated from *D. asper* (Schult with f.) Backer ex Heyne) plant root, 21 from *G. max* Merr. L., 25 from *S. officinarum* L., 19 from *T. cacao* L., 21 from *A. chinensis* L., 21 from *P. merkusii* L., 26 from *G. sepium* ((Jacq.) Kunth ex Walp.), 18 from *P.gajava* L., and 21 from *S. lycopersicum* L. The total number of isolated endophytic bacterial was 198 isolates. Almost all endophytic bacteria that infected higher plants could be isolated from the roots or other parts of the plant. This research finding was supported by Yuan et al. (2015). Eighty-two endophytic bacteria were isolated from bamboo root, rhizome, stem, and leaves. Zhao et al. (2018) found 276 endophytic bacteria isolated from root nodules of soybean. Arthee and Marimuthu (2017) isolated 22 endophytic bacteria from root and stem of sugar cane. Also, Konate et al. (2015) isolated 24 endophytic bacteria from cacao roots. Furthermore, Manikandan et al. (2016) isolated 54 endophytic bacteria from roots and stems of *A. lebbeck*. Abbamondi et al. (2016) recorded 23 endophytic bacteria isolated from tomato roots.

Pathogenicity test on tobacco plant

Based on the results of isolation, from 198 isolates, 5 endophytic bacteria were shown as pathogens. Three isolates endophytic bacteria from *G. maculata* plant root and 2 from *S. lycopersicum* plant roots showed the necrosis symptoms. These 5 isolates could not be used at further experiment because they showed pathogenic activity. Hundred and ninety-three isolates endophytic bacteria were nonpathogenic bacteria without showing necrotic symptom.

Tobacco plants were used as an indicator for hypersensitive testing. The hypersensitive response was indicated by the occurrence of browning on the area inoculated by bacteria. The browning indicated the death of local leaf tissue (necrosis). Hypersensitivity reaction (HR) was expressed as positive (+) when necrotic symptoms were formed on leaf tissue, while unchanged leaf tissue was negatively (−) reacted (Umesha et al. 2008). Nawangsih et al. (2011) reported that there were 49 endophytic bacteria isolated from the tomato plant root, and 8 showed chlorotic or necrotic zones when injected in tobacco leaves.

Antagonistic activity of endophytic bacteria

Pathogenicity test founded 193 isolates. The result of antagonistic activity test showed that there were 13 isolates showed activity > 50% in suppressing FOL. The percentage of inhibition of endophytic bacteria isolated from guava root (AJ_{14}) were the highest activity to FOL (87.30%), isolate from bamboo showed 60.20%, and all other isolates showed 50% inhibition activity (Table 1). Twenty-five isolates of endophytic bacteria from sugarcane roots were not able to inhibit FOL; otherwise, there were isolates from the pine, cacao, and sengon roots that had an average moderate inhibition (50%). Endophytic bacteria isolated from tomato also had the highest inhibition activity (ranged from 83.3 to 80.2%), moderate around 50%.

Inhibition of endophytic bacteria to FOL was estimated by the ranges of inhibition activity following Soytong (1988). The activity > 75% was the highest one, followed by 60–75% a high inhibition, and 50–60% was a moderate inhibition, then <50% was a low inhibition and (−) indicated no inhibition activity. Results of inhibition activity test indicated that 13 isolates showed activity > 50% in suppressing FOL (Table 1).

Endophytic bacteria had a potential effect on suppressing the growth of pathogenic fungi by producing

Table 1 Percentage of antagonistic activity of 13 endophytic bacteria in suppressing *Fusarium oxysporum* f. sp. *lycopersici* (FOL)

No.	Host plant	Isolated code	Percentage isolates against FOL (%)
01	*S. lycopersycum* L.	AM_{02}	83.3
02	*S. lycopersycum* L.	AM_{08}	50.0
03	*S. lycopersycum* L.	AM_{14}	80.2
04	*P. gajava* L.	AJ_{01}	50.6
05	*P. gajava* L.	AJ_{14}	89.2
06	*P. gajava* L.	AJ_{18}	87.3
07	*P. merkusii* L.	AP_{04}	50.2
08	*P. merkusii* L.	AP_{12}	50.9
09	*P. merkusii* L.	AP_{17}	50.2
10	*D. asper* (Schult with f.) Backer ex Heyne	AB_{06}	60.2
11	*D. asper* (Schult with f.) Backer ex Heyne	AB_{18}	50.2
12	*A. chinensis* L.	AS_{09}	50.3
13	*T. cacao* L.	AK_{08}	50.7

secondary metabolites, such as antibiotic, enzyme, hormone, toxins, and volatile compounds, and could be a source of plant resistance. The process of inhibiting endophytic bacteria on the growth of pathogenic fungi caused by several factors, such as space and nutritional competition, antibiotic compounds, and lytic enzymes produced to inhibit the growth of pathogens and to provide resistance to plants (Maksimov et al. 2018).

Strain *B. amyloliquefaciens* was able to produce secondary metabolites (iturin and bacillomycin D), which effectively inhibited *F. oxysporum* (Wang et al. 2016). There were 300 strains of bacterial antagonists isolated from South Korean mining soils that were filtered using multiple culture tests; there were 2 potential antagonistic strains found: *P. aeruginosa* and *B. stratosphericus*; both strains were optimal in inhibiting the mycelial growth of *Fusarium* sp. (Durairaj et al. 2018). According to Altaee et al. (2017), *P. aeruginosa* was able to inhibit 50% *Fusarium* sp. Under the present study, both *Pseudomonas* and *Bacillus* species were found as endophytic bacteria that could affect the growth of *Fusarium* mycelial growth.

Molecular identification of endophytic bacteria

Through this study, 13 endophytic bacterial isolates that had the potential as biocontrol agents against FOL pathogens were identified molecularly, using 16S rRNA (Table 2) and 4 bacterial genera, i.e., *Pseudomonas*, *Bacillus*, *Arthobacter*, and *Serretia* were obtained. Endophytic bacteria isolated from tomato roots were *P. aeruginosa* and *Arthrobacter* sp., from guava roots was *P. aeruginosa*, from pine roots were *P. aeruginosa* and

Table 2 Sequencing DNA from 13 endophytic bacteria isolates

No.	Isolate code	Species	Plant	Assession No.	Length of DNA fragment
01	AM_{02}	*P. aeruginosa*	*S. lycopersycum* L.	MT598016	1.431 bp
02	AM_{08}	*Arthrobacter* sp.	*S. lycopersycum* L.	MT598017	454 bp
03	AM_{14}	*P. aeruginosa*	*S. lycopersycum* L.	MT598018	1.423 bp
04	AJ_{01}	*P. aeruginosa*	*P. gajava* L.	MT598019	1.294 bp
05	AJ_{14}	*P. aeruginosa*	*P. gajava* L.	MT598020	1.430 bp
06	AJ_{18}	*P. aeruginosa*	*P. gajava* L.	MT598021	1.433 bp
07	AP_{04}	*P. aeruginosa*	*P. merkusii* L.	MT598022	1.420 bp
08	AP_{12}	*B. cereus*	*P. merkusii* L.	MT598023	1.465 bp
09	AP_{17}	*P. aeruginosa*	*P. merkusii* L.	MT598024	1.448 bp
10	AB_{06}	*P. moselii*	*D. asper* (Schult with f.) Backer ex Heyne	MT598025	1.424 bp
11	AB_{18}	*P. aeruginosa*	*D. asper* (Schult with f.) Backer ex Heyne	MT598026	1.446 bp
12	AS_{09}	*S. marcescens*	*A. chinensis* L.	MT598027	1.410 bp
13	AK_{08}	*B. thuringiensis*	*T. cacao* L.	MT598028	1.454 bp

bp base pair

B.cereus, from bamboo roots were *P. moselii* and *P. aeruginosa*, from sengon roots was *S. marcescens*, and from cacao roots was *B. thuringiensis.* A phylogenetic tree constructed, using 16S rRNA sequences of the suspected endophytic bacteria isolates related taxa, was generated by the maximum likelihood method (presented in Fig. 1).

Egamberdieva et al. (2017) reported that endophytic bacterial isolates from *Cicer arietinum* L. plant roots and identified using 16S rRNA as *B. cereus*, *B. subtilis*, and *B. thuringiensis* were able to inhibit the pathogen *F. oxysporum*. Based on the research of Bredow et al. (2015), endophytic bacteria associated with coffee plant (*Coffea arabica*), sugar cane (*Saccharum officinarum*), bean *(Pisum sativum)*, corn (*Zea mays* L.), soybean (*Glycine max*.), tomato (*S. lycopersicum*), and grape (*Vitis venifera* L.) analyzed using 16S rRNA showed that *Bacillus*, *Pseudomonas*, and *Mycobacterium* were the most common genera that colonized these plants. Arthee and Marimuthu (2017) reported that *Bacillus* sp. and *Burkholderia* sp. were identified using 16S rRNA as endophytic bacteria in sugarcane plant. Yuan et al. (2015) stated that *Arthrobacter*, *Staphylococcus*, *Bacillus*, and *Enterobacter* were the dominant bacterial strains as endophytic bacteria that colonized bamboo plant. Obtained results were compatible with that reported by these workers and shared the same types of bacterial species, such as the genera *Bacillus*, *Pseudomonas*, and *Serretia* which were dominantly present from isolation in plant roots (Table 2).

GC-MS analysis

Methanolic extraction was characterized and identified by GC-MS analysis. The interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The active principles with their retention time (Rt),

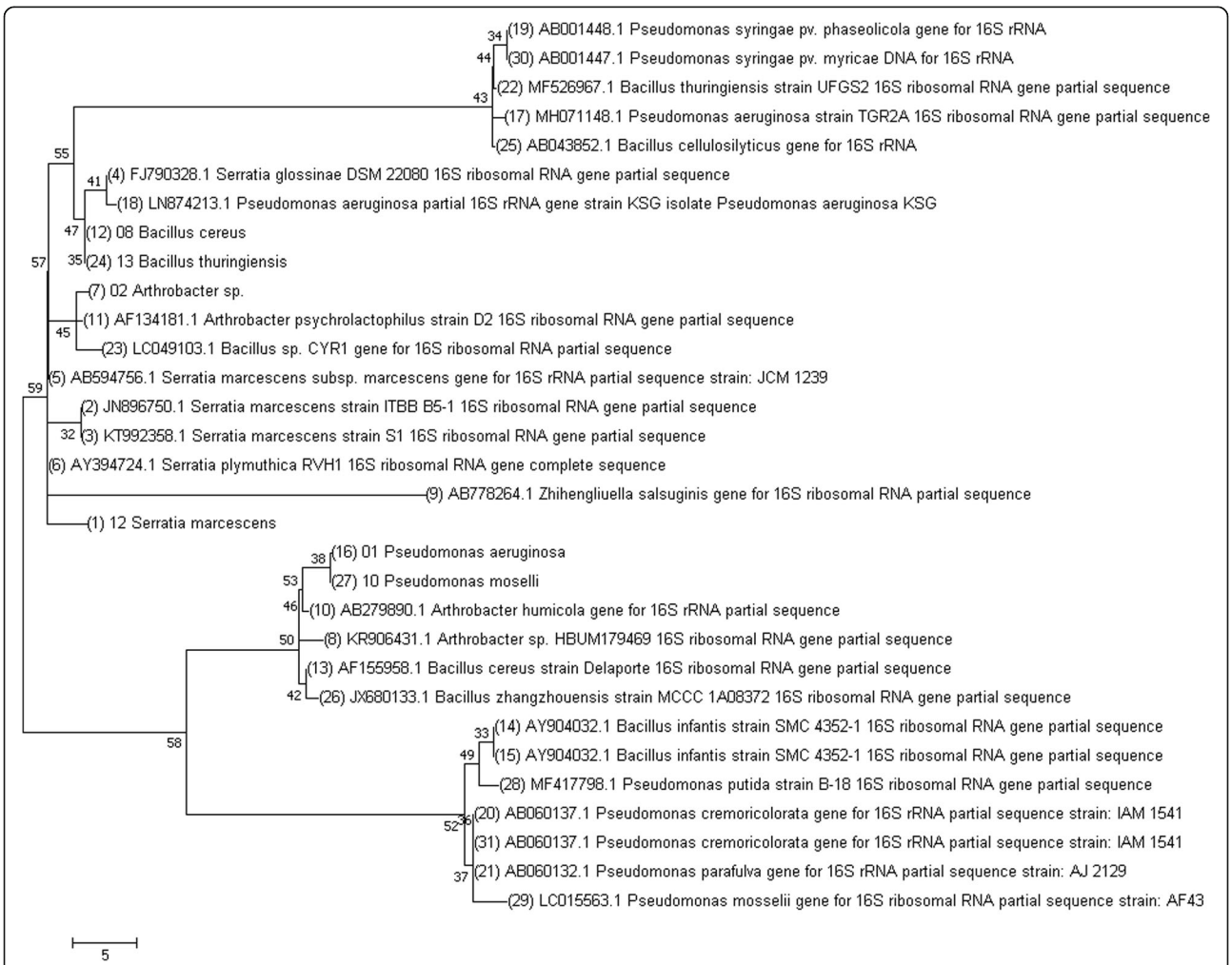

Fig. 1 Phylogenetic tree analysis of 16SrRNA gene by using Tamura-Nei model

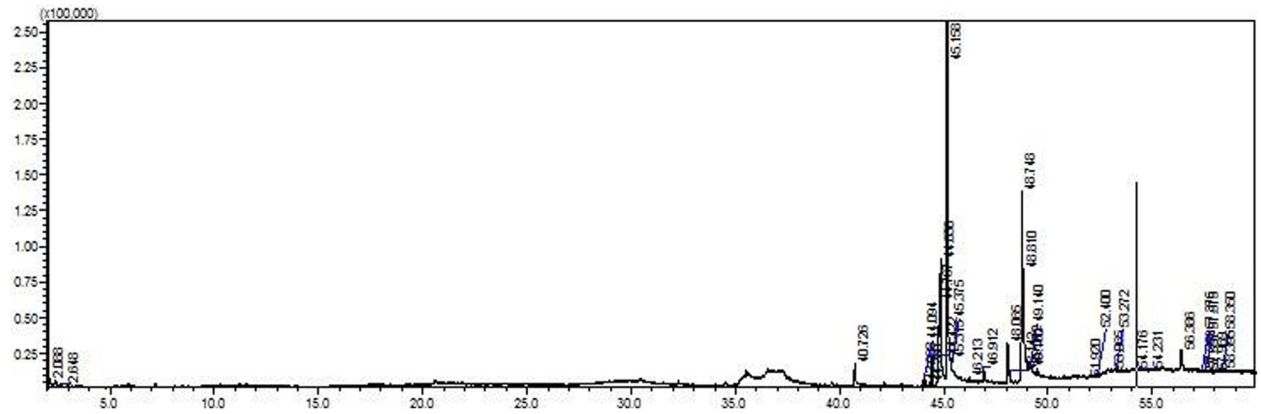

Fig. 2 GC-MS chromatogram of methanol extract of *Pseudomonas aeruginosa*

molecular formula, molecular weight, and concentration percentage (area %) are represented in Fig. 2. The major compounds in GC-MS analysis of *P. aeruginosa* was Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (0.33%).

Conclusion

One-hundred and ninety-eight isolates were isolated from 9 higher plants in Aceh, Indonesia. Thirteen of them were able to inhibit FOL at moderate and high rates. The 13 isolates were identified using 16S rRNA. One of the bacterial strains isolated from guava root (*P. aeruginosa*) achieved 89.2% inhibition and showed a superior ability to inhibit FOL.

Abbreviations

FOL: *Fusarium oxysporum* f. sp. *lycopersici*; 16S rRNA: 16S ribosomal ribonucleic acid; GC-MS: Gas chromatogram mass spectrometry; DW: Distilled water; NA: Nutrient agar; OD: optical density; LB: Lysogeny Broth; DNA: Deoxyribonucleic acid; PCR: Polymerase chain reaction; NCBI: National Center for Biotechnology Information; ML: Maximum likelihood; DMSO: Dimethyl sulphoxide; NIST: National Institute of Standards and Technology; TSQ: Thermo Scientific Gas; TG: Thermo gravimetric

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Authors' contributions

The concept and design of the experiments were prepared by all authors. VM conducted the experiments, analyzed the results, and wrote the manuscript. TH and KH performed the experiments and analyzed the data. RS, LS, S, and KH conceived the experiment and wrote and verified the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

Not Applicable.

Ethics approval and consent to participate

This manuscript is written in accordance with the guidelines for the authors available at journal website. In addition, this work has never been published before and now approved by all authors and host authorities.

Consent for publication

Not Applicable.

Competing interests

No potential conflict of interest was reported by the authors.

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