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Isolation of Endophytic *Pseudomonas* Strains from Papaya Leaves and Their Extracellular Enzyme Production and Antioxidant Profile

Purbowatiningrum Ria Sarjono[1*], Nur Fadilla Choirunnisa[1], Yunita Triwijayanti[1], Salsabila[1], Mukhammad Asy'ari[1], Ngadiwiyana[1] , Ismiyarto[1], Nor Basid Adiwibowo Prasetya[1], Yosie Andriani[2]

[1]*Department of Chemistry, Faculty of Science and Mathematics, Diponegoro University, Semarang, 50275, Indonesia*
[2]*IMB/ICAMB, Universiti Malaysia Terengganu*, Malaysia

Email: purbowatining@live.undip.ac.id

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Abstract

Endophytic bacteria, symbiotic microorganisms residing in plant tissues, produce bioactive compounds similar to host plants, such as antioxidants. These antioxidants are crucial in combating free radicals linked to degenerative diseases. This study isolates and characterizes two endophytic bacterial strains from papaya leaves, exploring their enzymatic and antioxidant activities. Two isolates of endophytic bacteria from papaya leaves were obtained, F1-A and F1-B. F1-A endophytic bacteria are types of monobacilli, Gram-positive bacteria. F1-B endophytic bacteria are types of bacilli. Using 16S rRNA analysis, both isolates were predicted to belong to the Pseudomonas bacterial strain. Research on optimizing their growth under various temperatures and pH conditions showed that both isolates grow best at 37°C. F1-B provides a better opportunity as a source of industrial enzymes because it can excrete amylase, urease, cellulose, and protease enzymes compared to F1-A, which can only produce amylase and protease enzymes. Nevertheless, F1-A can act as a potent antioxidant with an IC_{50} of 34.18 ppm compared to F1-B, which has an IC_{50} value of 292.31 ppm. The IC_{50} value of the F1-A isolate was not much different from the IC_{50} of quercetin, which was 12.50 ppm. The ability of F1-A as an antioxidant is also influenced by the results of phytochemical screening, which can contain more secondary metabolites than F1-B. These results highlight the potential of Pseudomonas strains as sources of industrial enzymes and natural antioxidants, warranting further investigation.

Keywords: Endophytic bacteria, genotypic, papaya leaves, phenotypic

1. INTRODUCTION

Free radicals are highly reactive molecules because they have unpaired electrons and an unstable nature. They will try to find their electron pairs from important body molecules such as DNA, proteins, and lipids. This process is called an oxidative reaction and can damage the structure of these biomolecules, ultimately contributing to aging, cancer, atherosclerosis, and other neurodegenerative disorders. Antioxidants are compounds that can neutralize free radicals by donating electrons or hydrogen atoms to them so that free radicals become stable and no longer damage other molecules. This mechanism directly reduces oxidative damage to biomolecules[1].

Each plant species provides different potential bioactive compounds [2], but opportunities for producing different bioactive compounds are not closed due to the influence of temperature and the environment [3]. Endophytic bacteria live symbiotically within the tissues of their host plant and do not cause adverse effects on the plants [4]. Endophytic bacteria generate metabolites similar to their host plants through various mechanisms that implicate complex interactions and adaptations. Endophytic bacteria can influence the metabolite profile of their host plants, thereby potentially producing similar bioactive compounds [5]. Like its host, the population of

endophytic bacteria is also strongly influenced by environmental conditions and the location of the host plant. The symbiosis of mutualism between endophytic bacteria and host plants allows endophytic bacteria to produce bioactive compounds, such as host plants, which are useful for the growth and self-protection of the bacteria and the host plants against the threat of environmental conditions [3]. Endophytic bacteria assist host plants in resisting pathogenic bacteria and enduring various stresses such as drought, salinity, and other biological and abiotic challenges [6]. Bioactive compounds from endophytic bacteria have the potential to be developed in the medical, industrial, and agricultural fields [6].

Research on the phytochemical content of papaya leaves has been widely reported. According to [7] papaya leaves contain secondary metabolites such as alkaloids, flavonoids, tannins, steroids, triterpenoids, and saponins. Secondary metabolites found in papaya leaves can function as antioxidants Click or tap here to enter text.[8,9], antibacterial [10], anti-inflammatory [11], analgesics [12], and anticancer [9,13]. Production of bioactive compounds from plants, in bulk, requires large amounts of plants, resulting in a wider area and a longer harvest time. While previous studies have demonstrated the bioactive potential of papaya leaves, the role of endophytic bacteria in producing similar compounds remains underexplored. Given the therapeutic significance of papaya leaves, investigating their associated endophytic bacteria may reveal new bioactive sources. These constraints can be overcome by utilizing endophytic bacteria in plant tissue to produce secondary metabolites similar to their host plants [14]. This study hypothesizes that endophytic bacteria from papaya leaves can produce secondary metabolites with significant antioxidant activity comparable to the host plants. This makes a big opportunity because bacteria are easily grown organisms, can be reproduced, have a short life cycle, use relatively few simplicia, and can produce large amounts of bioactive compounds [15,16]. Given the bioactive potential of endophytic bacteria, this study aims to isolate and characterize bacterial strains from papaya leaves, assess their enzymatic and antioxidant capabilities, and identify their phylogenic relationships.

2. RESEARCH METHODS

Materials

Peptone, yeast extract, nutrient agar, ethanol, methanol, Gram stain (crystal violet, lugol iodine, acetone, safranin), H_2SO_4, $BaCl_2$, $C_6H_8O_7.H_2O$, $Na_3C_6H_5O_7.H_20$, $Na_2HPO_4.2H_2O$, $NaH_2PO_4.H_2O$, $Na_2B_4O_7.10\ H_2O$, HCl, NaOH, carboxymethylcellulose, skim milk, starch soluble, red methyl, iodine solution, olive oil, tween-80, Hubl A Solution, H_2O_2, urea, phosphate buffer saline (PBS), agarose, tris acetate EDTA (TAE 1x), FluorSave obtained from Merck and use as it is without purification. DNA isolation kits (BIO-RAD "Chelex® 100), Primer 27F and Primer 1492R, Taq polymerase from Sigma Aldrich, and ddH_2O from Integrated Laboratory of Diponegoro University.

Instruments

Analytical balance (Ohauss), laboratory glasswares (Pyrex), refrigerators (Panasonic), incubators (Memmert), ose needles, Laminar Air Flow (Innotech), glass preparations, microscopes (Olympus), pH meters (Scientific), micropipettes (Thermoscientific), microtube, microcentrifuges (Scanspeed), microwave (Samsung), gene machine amp PCR system 2400 (Multigen Optimax), ThermoScientific Nanodrop 2000 spectrophotometer (Thermo Scientific), UV transilluminator (Cambridge), vortex, UV-VIS spectrophotometry (T60U).

Papaya Leaves Sterilization

This study used papaya leaves to obtain endophytic bacteria, fresh papaya leaves taken from the Tembalang area, Semarang City, Indonesia. The sterilization method used was the surface sterilization method [17]. Papaya leaves were cut into several parts; the surface was sterilized by soaking it in 70% ethanol for 60 seconds. Furthermore, the sample was soaked with $Ca(OCl)_2$ for 3 minutes and then with 70% ethanol for 60 seconds. The final step was rinsing using sterile distilled water three times [15].The final rinse with distilled water served as a negative control. If no bacteria grow, it indicates that the surface of the papaya leaves is sterile. Sterilized papaya leaves were used to isolate endophytic bacteria.

Isolation and Identification of Endophytic Bacteria

Sterile papaya leaves were then inoculated aseptically on Zobell solid growth media made in 3 Petri dishes and incubated at room temperature until a colony of endophytic bacteria grew. Based on differences in morphological appearance, colonies of growing endophytic bacteria can be separated to obtain a single isolate of endophytic bacteria. The single isolate of endophytic bacteria obtained was stored as a stock for further steps.

Morphological characterization with Gram staining

The endophytic bacteria obtained were stained with Gram stain violet crystal, lugol iodine, acetone alcohol, and safranin, and then they were observed using a microscope with 1000x magnification.

Physiological Characterization

Effect of temperature on the growth of isolates

A total of 1 mL of bacterial culture with a bacterial concentration of 0.5 McFarland or equivalent to a bacterial density of 1.5×10^8 CFU/mL was inoculated in a new Zobell liquid media, and then the samples were incubated at 4, 25, 30, 37, 40, 45, and 50 ˚C for 6 hours. The absorbance of the sample was measured using a UV-VIS spectrophotometer at 600 nm.

Effect of pH on the growth of isolates

A total of 1 mL of bacterial culture with a bacterial concentration of 0.5 McFarland or equivalent to a bacterial density of 1.5×10^8 CFU/mL was inoculated in a new Zobell liquid media, and then the samples were incubated at pH of 4, 5, 6, 7, 8, 9, and 10 for 6 hours. The absorbance of each sample was measured using a UV-VIS spectrophotometer at a wavelength of 600 nm.

Effect of time on growth isolates

A total of 1 mL of bacterial culture with a bacterial concentration of 0.5 McFarland or equivalent to a bacterial density of 1.5×10^8 CFU / mL was inoculated in a new Zobell liquid medium, then incubated at 37 °C for 40 hours. The number of bacteria growing was observed every 2 hours from 0 to 40 hours, through absorbance measurements with a UV-VIS spectrophotometer at a wavelength of 600 nm.

Biochemical characterization enzyme test and phytochemical screening test

Amylase enzyme test: Zobell solid media was enriched with starch added. 50 µL of bacteria was inoculated using the spread method and incubated for 6 hours. After that, the amylase enzyme was tested in addition to iodine. The amylase enzyme test is positive if a clear zone is formed.

Cellulase enzyme test: Zobell solid media was enriched by adding CMC. 50 µL of bacteria was inoculated using the spread method and incubated for 6 hours. The cellulase enzyme produced was tested in addition to Hubl A. The cellulase enzyme test is positive if a clear zone is formed.

Protease enzyme test: Zobell solid media was enriched by adding skim milk. 50 µL of bacteria was inoculated using the spread method and incubated for 6 hours. The test of the protease enzyme is positive if a clear zone is formed.

Lipase enzyme test: Zobell solid media was enriched with olive oil, Tween 80, NaCl, and methyl red. Then, the bacteria were inoculated using the disk method and incubated for 6 hours. Positive results if there is a clear zone around the colony. Urease enzyme test: Zobell solid media was enriched by adding urea. A total of 50 µL of bacteria was inoculated using the disc method. The urease test is positive if the media changes color to pink [18].

Catalase enzyme test: The test was carried out on a bacterial culture from Zobell sloping agar, which had been incubated for 6 hours and then exposed to H_2O_2. The catalase enzyme test is positive if bubbles occur [17].

Phytochemical screening was carried out based on [19]. The phytochemical screening test included alkaloids, flavonoids, saponins, triterpenoids, and steroids.

Alkaloid test: 2 mL of supernatant was mixed with 10 drops of 2 N HCl. It was divided into two different reaction tubes. The first tube was dripped with three drops of Mayer's reagent, while the second tube was dripped with three drops of Dragendorff's reagent. A positive test was evidenced by forming a cloudy white precipitate in the first tube and a brick-red precipitate in the second tube.

Flavonoid test: 5 mL of filtrate was taken, and magnesium powder, 2 mL of concentrated hydrochloric acid, and 5 mL of amyl alcohol were added. The sample was shaken and allowed to separate until an amyl alcohol layer was formed. A red, yellow, or orange precipitate on the amyl alcohol layer indicates a positive test.

Saponin test: 5 mL of the sample was cooled and shaken vigorously for 10 seconds. Positive test results are indicated by the presence of stable foam ± 5 minutes after adding HCl.

Terpenoid/steroid test: The filtrate was placed on a drip plate and allowed to dry. Then, add two drops of anhydrous acetic acid and one drop of sulfuric acid. A positive test is indicated by a color change to blue, purple, and green for steroids.

Tanin test: 3 mL of the filtrate was taken, and a few drops of 1% $FeCl_3$ were added. Positive test results are indicated by a color change to dark blue or black.

Quinon Test: Two mL of the filtrate was taken, and a few drops of 1 N sodium hydroxide were added. A color change to red indicated a positive test.

Genotypic identification

DNA Isolation and Purification

DNA isolation was performed using the Chelex method (BIO-RAD "Chelex® 100 kits) [20] As much as 2-3 ose of bacterial culture were put into a microtube containing 500 µL ddH_2O and 1000 µL 5% saponin in Phosphate Buffer Saline (PBS) and then stored at 4˚C for 24 hours. Then, the mixture was centrifuged at 12000 rpm for 10 minutes. The supernatant was discarded while 1000 µL ddH_2O was added to run it into the second centrifugation. The supernatant of the

centrifuge was then discarded, while into the pellet, 100 µL ddH_2O, and 50 µL Chelex 20% were added, and the mixture was heated in water at 60 °C for 10 minutes and homogenized using a vortex every 5 minutes and centrifuged again at 12000 rpm for 5 minutes. The supernatant from the third centrifuge (DNA sample) was transferred to a new microtube. Then the concentration and purity of the DNA were tested using a Thermo Scientific nanodrop 2000 spectrophotometer. Furthermore, DNA samples were stored in the freezer and can be used for the 16S rRNA gene amplification process. The 16S rRNA gene was amplified using the polymerase chain reaction (PCR) technique. The PCR mixture consisted of 15 µL ddH_2O, 13 µL template DNA, three µL primer 27F, three µL primer 1492R, 25 µL MyTaq mix, and six µL DNA samples—sequence order of each primer, i.e Bact27F (AGAGTTTGATCATGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The PCR process was carried out in the following steps: predenaturation (94 ^{0}C for four minutes), denaturation (94 ^{0}C for 30 seconds), annealing (53 ^{0}C for one minute), and extension/propagation (72 ^{0}C for two minutes). The cycle from denaturation to extension was repeated as many as 30 times.

The PCR products were verified by electrophoresis, which was conducted on 1% agarose in buffer solution TAE 1x using a submerged horizontal electrophoresis cell (BioRad) for 50 minutes at 70 volts. PCR products were verified by electrophoresis on 1% agarose in TAE 1x buffer using a submerged horizontal electrophoresis cell (BioRad) for 50 minutes at 70 volts. For each sample, complete 16S rRNA gene sequences were obtained through direct sequencing of PCR products using the dideoxy Sanger method. This process utilized one primer pair (Bact27F and 1492R) and was carried out using an automated DNA sequencer provided by DNA Sequencing Services (1st Base, Singapore). Sequence validation was performed by analyzing electropherogram data with Sequence Scanner 2 (Applied Biosystems, 2012). DNA Baser Sequence Assembler v3 (Heracle BioSoft, 2012) combined sequences. The sequence of each isolate's 16S rRNA partial genes was sequenced and presented in the FASTA format. Finally, the 16S rRNA sequence of endophytic isolates was compared with other bacterial sequences by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The results were compared with the GenBank sequence based on a partial 16S rRNA sequence to examine the relationship and similarity with endophytic isolates.

The phylogeny study was carried out following [21] by aligning using the MEGA 6 program. The 16S rRNA gene nucleotide sequence of samples with other comparative bacteria from the BLAST results found in NCBI were aligned using the Alignment by Clustal W alignment tool to obtain output data. Furthermore, the phylogenic tree construction was carried out from the alignment using the Mega 6 program through the Phylogenetic Analysis menu. The alignment results of Alignment by Clustal W were entered as input data. Furthermore, construct/test Neighbour-Joining Tree with phylogeny test using Bootstrap methods as many as 1000 to obtain a phylogeny tree construction.

Production of Endophytic Bacteria Metabolites

Using the growth curve data obtained (Effect of time on growth isolates) of endophytic bacteria. Logarithmic, stationary, and death phases were obtained based on the bacterial growth curve data. Metabolite production was started by making a starter in log phases. The starter is inoculated into a Zobell liquid medium. Secondary metabolite production begins with adding 10% starter to the production medium. For each bacterium, the incubation time for secondary metabolite production was carried out in the stationary and death phases. Bacterial cultures were centrifuged at 6000 rpm for 15 minutes. The filtrate containing secondary metabolites was concentrated by freeze-drying.

Antioxidant Activity Test using the DPPH method [22]

The DPPH parent solution was diluted with 2 mg of DPPH powder in a 50 mL volume flask with p.a methanol and stored in a dark bottle. The control was the DPPH solution, and the blank was methanol p.a. The maximum wavelength of the control is determined by measuring the maximum absorbance at a wavelength of 400-600 nm. The sample was dissolved in p.a methanol with various concentration variations. A total of 1 mL of metabolite samples from each concentration variation was added to 3 mL of DPPH 0.1 mM and incubated for 30 min in the dark. The next step measured absorbance at a maximum wavelength of 517nm and performed the same on ascorbic acid as an antioxidant comparator. Absorbance control and absorbance of test samples were used to calculate % inhibition using the following formula:

$$\%\text{inhibition} = (Abs\ control - Abs\ solution\ test)/abs\ control\ x100\% \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

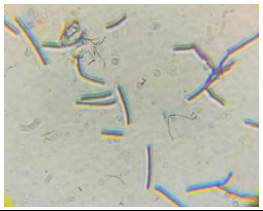
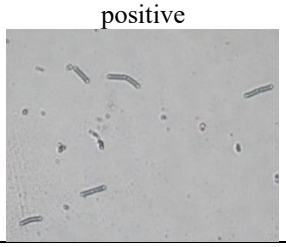
3. RESULTS AND DISCUSSION

Isolation and Identification of Phenotypic Endophytic Bacteria

Two bacterial colonies were found in papaya leaves, which are F1-A and F1-B bacteria. The morphological characteristics of gram staining of the two bacteria are shown in **Table 1**, which shows that F1-A endophytic bacteria are monobacilli and Gram-

positive bacteria. Macroscopic analysis showed that the bacteria form a colony of stems, white in color, with smooth edges and flat elevation. On the other hand, F1-B endophytic bacteria are types of bacilli and Gram-positive bacteria. Macroscopic analysis showed that the bacteria form a colony of stems, white in color, with smooth edges and convex elevation. Bacillus bacteria are bacteria in the form of cylindrical, short sticks or small rods that live alone, not clustered.

Table 1. Identification of Phenotypic Endophytic Bacteria

Colony character	F1-A	F1-B
Color	White	white
Colony shape	Circular	circular
Consistency	Soft	soft
Elevation	Flat	convex
Cell shape	Monobacillus	Bacillus
Types of Gram Bacteria	Positive	positive
Figure of Bacteria		

Physiological Characterization

Effect of temperature on the growth of isolates

Temperature significantly affects the metabolic rate and nutrient absorption of bacteria, thereby impacting the growth and survival of bacteria. Temperature influences enzymatic activity and membrane fluidity, and temperature plays an important role in determining bacterial metabolic processes and nutrient acquisition strategies. The implications of this Effect are enormous, affecting bacterial growth rates and survival strategies in various environments [23,24].

The Effect of temperature on bacterial growth is shown in **Figure 1**. Endophytic bacteria F1-A and F1-B have optimum growth temperatures at 37 °C. At higher temperatures than 37 °C, the number of endophytic bacteria that live has decreased. This is because endophytic bacteria have passed their optimum temperature and have reached their maximum growth temperature. An increase in temperature will cause an increase in bacterial growth until an increase in temperature is not followed by an increase in bacterial growth or a bacterium has reached its maximum temperature [25]. It can also be concluded that the two isolates of endophytic bacteria belong to the mesophyll bacteria category, which can live in a temperature range between 15 ºC and 45 ºC [26].

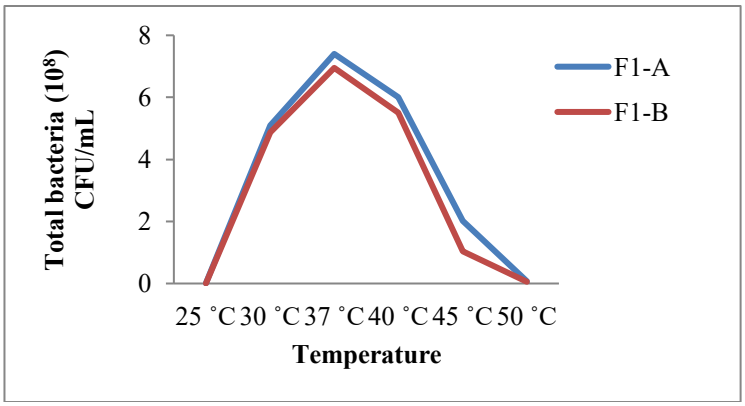


Figure 1. Effect of temperature on the growth of endophytic bacteria

Effect of pH on the growth of isolates

The Effect of pH on the growth of both endophytic bacteria is shown in **Figure 2.** The Effect of changes in pH on bacterial growth is an important factor that influences microbial activity and metabolism. Different bacteria show varying growth responses to changes in pH, which can significantly impact the proliferation and metabolic function of the bacteria. The highest number of living bacteria is reached at pH 7, so the two endophytic bacteria are neutrophils, which can live at pH between 5 and 8 [27]. Changes in pH outside the pH range of growing

neutrophil bacteria can damage the metabolic enzymes these bacteria produce. Denaturation of the enzyme allows for conformational changes in the tertiary protein structure [27]. The number of endophytic living bacteria is low at pH 4 (acidic conditions) and pH 10 (alkaline conditions). Too acidic or too basic environments will affect the rate of bacterial growth. According to [28], a cell environment that is too acidic or basic causes protons to enter and exit into the cytoplasm and decrease or increase the cell's internal pH, so that it can denature protein cell components, including enzymes, resulting in inhibiting or even stopping the bacterial cell growth.

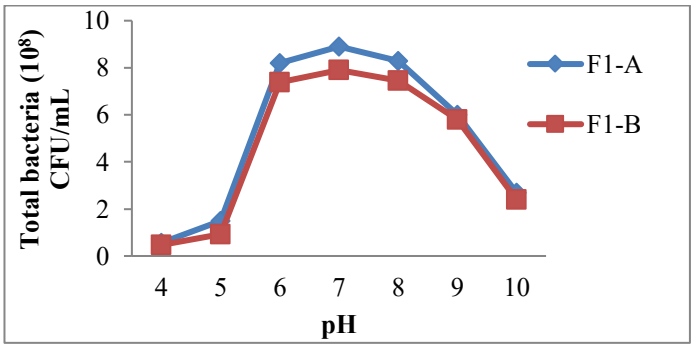


Figure 2. Effect of pH on the growth of endophytic bacteria

Effect of time on the growth of isolates

The Effect of time on bacterial growth was measured to determine the growth phase of bacteria. The growth of bacterial cells was measured indirectly by measuring the turbidity of endophytic bacteria using a spectrophotometer. The results of measurements of the absorbance value of bacterial growth in every 2-hour range are shown in **Figure 3**.

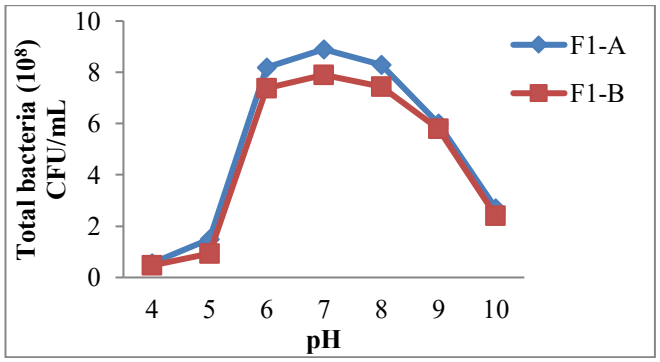


Figure 3. The Effect of time on the growth of endophytic bacteria

Both isolates of bacteria have different growth phases. F1-A bacterial isolate has a faster growth phase than F1-B isolate, as seen from the number of bacteria present. Despite having different periods, both bacteria show logarithmic, stationary, and death phases. F1-A bacterial isolate has a more extended growth phase than F1-B. This can be seen from achieving a more extended phase of death. The final phase of bacterial growth is the phase of death. The F1-B death phase occurred at the 24th hour, while the F1-A death phase was at the 38th hour. In this phase, the number of bacterial cells decreases, where cells die faster than living cells. The leading causes of bacterial death are nutrients in the medium, energy reserves in the cells being depleted, and cell autolysis. In the autolysis process, enzymes will exit the cell so that cell components will be damaged and dispersed into the media. Based on the growth phase, the next stage is the production of metabolites, which are obtained in several phases. **Figure 3** determines the production time of secondary metabolites at the phytochemical screening stage.

Biochemical characterization enzyme test and phytochemical screening test

Enzyme Activity

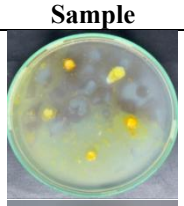
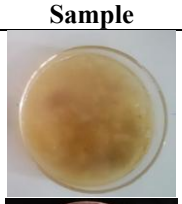
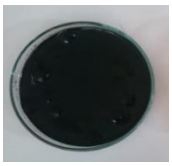
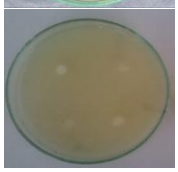
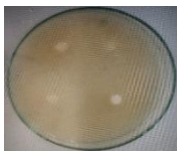
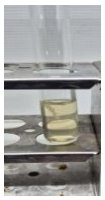
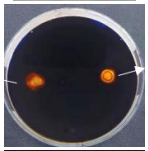
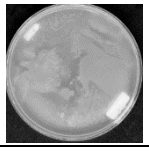
The analysis showed that isolates of F1-B bacteria had more extracellular enzyme activity than F1-A, as shown in **Table 2**. Isolate F1-B produces amylase, urease, cellulose, and protease enzymes, while isolate F1-A only produces amylase and protease. Samples in phytochemical screening were taken based on data on the Effect of time on the growth of endophytic bacteria, which provides information

about the bacterial growth phase. Testing was done in the stationary phase and the initial phase of death because the secondary metabolites are produced in those phases, and when the nutrients have started to run out.

Phenotypic Characterization

While not critical for an organism's growth, secondary metabolites serve an adaptive role by acting as defense molecules against pathogens, protection, and adaptation to environmental stress. Protection against ultraviolet light appears when there is pressure from the environment, such as nutritional problems during growth. Some secondary metabolites are steroids, peptides, flavonoids, phenols, terpenoids, alkaloids, polyketons, and quinols. These substances, such as anticancer, antioxidants, antimicrobials, anti-inflammatory agents, and immunosuppressive agents [29], are crucial in therapeutic contexts.

Table 2. Extracellular enzyme activity of endophytic isolates

Enzyme activity	Colony				Negative control
	F1-A	Sample	F1-B	Sample	
Amylolytic	+		+		
Urease	-		+		
Lipase	-		-		
Catalase	-		-		
Cellulolytic	-		+		
Protease	+		+		

Based on **Figure 3**, this secondary metabolite production was carried out in the stationary and early death phases. For the F1-A isolate, the metabolites were produced at the incubation times of the 18th (EM-18) and 38th (EM-38) hours, while for the F1-B isolate, at the 16th (EM-16) and 24th (EM-24) hours. The products were concentrated using the freeze-drying method to obtain thick extracts as a brown paste.

As shown in **Table 3**, secondary metabolites from endophytic bacteria are produced only from one bacterial cell, while papaya leaves are high-level multicellular plants. F1-A isolate can produce more secondary metabolites than F1-B isolate. According to [30,31], endophytic bacteria have the potential to produce bioactive compounds that are similar to their host plants' bioactives. It is suspected that plants have a complex availability of primary metabolites, so they can have a variety of compounds. At the same time, bacteria are simple creatures and grow on simple media as well. Therefore, they produced only specific secondary metabolites. In addition, the enzymes

possessed by bacteria also influence the formation of the secondary metabolites. The enzymes that bacteria have in plants can be different. Therefore, secondary metabolites produced by endophytic bacteria can also be different, but there are still similarities with the host. Plants produce physiologically potent bioactive compounds. However, this production provides a more heterogeneous picture of environmental defenses than endophytes, resulting in more secondary metabolites than endophytes. Under controlled environmental conditions, microorganisms produce primary and secondary metabolites [32]. Plants associated with endophytic microorganisms have been shown to have the potential to be a high source of ingredients and medicinal products compared to plants alone [33].

Table 3. Phytochemical screening of endophytic bacteria

Parameter	F1-A			F1-B			Positive control
	EM-18	EM-38	Figure	EM-16	EM-24	Figure	Ethanol extract of Carica papaya leaves
Alkaloid	+	+		+	+		+ 
Flavonoid	-	-		-	-		+ 
Saponin	+	+		-	-		+ 
Tannin	-	-		-	-		+ 
Steroid	-	-		-	-		+ 
Triterpenoid	+	+		+	+		+ 
Quinone	+	+		-	-		+ 

Genotypic identification

DNA Isolation and Purification

Endophytic isolate strain identification was based on 16S rRNA gene sequencing using universal primers. Based on the phylogeny tree obtained, the endophytic bacterial isolate F1-A relates to *Pseudomonas azotoformans* strain NBRC 12693. F1-B endophytic bacterial isolate has the closest relationship with *Pseudomonas brenneri strain CFML 97-391*, *Pseudomonas proteolytica strain CMS 64,* and *Pseudomonas panacis strain CG2010*, but F1-B endophytic bacterial isolate forms a new branch that is not in one branch with comparative bacteria that have the closest relation.

Based on the **Table 4**, F1-A bacteria are closely related to *Pseudomonas azetoformans.* F1-B bacteria have the closest kinship to *Pseudomonas panacis* and *Pseudomonas proteolytica* because they have the same phenotypic characteristics as the closest bacteria in the phylogenetic analysis, namely, from the

temperature conditions, which are mesophilic bacteria, rod-shaped or rod-shaped bacteria, aerobic, and live symbiotically on plants. The difference between endophytic bacteria F1-B and the three closest bacteria in the phylogenetic analysis is in the type of bacterial gram and the enzymes produced.

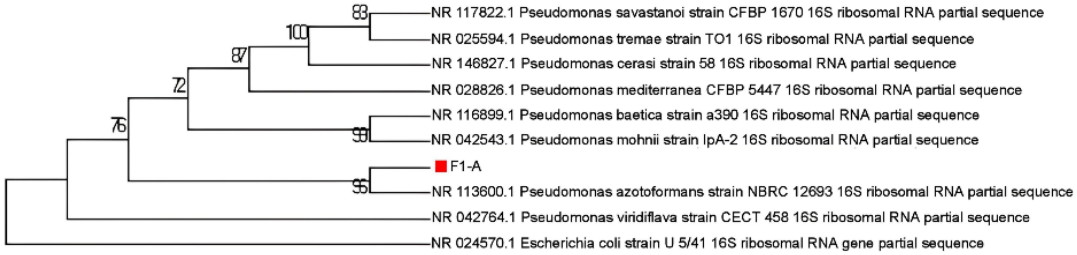


Figure 4. Construction of phylogeny tree of endophytic bacteria F1-A

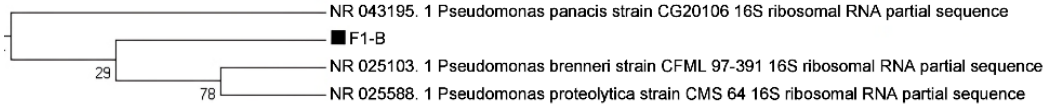


Figure 5. Construction of phylogeny tree of endophytic bacteria F1-B

Table 4. Comparison of Isolate F1 with Pseudomonas strains

No	Characteristics	Endophytic bacteria F1-A	Endophytic bacteria F1-B	*Pseudomonas proteolytica* [34]	*Pseudomonas panacis* [35]	*Pseudomonas azetoformans* [36]
1	**Growth characteristics**					
	Habitat	Papaya leaves	Papaya leaves	antarctic waters	Korean ginseng root	Tunisian soil
	Temperature	37 ˚C	37 ˚C	4-30 ˚C	4-35 ˚C	30 ˚C
	pH	7	7	7	NA	7.3
2	**Phenotypic characteristics**					
	Gram bacteria	+	+	–	–	–
	Cell shape	Stem	Stem	Stem	Stem	Stem
3	**Enzyme activity**					
	Amylase	+	+	–	NA	NA
	Cellulase	-	+	–	NA	NA
	Protease	+	+	+	+	+
	Lipase	–	–	+	–	–
	Urea	-	+	–	–	+
	Catalase	–	–	+	NA	+

Positive (+), Negative (–), Data not available (NA)

Antioxidant activity Test using DPPH method

Antioxidants have a molecular structure that can provide their electrons (electron donors) to free radical molecules without being completely disturbed in their function, and can break chain reactions [37]. DPPH molecules are organic nitrogen radical compounds giving a purple color in solution with maximum absorption (λ_{max}) at 517 nm. When receiving electrons or free radical species, the color changes from purple to yellow, accompanied by a reduction in absorbance at λ_{max}. This change in absorbance is used to quantify antioxidant activity [37–39]. The results of IC_{50} values are presented in **Table 4**.

Table 5. IC_{50} results from endophytic bacterial isolate extracts

Sample		IC_{50} (ppm)
F1-A	EM-18	41.27 ± 0.32
	EM-38	34.18 ± 0.25
F1-B	EM-16	376.37 ± 10.17
	EM-24	292.31 ± 7,53
Quercetin		12.50 ± 1.15

Table 5 data used is the significance value (sig.)
1. Sample variations affect antioxidant inhibition (IC_{50}) as indicated by the sig value. = 0.000 F1-A has antioxidant activity that is significantly different from F1-B as indicated by $p < 0.05$
2. Concentration affects antioxidant activity (IC_{50}), which the sig value indicates. = 0.048 variations in concentration affect antioxidant activity; the higher the concentration, the greater the antioxidant activity, as indicated by $p < 0.05$.

Table 5 shows that the F1-A isolate has better antioxidant activity than the F1-B isolate. Both isolates initial and death phases (EM-38 and EM-24) result in better antioxidant activity than the stationary phase (EM-18 and EM-16). In another Sarjono et al. (2023) study, the IC_{50} of EM 38 endophyte *Pseudomonas hibiscicola* W had an IC_{50} of 201.87 ppm. At the same incubation time, EM 38 isolate F1-A, compared with EM 38, has a greater IC_{50} value. These results prove that secondary metabolites are primarily produced in the early death phase. Although the data in **Table 3** show that the stationary phase and the initial phase of death produce the same type of secondary metabolite, the number of secondary metabolites in the death phase is greater than that in the stationary phase. The more secondary metabolites produced, the higher the antioxidant activity. Limitations of nutrients in the medium will stimulate the production of enzymes that contribute to the formation of secondary metabolites to maintain their survival [40].

Based on phytochemical screening results, the F1-A isolate showed that alkaloids, saponins, and terpenoids can reduce DPPH radical compounds. While F1-B isolate only contains alkaloids and terpenoids. The presence of saponins may strengthen the antioxidant activity of F1-A isolate since F1-A isolate has better antioxidant activity than F1-B. Saponin compounds have hydroxyl groups that can transfer electrons to free radicals. Saponins indicate their antioxidant properties in direct interaction with DPPH free radicals. They donate electrons or hydrogen atoms to neutralize free radicals [41]. Plants containing saponins act as natural antioxidants because these compounds can bind with free radicals in complex environments [42].

Alkaloids can act as antioxidants by reducing free radicals by giving the H atom. The antioxidant activity of terpenoid compounds is attributed to unsaturated terpenoids with conjugated double bonds, which enable them to donate hydrogen atoms. Terpenoid compounds are antioxidants because they can donate electrons and reduce free radicals [43].

4. CONCLUSION

The F1-A endophytic bacteria are types of monobacilli, gram-positive bacteria, and can produce amylase and protease enzymes. They can generate alkaloids, triterpenoids, saponins, and quinones. F1-B endophytic bacteria are bacilli, gram-positive bacteria, and can produce amylase, urease, cellulose, and protease enzymes. They can result in alkaloids and triterpenoids. Both isolates grow best at 37°C, pH 7, and are classified as mesophyll bacteria. Both bacterial isolates have a relationship with *Pseudomonas*. Both F1-A and F1-B isolate extracts have antioxidant properties, with vigorous activity obtained by F1-A. Further research is needed regarding the effectiveness of antioxidant abilities in vivo.

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