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Characterization of *Ralstonia solanacearum* Using Fourier Transform Infrared (FTIR) Spectroscopy

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KEYWORDS

Ralstonia solanacearum; FTIR spectroscopy; characterization; detection; biomarkers.

Abstract *Ralstonia solanacearum*, the causal agent of bacterial wilt disease is worldwide in distribution, and results in serious economic losses, particularly in the tropics. Detection and characterization of microorganisms by Fourier transform infrared spectroscopy (FTIR) technique promises to be of great value because of the method's inherent sensitivity, small sample size, rapidity, and simplicity. In this study, we used FTIR spectroscopy for the characterization of *Ralstonia solanacearum*. The bacteria were grown on Nutrient Agar (NA) at 28°C for 48 hours. The colonies of *Ralstonia solanacearum* on nutrient agar medium were smooth circular, raised, and dirty white. Cultures of bacteria were identified by molecular methods using PCR techniques. The DNA was amplified using a specific primer pair, 759f/760r (forward primer: 5'- GTCGCCGTCAACTCACTTTCC 3', reverse primer: 5'-GTCGCCGTAGCAATGCGGAATCG-3'). The PCR produced a single band of 280 bp from the isolated DNA of cultured bacteria. Bacterial spectra were obtained in the wavenumber range of 4000–400 cm^{-1} using FTIR spectroscopy. The identification of cell wall constituents in region 3000–2800 cm^{-1}, the proteinaceous structure of bacteria in region 1665–1200 cm^{-1}, and the fingerprint of bacteria in region 1200-800 cm^{-1} are all part of the spectra analysis in this study. Absorption bands obtained from bacteria *Ralstonia solanacearum* samples associated with protein, phospholipids, nucleic acids, and carbohydrates appear in the bacterial IR absorption spectra.

Introduction

Bacteria are the cause of serious infections and diseases in different organisms. Ralstonia solanacearum, the causal agent of bacterial wilt disease is worldwide in distribution, and results in serious economic losses, particularly in the tropics (Kago *et al*., 2016). Ralstonia solanacearum has a very wide range of host plants. More than 400 species of plants from 50 botanical families, including important plants that are cultivated in Indonesia such as potatoes, tomatoes, eggplants, tobacco, cloves, and bananas (Setiawan, 2019). Early and rapid detection and identification of the pathogen bacteria are crucial for effective control and treatment.

Currently, most of the methods used for the identification and classification of bacterial samples are time-consuming (24–48h), with low specificity. These methods are mainly based on physiological identification of the bacteria and involve visual and microscopic observations of the bacteria after 24 h growth in selective media. Other methods used for bacterial identification are biochemical, immunological, and molecular. Although these methods are fast and accurate, they

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are not effective for screening large numbers of samples. In addition, the immunological methods depend on the existence of specific antibodies for the examined bacteria. The molecular methods are expensive and available only for limited isolates (Salman *et al*., 2019).

Detection and characterization of microorganisms by Fourier transform infrared spectroscopy (FTIR) technique promises to be of great value because of the method's inherent sensitivity, non-destructive, small sample size, rapidity, simplicity, and the potential for complete computerization (Salman *et al*., 2010; Maquelin *et al*., 2002). Naumann *et al*. (1991) have shown that FTIR absorption spectra are highly specific fingerprints of microbial cells and that by using IR spectroscopy it is possible to differentiate between bacterial cells, even at the isolate level.

Each bacterial species has a complex cell wall/membrane composition which gives a unique IR fingerprint, due to the stretching and bending vibrations of molecular bonds or functional groups present in its proteins, nucleic acids, lipids, sugars, and lipopolysaccharides (LPS). The molecular composition varies from species to species and even at strain levels. Therefore each bacterium will have a unique and characteristic spectrum, and single microorganisms could be identified from an FT-IR spectrum (Davis & Mauer, 2010).

IR spectra measured for intact cells of bacteria are usually complex and the peaks are broad due to the superposition of contributions from all the biomolecules present in a bacterial cell (Davis & Mauer, 2010). In the absence of water, Naumann (1991) recommends that five major absorbance regions in IR spectra should be analyzed for the identification of bacteria: the 3000–2800 cm^{-1} spectral region is the fatty acid region (region I); 1700-1500 cm-1 contains the amide I and II bands of proteins and peptides (region II); 1500-1200 cm^{-1} is a mixed region of fatty acid bending vibrations, proteins, and phosphate-carrying compounds (region III); 1200-900 cm^{-1} contains absorption bands of the carbohydrates in microbial cell walls (region IV), and 900-700 cm^{-1} is the 'fingerprint region' that contains weak but very unique absorbances that are characteristic to specific bacteria (region V). Regions I and II are the most useful for routine bacterial identification; however, the other regions may be used to better understand minor variations in the structure and composition of the bacteria. The fingerprint region is significant for the discrimination of microorganisms at the strain level.

In the present study, FTIR spectroscopy was used to characterize the plant pathogen bacteria Ralstonia solanacearum. Investigation of bacteria samples by FTIR spectroscopy to determine the specific spectroscopic biomarkers useful for identification and discrimination of selected bacterial species.

Materials and methods

Cultivation of bacteria

This study was conducted on June to September 2022. In this study, bacteria Ralstonia solanacearum were supplied by the Laboratorium of Plant Quarantine, Agricultural Quarantine Standard Test Center, Jakarta. The bacteria were grown in Nutrient Agar (NA) at 28 °C for 48 hours. Cultivated bacteria were split into duplicate samples that were used for identification by FTIR spectroscopy and molecular characterization respectively.

Molecular characterization

The bacteria were grown in nutrient agar media for identification using PCR techniques, and the nucleotide sequence was determined as follows:

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DNA extraction

A culture of bacteria 5-10 mg of 48 hours old colonies was taken by a bacterial loop and transferred into a tube, suspended in 1000 µl of ddH2O, homogenized by the vortex. Bacterial suspensions were pelleted by centrifugation at 1000 rpm for 2 minutes. The pellet was resuspended with 500 ml of ddH2O and was ready for DNA extraction using CTAB methods (Doyle & Doyle, 1990) with modification. The modification was carried out by adding PVP (Polyvinylpyrrolidone) and β-mercaptoethanol compounds. The ingredients for the extraction buffer consisted of 2% (w/v) CTAB (Cetyl trimethylammonium bromide) 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, and 1% (w/v) PVP. The PVP compound was added to the buffer when the extraction activity was going to be carried out, while the β-mercaptoethanol compound was not added directly to the extraction buffer but was added separately because of its hazardous and pungent odor. The extraction buffer was preheated at 60°C while stirring to facilitate the homogenization of the components, then cooled at -20°C for 10 minutes before use.

PCR amplification and DNA sequencing

The DNA was amplified using a specific primer pair, 759f/760r (forward primer: 5'-GTCGCCGTCAACTCACTTTCC 3', reverse primer: 5'-GTCGCCGTAGCAATGCGGAATCG-3') which amplified at 280-bp fragment (Opina et al., 1997), using Dreamtaq green PCR master mix 2X (Thermo). The final volume of each PCR reaction mixture was 25 µl containing: 12.5 µl Dreamtaq green PCR master mix 2X, 1 µl (10 µM 759F Primer), 1 µl (10 µM 760R Primer), 1 µl template DNA (30 ng/µl), and 9.5 µl of nuclease-free water. The following cycling program was used in a thermal cycler: 96°C for 5 minutes, followed by 30 cycles of 94°C for 15 seconds, 59°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension period of 10 minutes at 72°C. The PCR product was subjected to electrophoresis on 1.5 agarose gel. Agarose gel electrophoresis of DNA was performed with TAE buffer, stained with ethidium bromide, and bands were visualized on a UV-transilluminator. This PCR amplifies the DNA on the 280-bp fragment.

Fourier Transform Infrared Spectra Measurement

IR bands depend upon the nature of the molecular bond, the atoms involved, and the strength of the intermolecular interactions. Thus, the IR spectrum of the examined molecules is considered a biochemical fingerprint (Salman *et al*., 2019).

Sample preparation

Bacterial cells were taken with a bacterial loop from the agar surface amount of 2 mg, suspended in 1000 µl of ddH2O, and pelleted by centrifugation at 1000 rpm for 2 min. Each pellet was resuspended with 50 µl of ddH2O. These preparation samples are based on the methods of Salman (2019) with modifications. A drop of 20 µl of the obtained sample was placed on a certain area on the Zinc-Selenide crystal that is transparent to IR radiation, air dried for 15 minutes, and then examined by FTIR microscopy.

Fourier Transform Infrared Spectra Measurement

IR absorption spectra were acquired from these samples in the 400 to 4000 cm-1 range with 4 cm-1 spectral resolution. Each sample (suspension) was taken at 20 µl. Then, the FTIR spectrophotometer (JASCO 6800) was blank with water as the sample measurement background. The sample was then measured for its FTIR spectrum with an FTIR spectrophotometer with a TGS detector and attenuated total reflectance (ATR, Pro One) at an incident angle of 45 deg and a scanning speed of 2 mm/second.

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Results and discussion

Characterization of Ralstonia solanacearum

The colonies of R. solanacearum on nutrient agar medium were smooth circular, raised, and dirty white (Figure 1). R. solanacearum was small straight rod-shaped, measuring 1.5–3.12µ X 0.25–2.5µ and Gram-negative with a single polar flagellum (Pawaskar, 2014). Bacteria are aerobic and their colonies on solid media are small, irregularly spherical, white in reflected light, and brown in transmitted light (Hayward, 1991).

Figure 1. Cultural appearance of *Ralstonia solanacearum* on Nutrient agar after 48 hours incubation.

With the species-specific primer (759F/760R), the PCR produced a single band of 280 bp from the isolated DNA of cultured bacteria (Fig. 2). This banding pattern was in agreement with the result of Opina *et al*. (1997).

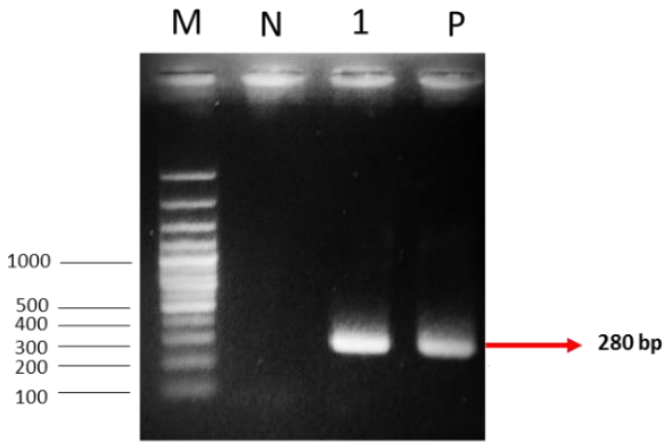

Figure 2. DNA products amplified by polymerase chain reaction (PCR) of *Ralstonia solanacearum.*
(M: 100 bp DNA ladder (loaded 3,5 µl), N: negative control, 1: genomic DNA of *R. solanacearum,* P: Positive control).

FTIR spectra of Ralstonia solanacearum

Samples of bacteria were investigated by FTIR spectroscopy to determine the specific spectroscopic biomarkers useful for identification and discrimination of selected bacterial species.

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The wave number positions of absorbance peaks, peak intensities, and peak widths are useful for a functional group, cell component, and sample identification. Several interesting peaks appear on an IR spectrum of bacteria, and most of them represent functional group vibrations in the main biomolecular constituents like protein, fatty acids, nucleic acids, and carbohydrates. Specific identification can be achieved from spectra by focusing on specific absorbance regions related to those compounds that are diagnostic for a specific pathogen (Davis & Mauer, 2010).

Identification by a spectral band of cell wall constituents, lipids, and proteins

The 2800-3000 cm−1 region is contributed mainly by phospholipids and proteins due to the functional group, CH2 and CH3 symmetric and asymmetric stretching vibrations (Salman *et al*., 2019). In this study, the bands of cell wall constituent were found at 2955 cm−1, 2923 cm-1, and 2852 cm−1 (Fig. 3). The band at 2955 cm−1 is assigned to C-H asymmetric stretching of -CH3 in fatty acids (Davis & Mauer, 2010). The bands at 2923 cm-1 and 2852 cm−1 are symmetric and asymmetric of CH2, respectively, representing a group of lipids (Maity *et al*., 2013).

Cell proteins are typically indicated by several amide bands (Maity *et al*., 2013). The present study from 1665 to 1200 cm-1 was assumed to be dominated by proteins (Figure 4). Many peaks were observed in this region. The band at 1646 cm−1 was attributed to protein amide-I (Huleihel *et al*., 2018). The NH2 stretching was associated with proteins of the amide II band at 1635 and 1541 cm−1 (Skolik *et al*., 2020). Proteins Amide-III contributes at 1231 cm−1 (Maity *et al*., 2013). Meanwhile, CH2 binding and C=O symmetric stretching of COO- assigned to lipids were found at 1455 and 1396 cm−1 (Davis &Mauer, 2010; Maity *et al*., 2013).

The 1200-900 cm^{-1} regions contain absorption bands of cell wall carbohydrates and polysaccharides, and also of nucleic acid due to the stretching vibration of PO_2^- groups found in nucleic acids, as well as C-O, C-O-H, and C-O-C deformation vibration and C-O-P stretching vibration (Zarnowiec *et al*., 2015). A large absorbance band at 1080 cm^{-1} is mainly contributed to carbohydrate and nucleic acid vibrations found in this region. The peak at 1080 cm^{-1} was attributed to PO_2^- symmetric stretching of nucleic acids and phospholipids (Salman *et al*., 2014). Also, the peaks at 1050 and 970 cm^{-1} were found and assigned as PO2 very strong stretching, representative of glycopeptides and ribose as shown in Figure 5 (Maity *et al*., 2013).

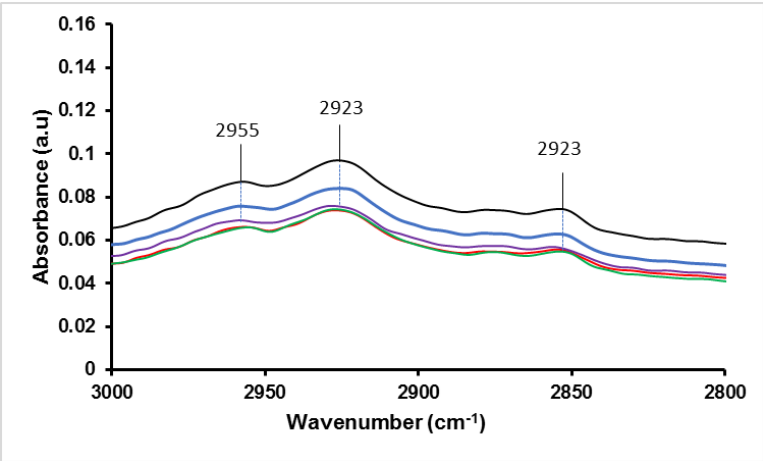

Figure 3. Cell wall constituents of bacteria in the FTIR spectral region of 3000-2800 cm^{-1} from bacteria *Ralstonia solanacearum* samples.

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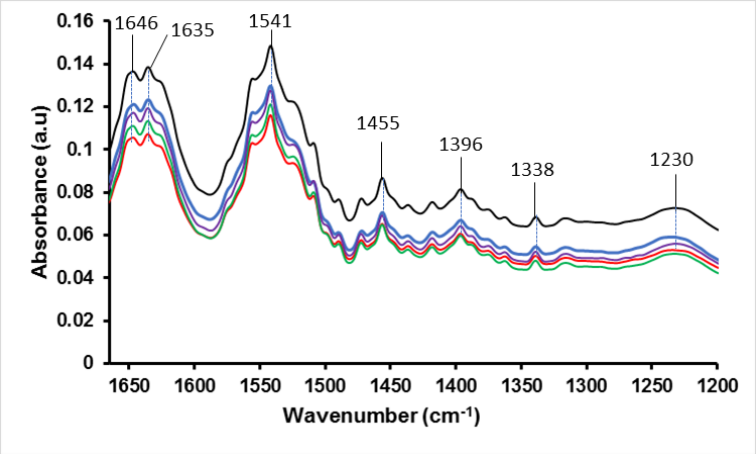

Figure 4. Proteinaceous structure of bacteria in the FTIR spectral region of 1665–1200 cm^{-1} from bacteria *Ralstonia solanacearum* samples.

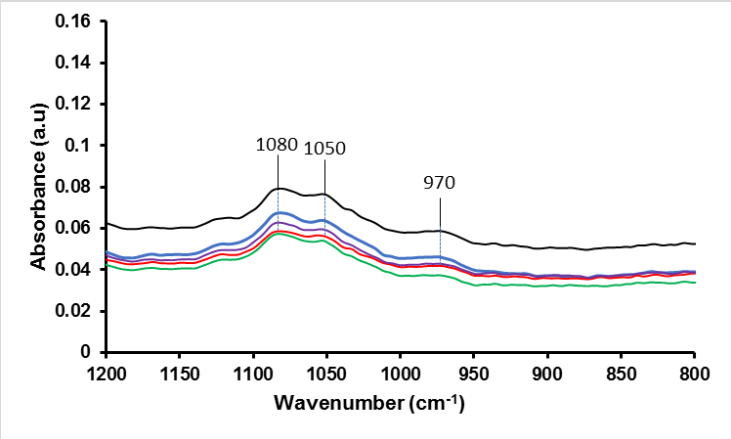

Figure 5. The fingerprint of bacteria in the FTIR spectral region of 1200–800 cm^{-1} from bacteria *Ralstonia solanacearum*.

The spectra obtained from the samples in this study are similar to one another. A unique and consistent spectral marker of *Ralstonia solanacearum* was discovered in this present study. The result could mean that it might be possible to use these biomarkers for rapid and easy detection and identification of bacteria.

Conclusions and suggestion

The most attractive features of FTIR spectroscopy methods for the detection and identification of bacteria are their rapidity, simplicity, and uniform applicability to any group of microorganisms. In this study, we examined the potential of FTIR spectroscopy as a rapid characterization method of *Ralstonia solanacearum.* FTIR spectroscopy can be used to identify bacteria with unique and consistent spectral markers. All the features of the absorption bands obtained from bacteria *Ralstonia solanacearum* samples associated with protein, phospholipids, nucleic acids, and carbohydrates appear in the bacterial IR absorption spectra.

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