Bioactive compound of Streptomyces capoamus as biocontrol of Bacterial Wilt Disease on Banana Plant

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Abstract. Banana is a plant that has a high economic value for human to fulfill his daily fruits need. The antagonistic test between nine isolates Streptomyces sp. and R. solanacearum showed that the Streptomyces sp. 9 has the highest resistance (19 mm), indicating a strong inhibition and has smallest concentrations of inhibition (MIC) of 10%. The glasshouse scale test demonstrated that the Streptomyces sp. 9 culture sprayed 4 times on a different day could suppress bacterial wilt disease by 100% from 11% in control. The research was to determine molecular identification of Streptomyces sp.9 using 16S rRNA and identification of active compound by Gass Chromatography Mass Spectra (GCMS). The results showed that the molecular identified Streptomyces sp.9 as a Streptomyces capoamus. The chemical compounds which have antimicrobial are Hexadeconoic acid methyl ester, Heneicosane, Hexacosane(CAS) n-Hexacosane, Limonene, Dotriacontane and Tetracontane as antimicrobial agent. The conclusion was Streptomyces capoamus showed various bioactive compounds, which is importance as potential biocontrol on bacterial wilt disease on banana plants. There could be probably of new bioactive compounds and this also provided a new insight towards the development of good candidates for bioactive natural products.

1. Introduction
Plants disease may result in losses in quantity and quality of the crop caused by bacterial wilt on tomatoes, peppers, potatoes, ginger and banana plants. Among the four plants, banana is the most widely attacked by R. Solanacearum pathogen. According to the interviews with farmers Mr I Wayan Diandra (March 2014) in the village of Pendem jembrana Bali Indonesia, bacterial wilt disease struck his entire banana crop area of 20 ha. Even more to that the whole banana plant cultivated in the village of Pendem, in Jembrana Bali Indonesia also suffered the same situation which total area of 4 ha. The symptoms of the bacterial wilt disease in banana plants are wilted leaves, brown spots on the vessel of the banana stems and the fruit become rotten and dried.

Streptomyces also lives in the most rhizosphere of some crops like pepper, tomato, onion and legume crops. The existence of Streptomyces in the rhizosphere of plants can serve to keep these plants from pathogens both fungi and bacteria [1]. The Genus of Streptomyces can also produced antibiotics to protect plants from pathogens that attack plants [2].

Many genus Streptomyces have been used as anti-fungi agents to control several pathogenic fungi on plants. The capability of Streptomyces in inhibiting growth of pathogenic fungi is due to its capability to produce both antifungal agent and extracellular hydrolytic enzyme those are able to degrade fungi’s cell wall [3]. Streptomyces sp. has the capability to produce antibiotics with wide
variation of chemical structure. Streptomyces sp. ANU 6277 produces 8-hydroxyquinoline with antifungal and antibacterial characteristics[4].

Nine isolates of Streptomyces sp were found in the banana plant rhizosphere soil in the village of Pendem, in Jembrana District with different characteristics to one another [5]. Furthermore, antagonistic test between Streptomyces sp. and R. solanacearum showed that the Streptomyces sp. 9 has the highest resistance (19 mm), indicating a strong inhibition with the resulting metabolite filtrate had the smallest concentrations of inhibition (MIC) of 10%. The glasshouse scale test demonstrated that the Streptomyces sp. 9 culture sprayed four times on a different day could suppress bacterial wilt disease by 100% from 11% in controls [6]. This research were to determine molecular identification of Streptomyces sp.9 using 16S rRNA, and identification antibiotic compounds by using GCMS analysis.

2. Material and method
This research was conducted in the Laboratory of Microbiology Department of Biological Science UNUD, Joint Laboratory, Faculty of Mathematics and Natural Sciences, University of Udayana. This study was conducted from March 2016 to September 2016. Streptomyces sp.9 molecular identification using 16S rRNA primers 20f (5'-G AGTTTGATCCTGGCTCAG) and 1500r (5'GTTACCTTGTTACGACTT) was conducted in the Laboratory of Microbiology, Bogor Agricultural Institute (Institut Pertanian Bogor/IPB).

2.1. Isolation of genomic DNA
Rejuvenation of bacterial isolates was done by using Yeast Salt Agar (ISP6) media and the cultures were incubated at room temperature for four days. Five colony circles (diameter 5 mm) were added to Yeast Salt Broth (ISP7) media and incubated in an incubator shaker at a temperature of 25°C for four days. A total of 1.5 ml bacterial culture was incorporated into 1.5 ml Eppendorf, it was then centrifuged at a speed of 800 rpm for 10 minutes. The supernatant was discarded and the formed pellets were washed with STE buffer (composition: 0.3 M sucrose; 25 mM Tris-HCl; 25 mM EDTA.2Na pH 8), then centrifuged at 8000 rpm for 10 minutes. Pellets were washed three times repeatedly. Furthermore, the supernatant was discarded and to the pellet was added 200 µl STE buffer and 45 µl lysozyme (20 mg/ml) and frequently turned slowly then incubated at 55 ° C for 1 hour to form protoplasts. A total of 20 µl proteinase K (20 mg/ml) was added to the mixture and incubated at 55 ° C for 1 hour. Followed by the addition of 400 µl of 10% CTAB in 0.7 M NaCl solution and then incubated at 65 ºC for 30 minutes. Then to the solution was added one time the volume of phenol: chloroform (25:24) and centrifuged at 12,000 rpm for 10 minutes. Clear phase was transferred to a new tube and added with 0.6 times the volume of isopropanol and 20 µl of sodium acetate, incubated at a temperature of -20ºC overnight. Then centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded while the pellet was washed using 1 ml of 70% alcohol. Deoxyribose Nucleic Acid (DNA) was wind dried for 1 hour to dispose of alcohol and then dissolved in 50 µl sterile ddH2O and stored at 4 ºC.

2.2. DNA amplification by PCR
16S rRNA gene was amplified by Polymerase Chain Reaction machines (Perkin Elmer GeneAmp PCR system 2400, Germany) using specific primers of prokaryotic actinomycetes, namely 20f (5'-GAGTTTTGATCCTGGCTCAG) and 1500r (5'-GTTACCTTGTTACGACTT). The composition of the PCR reaction consisting of Taq DNA polymerase enzyme La 0.5 mL, 25 mL 2X GC buffer, dNTP mixture 8 mL, 20f primer (10 pmol) 1.5 µl, 1387r primer (10 pmol) 1.5 µl, ddH2O 9.5 µl and 4 µl DNA template. PCR conditions used are pre-denaturation (94 ºC, 5 min), denaturation (94 º C, 1 min), annealing (57°C, 1 minute and 10 seconds), and the post-PCR (72°C, 7 min) with the number of cycles of 30 cycles. The separation of DNA PCR products is done on a mini-gel electrophoresis machine using 1% agarose on 75 Volt power supply voltage for 45 minutes. Visualization of DNA was done over the UV transilluminator using Ethidium Bromide (EtBr) dye.

2.3. DNA sequence and alignment
The amplified Deoxyribose Nucleic Acid (DNA) was partially sequenced to determine the nucleotide sequences using the services of PT Macrogen, Korea. The nucleotide sequences, as the result of the
sequencing process, would then be aligned with the GeneBank data using BLAST-N program (Basic Local Alignment Search Tool-Nucleotides) from the NCBI website (National Center for Biotechnology Information).

### 2.4. Preparation of Streptomyces sp.9 filtrate

Streptomyces sp.9 was grown for five days at YEMA media and incubated at 28 ± 2°C, then taken using cork borer (diameter of 5 mm) by 5 pieces and put in a glass bottle filled with 100 mL Yeast Malt Extract Broth media. Then it was incubated on a Rocking Platform Mixer at a speed of 80 rpm for 14 days. Centrifugation process was then performed at a speed of 10,000 rpm for 15 minutes to obtain a supernatant and pellet section. The supernatant was filtered using 0.45 µm filter paper. The filtrate was taken while the residue was discarded. The obtained filtrate was evaporated and then partitioned by introducing the filtrate into a 250 mL separator flask and was added with the solvent of ethyl acetate 125 mL plus 125 ml filtrate with a ratio of 1: 1 (v / v). Then it was homogenized and left undisturbed for 24 hours to separate between water phases and ethyl acetate phase. Each of these phases (water and ethyl acetate phase) was separated by separating funnel and then each phase was evaporated with an evaporator machine at a temperature of 40°C until its concentration becomes one fifth.

### 2.5. Compounds analysis of the actinomycetes filtrate by thin layer chromatography (TLC) and Gas Chromatography-Mass Spectrometry (GCMS)

The results of each phase at 4.8.5 which had the highest inhibitory capability will be tested by using TLC. The filtrate was spotted on a TLC plate (silica gelplat Merck 60 F254) inflated with a mixture of chloroform-ethyl acetate-acetic acid (7: 3: 1, v / v). Active spot was visualized under UV light λ254 and λ365 nm [7]. o purify the compounds, column chromatography was performed and to identify the active compounds of the Actinomycetes filtrate, GCMS was performed. Gas chromatography-mass spectrometry was carried out to obtain molecular weight of the active compound.

### 2.6. The activity test of the active compound in the antibiotic filtrate of Streptomyces sp.9 with the well diffusion method

The testing was done by examining the filtrate activity of the Streptomyces sp. against R. solanacearum. Petri dish containing 10 ml of NA medium and 200 µl R. solanacearum suspension was allowed to solidify. Then diffusion well was made in the center of the Petri dish using a cork borer. 20 µl of the filtrate were added into the diffusion well.

### 3. Results and discussion

#### 3.1. Molecular Identification of Streptomyces sp.9 using 16S rRNA

Genome Amplification of bacterial samples using PCR with primers 63F and 1387r produced DNA fragments measuring approximately 1300 bp.

#### 3.2. Phylogenetic tree construction of Streptomyces sp.9

Based on these data, the Streptomyces sp.9 can be identified as Streptomyces capoamous with 99% similarity (Figure 1).
3.3. Isolation and identification of active compounds from the antibiotic produced by *Streptomyces capoamus*

The result of the antibiotic fractionation using a hexane solvent, Ethyl acetate and n butanol, was 6 fractions were found. Followed by the Thin Layer Chromatography (TLC) test and observed using ultraviolet light, four active compounds were found. To the four active compounds, antagonist test was conducted with pathogen *Ralstonia solanacearum*. The result is shown in Table 1 and Figure 2. The
result showed that RF mixture (RFG1, RFG2, RFG3 and RFG4) has the highest inhibitor (24 mm) and namely RFG4 and then analyzed for the compounds by using GCMS as indicated on Table 2 and Figure 4.

Table 1. The antagonist test of the active compound in R. solanacearum.

| No | Active compound              | Inhibitory (mm) |
|----|------------------------------|-----------------|
| 1  | RFG1                         | 7               |
| 2  | RFG2                         | 20              |
| 3  | RFG3                         | 22              |
| 4  | RFG4                         | 19              |
| 5  | RF mixture (RF1,RF2,RF3,RF4) | 24              |

Gas chromatography-mass spectrometry (GC-MS) analysis showed 33 chemical compounds. The chemical compounds which have antimicrobial properties identified in the n butanol extract of S. capoamus are Hexadeconoic acid methyl ester, Heneicosane, Hexacosane(CAS) n-Hexacosane, di Limonene, Dotriacontane and Tetracontane. Several substances were found namely 3,5-Dichloro-2pyridone, Benzeneacetic acid, Heptadecane, Phenol, 2,4-bis(1.1-dimethylethyl), Dodecanoic Acid Methyl, Ester, Hexadecanoic Acid Methyl Ester, 1-(+)-Ascorbic Acid 2,6 Dihexadecanoate were identified by GC-MS characterization technique. These chemical compounds are the isolate substance derived from Streptomyces galbus TP2 and Streptomyces humidus, which are recognized as antifungal and have a significant role in medical. Furthermore Analysis by GCMS of extract Streptomyces cacaoi strain SU2 were produce Heneiconsane, Dodecane, Eicosane, and Cetene that have antimicrobial activity to pathogenic bacteria and fungi [8].
Ethanol leaves extract of Ehretiaabyssinica contained mainly Octadecenamide (5.77%); Lucenin 2 (5.46%); Docosane and Nonacosane (3.75%); Cyclopropene (3.50%); Hematoporphyrin (2.68%); Tetratetracontane (2.36%); Dotriacontane (1.57%); Acetic acid (1.53%); Nmethylglycine (1.49%); Propyne antimicrobial (1.41%). All identified compounds are known to have antimicrobial activity. Those compound can inhibit the growth of Pseudomonas aeruginosa, Staphylococcus aureus and Micrococcus [9]. Streptomyces strain KX852460 produced Eicosane (C20H42) and dibutyl phthalate (C16H22O4) as antifungal activity and might have potential biocontrol antagonist against R. Solani AG-3 KX852461 [10].

Table 2. Active compounds from the antibiotics filtrate of Streptomyces capoamu by using GCMS analysis with n-butanol solvent.

| No | Active compound name | Molecular formula | Molecular weight | Retention time (minute) |
|----|----------------------|-------------------|------------------|------------------------|
| 1  | 3,3-Dimethoxy-2-butane | C6H12             | 89               | 3.115                  |
| 2  | Di-sec-butyl ether    | C6H22O3           | 103              | 3.204                  |
| 3  | Nonane(CAS)n-Nonane   | C9H20             | 57               | 3.655                  |
| 4  | Decane (CAS) n-Decane | C10H22            | 43               | 5.212                  |
| 5  | 1-Hexan-2-ethyl-(CAS)2ethyl hexan | C8H18O | 57 | 5.739 |
| 6  | Butane,1,1-dibutoxy   | C12H26O2          | 57               | 9.167                  |
| 7  | n-Butyric acid 2-ethylhexyl ester | C12H24O2 | 71 | 10.168 |
| 8  | Octane,1,1-oxib       | C16H34O           | 57               | 10.422                 |
| 9  | 3,3-Dimethoxy-2-butane | C7H16FO2P         | 127              | 12.752                 |
| 10 | Cycloheptaxoylone, tetradecaethylm | C14H42O7Si7 | 73 | 14.741 |
| 11 | Hexadeconic acid, methyl ester | C7H34O2 | 74 | 17.367 |
| 12 | Octadecamethylocylonnosilox | C18H48O6Si7 | 73 | 17.910 |
| 13 | 9-Octadecenoic acid,methyl ester | C19H36O2 | 55 | 19.080 |
| 14 | Octadecanoic acid, methyl ester | C19H38O2 | 74 | 19.316 |
| 15 | Heneicosane           | C20H42            | 57               | 21.718                 |
| 16 | Tetracosamethylocyclodecasi | C18H54O9Si9 | 237 | 20.560 |
| 17 | Hexacosane(CAS)n Hexacosane | C20H45 | 57 | 22.562 |
| 18 | 1,2-Benzenehexanoylcarboxylic acid, ditridecyl ester | C3H4H8O4 | 149 | 22.683 |
| 19 | Tetrocontaine         | C40H82            | 57               | 23.523                 |
| 20 | Triacontane,1-bromo   | C40H82            | 57               | 24.332                 |
| 21 | Dotriacontane         | C32H66            | 57               | 26.046                 |
| 22 | dl-Limonene           | C10H16            | 68               | 5.803                  |
| 23 | 3,1-Hydroxy-3-methylvaleric acid | C7H14O | 43 | 4.168 |
| 24 | Hexane,2,3,5-trimethyl-(CAS)2,3,5-Trimethyl | C4H2O | 43 | 4.552 |
| 25 | Ozirane-2-Carboxylic acid ethyl | C5H8O3 | 43 | 4.967 |
| 26 | 3-Hexen-2-one(CAS)1-Butenyl methyl keton | C7H10O2 | 43 | 4.805 |
| 27 | Cyclopentaxylone, decamethyl-(CAS) Dime | C10H30O5Si5 | 73 | 10.785 |
| 28 | Oxalic acid, cyclohexymethyl tridecyl ester | C22H4O4 | 97 | 14.494 |
| 29 | Cyclooctasiloxane, hexadecamethyl | C16H48O8Si8 | 73 | 16.400 |
| 30 | Hetexhexane, hexadecamethyl-(CAS) Hexadecamethyl | C16H48O6Si7 | 73 | 17.910 |
| 31 | Tetracosamethylocyclododecasi | C24H72O12Si12 | 237 | 20.560 |
| 32 | 2-Hexanone,3,3-dimethyl-(CAS)3,3-Dimethy | C8H16O | 43 | 3.906 |

Chemical composition of the essential oil of Eucalyptus globulus Labill., grown in Montenegro, was analyzed by GCMS and it has antimicrobial activity. Other compounds identified in the oil were β-pinene, limonene, α-phellandrene, γ-terpinene, linalool, pinocarveol, terpinen-4-ol, and α-terpineol. The results of the antimicrobial activity tests revealed that the essential oil of E. globulus has a strong antimicrobial activity, especially against Streptococcus pyogenes, Escherichia coli, Candida albicans, Staphylococcus aureus, Acinetobacter baumannii, and Klebsiella pneumoniae [11]. Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) analysis of S. thermocarboxydu
filtrate can inhibited the growth of *Fusarium oxysporum* by damaging cell wall and plasma membrane on macroconidia, microconidia cell, hypha and chlamydospora [12].

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4. Conclusion

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Acknowledgement

On this occasion, the authors would like to thank profusely to the Institute for Research and the Community Services University of Udayana which facilitates to obtain the Competitive Research Grant funds from the Ministry of Education and Culture of the Republic of Indonesia based on the Letter of Assignment No; 311-39 / UN14.2 / PNL.01.03.00 / 2015. Grant International Conference from Ministry of Education and Culture of the Republic of Indonesia based on the Letter of Assignment No; 1442/E.5.3/PB/2016 in Faculty of Agriculture Ryukoku University Kyoto Japan September 2016.