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Research Article

Isolation and Identification of Endophytic Cellulolytic Bacteria in *Syzygium aqueum* Fruit

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ABSTRACT
Some endophytic bacteria are known to have hydrolytic activity by producing hydrolase enzymes. Endophytic bacteria can be found in plant tissue. Exploration of endophytic cellulolytic bacteria in water apple (Syzygium aqueum) fruit has not been widely carried out, so this research is the first to be conducted. This study aimed to isolate, screen, and identify endophytic bacteria from water apple fruit that could produce cellulase enzymes. The research began with bacterial isolation. Then, cellulolytic screening was carried out by inoculating the isolates into a differential medium containing cellulose, Bushnell-Haas agar (BHA). The screening results were characterized and identified through 16S rDNA gene sequence analysis. The isolation results indicated that seven bacterial isolates were screened for cellulolytic activity by cultivating them on BHA medium. The screening revealed that one isolate encode CSZA2 had cellulolytic activity with a cellulolytic index of 0.87. Biochemical characterization and molecular identification of the CSZA2 isolate showed a 99.93% similarity to the Pseudomonas putida RTI2, a bacterium known for producing cellulase enzymes. The discovery of bacterial cellulolytic activity can be developed for biomass degradation, bioethanol production, and agricultural biotechnology.

Keywords: *Bacteria; Cellulolytic; Endophytic; Fruit; Syzygium aqueum.*

Introduction

Bacteria that live in plant tissue are called endophytic bacteria. These bacteria live in plant tissue without causing negative impacts on their host plants [1]. The endophytic bacterial community is specific regarding its nutritional needs and can vary in type between bacteria that live on one plant and another. This is because endophytic bacteria have adapted to the chemical conditions of the metabolites of the plant. Exploration of endophytic bacteria for the production of bioactive compounds in the form of extracellular and intracellular metabolites is known to be quite efficient, so it is often done using many different types of plants and exploring different abilities [2], [3].

Some endophytic bacteria are known to be able to produce hydrolytic enzymes. Hydrolytic bacterial enzymes or hydrolases that are commonly produced by endophytic bacteria are generally cellulases, pectinase, protease, amylase, lipase, and xylanase. The production of hydrolase enzymes is influenced by substrate conditions where the bacteria originate [4], [5]. In general, it can beexplained that hydrolase enzymes haveimportant functions in agriculture orindustry.

Cellulolytic enzymes are divided into several groups based on their structure, shape, and activity. Types of cellulolytic enzymes include Cellulose Binding Module (CBM), endo-β-1,4-gluconase, exo-β-1,4-gluconase, and β-1,4-glucosidase [6].In industry, cellulase enzymes are used to process agricultural by-products containing a lot of cellulose to produce glucose which is often used in bioethanol production. Bioethanol is a potential energy source that can produce fossil fuels [4], [7].

Syzygium aqueum known as water apple is a plant from the Southeast Asian region. Water apple fruit has benefits for increasing immunity and body energy because of its nutritional content [8], [9]. This fruit contains approximately 4.5 g carbohydrates, 0.7 g protein, 0.2 g fat, 1.9 g fibers per 100 g. Several secondary metabolites, antioxidants and vitamins also constitute these fruit nutrient, such as phenolic contents ranging from 28.8 - 30.7 mg, flavonoids ranging from 62.03 - 62.07 µg, β-carotene, ascorbic acid, thiamin, and riboflavin[10], [11]. The nutrients in this fruit are suitable for the living environment of various microbes, one of which is bacteria.

Previous research on endophytic bacteria has only been reported on the stems of *S. aqueum* plants, but never on the fruit. Main focus of the previous study is to isolate endophytic bacteria that have antagonistic ability against pathogenic fungi, which is different from this research purpose [12]. Another study about their endophytic microbes also isolated endophytic fungi from the bark, root bark, and leaves of *S. aqueum* [13].

The insufficient research on endophytic bacteria derived from water apple fruit, particularly regarding cellulase production, is the main reason for this study. The cellulolytic activity of these bacteria, which can also be applied in industry and the decomposition of agricultural by-products, may lead to valuable discoveries. Therefore, this research aimed to isolate and identify endophytic cellulolytic bacteria from water apple fruit (*S. aqueum*).

Materials and Methods

This type of research is observational research. The results of this study were analyzed descriptively by explaining the tabulated data. This research was conducted from August to November 2024. The isolation and screening stages of cellulolytic potential were carried out in the microbiology laboratory, while the DNA isolation, PCR, and electrophoresis stages were carried out in the molecular biology laboratory of the Faculty of Health Sciences, Maarif Hasyim Latif University.

Materials

The tools used in this research include Petri dishes, Erlenmeyer flasks, Test tubes, inoculating loop needles, micropipettes, cotton swabs, mortar, pestle, scalpel knife, Biosafety Cabinet 1300 Series A2 (Thermo Scientific), Incubator (Memmert IN110), PCR (Bio-Rad), Waterbath (Benchmark Scientific, USA), centrifuge (Thermo Scientific), Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific), bluegel electrophoresis. The sample used in this research are fruits of *Syzygium aqueum* plant. Material needed include deionized water, 2% sodium hypochlorite, 70% ethanol, nutrient agar (Merck), bacto agar powder (Himedia), K_2HPO_4, KH_2PO_4, $FeCl_3.6H_2O$, NH_4NO_3, $MgSO_4.7H_2O$, $CaCl_2$, carboxymethyl cellulose (CMC) (Himedia), Congo red, tripton water (Merck), peptone water (Merck), MR-VP medium (Merck), Simmon's citrate medium (Merck), triptic sugar iron agar (Merck), lysine iron agar (Merck), glucose, sucrose, lactose, maltose, ddH_2O, Wizard Genomic DNA Purification Kit Promega, Green Gotaq PCR master mix Promega, 16S rDNA primers 27F (aga gtt tga tcc tgg ctc ag), 1492R (ggt tac ctt gtt acg act t) (IDT Oligo), and agarose gel (Mini-Sub® Cell GT Cell Tank and Lid).

Methods

1. solation of Endophytic Bacteria of S. aqueum Fruit

Isolation of endophytic bacteria from *S. aqueum* fruit began by sterilizing the fruit surface. The fruit used was first washed with sterile deionized water. The fruit was then soaked in 2% sodium hypochlorite solution for 5 minutes and then rinsed with 70% ethanol for 30 seconds. The fruit was then rinsed with sterile deionized water and allowed to dry before being processed. The fruit dried in a *Beaker glass* contains Whatman filter paper no. 1, until the rinsed water drained into the paper, approximately 15 minutes. Dried fruit was then processed by cutting it into small pieces first and weighing 1 g. The fruit was then suspended with 9 ml of phosphate buffer and then 0.1 mL was taken and spread on nutrient agar (NA) media and repeated 5 times [12–15] .

2. Screening of Cellulolytic Bacteria Isolate

The cellulolytic screening was carried out using Bushnell-Haas agar (BHA) medium prepared with ingredients in the form of (g/L) agar, K_2HPO_4 1; KH_2PO_4 1; $FeCl_3.6H_2O$ 0.05; NH_4NO_3 1; $MgSO_4.7H_2O$ 0.2; and $CaCl_2$ 0.02 then added 1% carboxymethyl cellulose (CMC) [16].Bacteria were inoculated on BHA medium containing 1% CMC using a streak technique to form a line. Furthermore, the isolate results were incubated for 24 hours at 32ºC. Observations were made on the media by looking at the visible clear zone. Congo red reagent was added to the media to clarify the clear zone produced by bacteria so that it was easy to observe.

Observation was continued with a quantitative screening test to determine the cellulolytic activity capability of bacteria. The test was conducted using the spot in lawn method, by inoculating cellulolytic bacterial isolates on BHA + cellulose media by spotting at one point only. The medium were then incubated at 32ºC for 24 hours. Congo red reagent was added to media to clarify the clear zone around the colony of bacteria. Cellulolytic index obtained by calculating with the formula.

$$CI = \frac{CZ\ CD}{CD}$$

CI : Cellulolytic index (mm)
CZ: Clear zone (mm)
CD: Colony diameters (mm)

The category of cellulolytic index determines their capabilities to degrade cellulose. CI ≤ 1 was low cellulolytic activity, 1 < CI ≤ 2 was medium cellulolytic activity, CI > 2 was high cellulolytic activity [17].

3. Morphological and Biochemical Characterization

All cellulolytic bacteria that were successfully isolated were characterized by their colony and cell morphology. Endophytic bacteria that were known to have cellulolytic activity were characterized biochemically by growing them on test media, including IMVIC, TSIA, Lysine and sugar fermentation. The results were observed 24 hours after inoculation

4. Identification of Cellulolytic Bacteria Isolate

All potential bacteria were identified molecularly using 16S rDNA primers 27F (aga gtt tga tcc tgg ctc ag) and 1492R (ggt tac ctt gtt acg act t). Detection of cellulolytic bacterial isolate DNA began with the extraction of total DNA from bacterial isolates that had been prepared on nutrient agar slants, which were then taken in 2 full loops suspended in 200 µL of ddH2O, then vortexed. The suspension was then heated at 95 °C for 20 minutes using a water bath (Benchmark Scientific, USA). After that, the culture suspension was centrifuged at 10,000 rpm for 5 minutes at 4 °C. 180 µL of supernatant was separated from the pellet to be used as a DNA template in the PCR reaction [18].

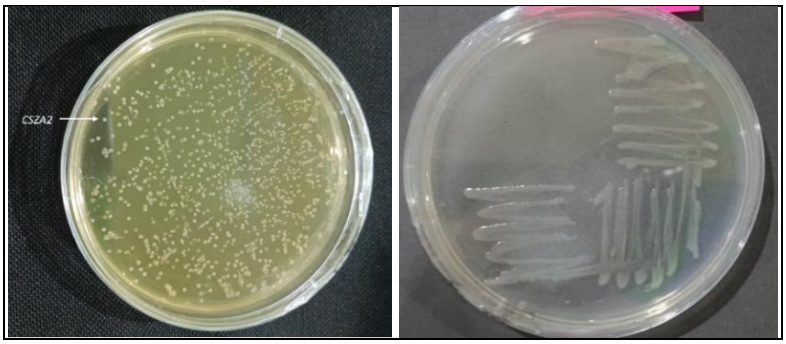
DNA concentration and purity were measured by observing the absorbance value with the help of Genesys 10S UV-Vis Spectrophotometer. DNA template

amounting to 10µL was added with sterile distilled water until the volume reached 1000 µL. The DNA was slowly mixed with the help of a micropipette. The diluted DNA was then inserted into a cuvette, and its absorbance was measured at a spectrophotometer wavelength of 260 nm. The absorbance was measured again using a wavelength of 280 nm [19].DNA concentration was calculated using the following equation *(eq1)*. DNA purity is further measured using the following equation *(eq2)*.

DNA Concentration = Å260×50×dilution factor ……*(1)*
DNA Purity = Å260/Å280 ……(*2*)

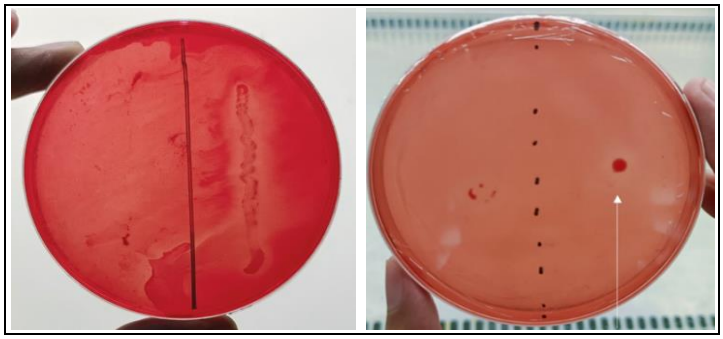
Good DNA purity is indicated when the absorbance ratio shows a figure of 1.8–2 and the concentration is above 100 µg/mL. The PCR reaction was made with a composition of 50 µL containing 25 µL Gotaq green, 5 µL primers 27F and 1492R, 5 µL DNA template and 10 µL ddH2O. Then inserted into the PCR machine and the stages were arranged, namely pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. The results of the amplification were visualized using 0.8% (w/v) agarose gel electrophoresis [18].

The amplicons were then sequenced at 1st BASE DNA Sequencing Malaysia. The sequencing results were assembled into contigs using Bioedit software version 7.2. The 16S rDNA sequences obtained were compared with the database available at NCBI using the BLAST search tool, which can be accessed via the link https://blast.ncbi.nlm.nih.gov, and phylogenetic tree analysis using MEGA software version 11. Pylogenetic tree generated through Neighbor-Joining method and 1000 replicate of boot-straps.



A B

Figure 1. CSZA2 colony morphology from bacterial isolation (A). CSZA2 colony morphology from the purification process (B).



A B

Figure 2. Qualitative screening result of CSZA2 cellulolytic activity (A). Quantitative screening result of CSZA2 cellulolytic activity using spot inoculation methods (B).

Table 1. Phenotypic characteristics of water apple fruit (*Syzygium aqueum*) bacterial isolates

Phenotypic characteristics	Isolate code					
	ASZA1	**ASZA2**	**BSZA1**	**BSZA2**	**CSZA1**	**CSZA2**	**ESZA1**
Colony Shape	Circular	Irregular	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Undulate	Entire	Entire	Entire	Entire	Entire
Elevation	Convex	Flat	Convex	Raised	Raised	Raised	Raised
Surface	Smooth	Wrinkle	Smooth	Smooth	Smooth	Smooth	Smooth
Optical	Opaque	Translucent	Opaque	Opaque	Opaque	Opaque	Opaque
Color	White	Pale White	White	Pale White	Yellowish White	White	White
Cell Shape	Short Rod	Short Rod	Rod	Circle	Short Rod	Rod	Short Rod
Cell Structure	Diplo bacilli	Triplo bacilli	Mono bacilli	Mono bacilli	Diplo bacilli	Diplo bacilli	Diplo bacilli
Gram	Negative	Positive	Negative	Positive	Positive	Negative	Negative

Results and Discussion

Result

The results of the bacterial isolation process that has been carried out obtained 7 bacterial colonies with various characteristics. The characteristics of the 7 bacterial colonies are presented in Table 1. Seven bacterial colonies come from different plates with the same media. Based on the table, 3 Gram-positive bacteria and 4 bacteria with Gram-negative were obtained.

The seven bacterial isolates that have been selected based on their character differences were finally screened using Bushnell-Haas hydrolytic media. Based on the results of the cellulase activity screening test, it was found that there was only one type of bacterial isolate, namely CSZA2 (Figure 1), which showed cellulolytic activity. Bacterial cellulolytic activity is known from the appearance of a clear zone around the colony, after the administration of congo red reagent in a medium containing cellulose (CMC) (Figure 2). The cellulolytic index (CI) of the CSZA2 isolate was 0.87. These results cellulolytic index (CI) $\leq$ 1, so the cellulolytic activity of CSZA2 bacteria is included in the low activity category.

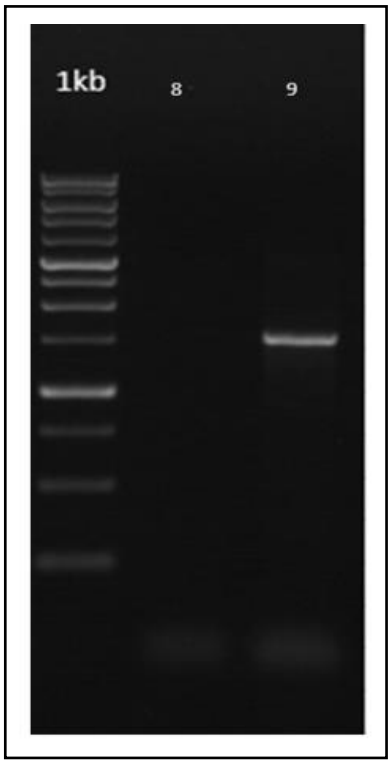


Figure 3. PCR Analysis Results of CSZA2 Isolate. 1kb: Marker; 9: CSZA2 Isolate.

Biochemical reaction tests conducted on the CSZA2 bacterial isolate produced the characteristics presented in Table 2. Catalase, Indole, MR, VP, and

sugar fermentation tests (glucose, lactose, sucrose, maltose) yielded negative results. Positive results were obtained in the motility test, lysine test, and citrate test. The TSIA test results were alkaline slant and butt, and negative for both gas production and H_2S. The TSIA test describes that no sugar were fermented. The CSZA2 isolate was then prepared on NA slant media for molecular identification.

Table 2. Biochemical characterization of bacterial isolate CSZA2

Test	Indol	MR	VP	Citrate	Motil	Lysine	Catalases	TSIA	Carbohydrates Fermentation			
									Glucose	Lactose	Sucrose	Maltose
Isolate CSZA2	—	—	—	+	+	+	—	Slant Ak, Butt Ak, Gas (—), H2S (—)	—	—	—	—

Notes: Positive results (+); Negative results (-); Alkaline (Ak).

The concentration of CSZA2 isolate DNA was 50 µg/mL, while the purity of DNA was 0.5. These results indicate that the DNA template is suspected to be contaminated with protein, but we decided to continue the identification process using the DNA template sample. The results of the amplification were visualized using 0.8% agarose gel electrophoresis. The presence of DNA bands with a molecular weight of ± 1500 bp can be seen in Figure 3. indicating that 16S rDNA was amplified. A phylogeny tree was created to describe the relationship of isolates suspected of having the potential to produce cellulase enzymes. The identification results based on 16S rDNA showed that the CSZA2 isolate was *Pseudomonas putida* RT12 with a similarity of 99.93% (Figure 4).

Discussion

Based on the results obtained, it is known that from 5 repetitions of isolation carried out, only 7 different bacterial colonies were obtained (Table 1). This number is not too much. Previous studies showed that endophytic bacteria in the upper body tissue of plants are not numerous. This result was also obtained in a study to isolate endophytic bacteria in rambutan fruit (*Nephelium lappaceum*), which obtained 9 isolates. Other studies isolating bacteria from avocado and black grapes only obtained 3 isolates each [12], [16], [17].

Several things, including the level of fruit ripeness, fruit health, weather, and season, can influence the number of bacteria in the fruit [22]. Bacteria in the fruit can come from the carposphere or the air around the fruit and enter through the pores in the fruit [23]. This number is not too much compared to bacteria from the soil or rhizosphere due to differences in nutrition. Some of the bacteria found in the fruit also come from root bacteria that have cellulolytic enzymatic reactions that are used to enter other tissues of the plant including the fruit [16], [19].

The morphological diversity of isolated bacterial isolates (Table 1) can be influenced by the environmental conditions of the bacteria's origin. In addition, morphogenetics is determined by gene expression from bacterial species. Its shape can affect important physiological functions such as nutrient acquisition, motility, interaction and resistance to pressure [24]. Endophytic bacteria can be Gram negative or Gram positive [20], [25].

The CSZA2 bacterial isolate that has been identified as *P. putida* generally has a milky white, round, sticky, moist, opaque colony morphology (Figure 1). Meanwhile, the cell morphology is rod-shaped and Gram - negative [26].

Biochemical test of CSZA2 isolate shows that most carbohydrates fermentation tests have negative results (Table 2). These results are similar to *P. putida* strain ST3 characteristics, which obtaining negative results for rhamnose, N-acetyl-glucosamine, D-sucrose, mannitol, maltose, L-fucose, sorbitol, and many other sugars [27].

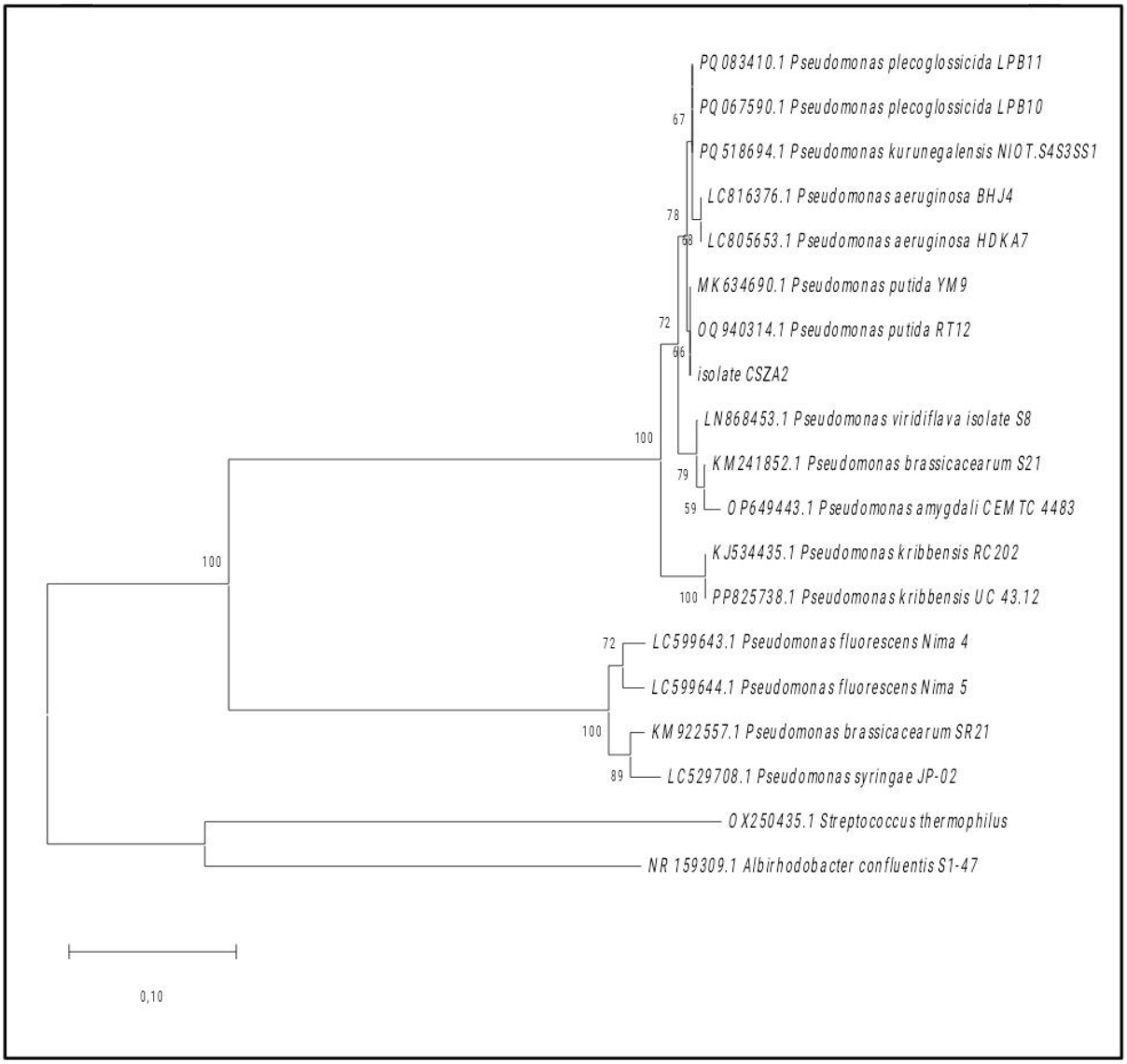


Figure 4. Phylogeny Tree of Bacterial Species Potentially Producing Cellulase Enzymes Based on 16S rDNA Sequence Data Neighbor Joining Bootstrap Algorithm 1000.

Based on the screening results (Figure 2), one bacteria was found to have hydrolytic activity. The hydrolytic activity found was cellulolytic. This is because the bacteria produce cellulase enzymes, however, the results found that the CSZA2 isolate has low cellulolytic activity. Bacteria usually can produce cellulase enzymes if cellulose is available in the substrate. Plants are the main source of cellulose. Generally, fruit contains 0.4 to 4.2% of plant cellulose, which is relatively low compared to leaves that have 15-20% cellulose and tree branches that contain 40-50% cellulose. This might affect the cellulase production of CSZA2 [6].

The low cellulolytic activity of the CSZA2 isolate is expected because endophytic bacteria tend to produce plant cell wall-degrading enzymes in small

amounts to move from one tissue to another in a plant. These cell wall-degrading enzymes include cellulase and pectinase. If the degrading enzyme is secreted in large quantities, the plant will provide an immune response, because the plant may consider this bacteria as a pathogen. This will endanger the survival of endophytic bacteria [28].

Previous research found that the genus *Pseudomonas* has the highest CI index of 1.3. Each type of isolate may have different cellulolytic activity when screened on CMC agar media. Cellulolytic bacteria may have a higher CI index value if they grow in their original habitat compared to when inoculated on CMC agar media [17].

The journey of endophytic bacteria throughout plant tissues is due to high motility and enzymatic ability, namely the production of cellulase to help degrade cellulose in plant tissues [29]. Secretion of bacterial cellulase usually requires the concerted action of c-di-GMP-responsive inner membrane synthase (BcsA), membrane-anchored accessory protein (BcsB), and several additional Bcs components. Cellulase breaks down glucosidic bonds using acid-based catalysis. Two catalytic residues of the enzyme carry out hydrolysis: a general acid (proton donor) and a nucleophile/base [21], [22]. Bacteria that are unable to produce hydrolytic enzymes are due to several factors. The main factor is that bacteria do not have genes encoding hydrolytic enzymes [30–32].

Cellulase is an enzyme complex consisting of endoglucanase, exoglucanase, and β-glucosidase. This enzyme complex hydrolyzes β-1,4-glycosidic linkages in cellulose in synergy [33]. There are a few numbers of bacteria that possess all of the cellulase enzyme complex genes. Only a few bacteria can hydrolyze the natural form of cellulose, which is the crystalline form. Endoglucanase is the enzyme that has high capabilities to hydrolyze CMC in artificial media. Bacteria that show low cellulolytic activity on the CMC media might be due to the lack of the endoglucanase enzyme produced. Truly cellulolytic bacteria that can produce three cellulase complexes usually utilize cellulose for metabolism, otherwise cellulase only produced for pathogenesis or cellulose production [34], [35].

The identification process shows that the DNA sample may have protein contamination because the purity ratio is below 1.8-2.0 and the concentration below 100 µg/mL, however, the DNA still can be used as a template for PCR amplification [19]. Protein contamination may slow PCR amplification process [36]. The results of the PCR analysis showed that the CSZA2 bacterial isolate was a species of *Pseudomonas putida* RT12 bacteria with a similarity of 99.93% (Figure 4). *P. putida* has the characteristics of not fermenting sugars. Biochemical test reactions that generally have positive results are citrate tests [24], [25]. *P. putida* is a species of bacteria that can be isolated from soil or endophytes in several plant tissues. *P. putida* has been reported to be found in pepper and plant roots [26–28]. *Pseudomonas* is one of the most common taxa found as endophytes, followed by *Bacillus, Erwinia, Enterobacter,* and *Flavobacterium.* The endophytic bacteria have over 40 genera of diversity [21]. The genus Pseudomonas is classified as a bacteria that can produce cellulase [37]. *P. putida* has been reported to be able to produce cellulase enzymes and can be used to break down cellulose from palm oil mill wastewater [38].

Conclusion

Based on the results, only one isolate which had cellulolytic ability among seven others. That isolate encode CSZA2 which has been proven to produce cellulase activity with cellulolytic index 0.87. The results of morphological and biochemical characteristics, as well as molecular identification indicate that isolate CSZA2 is a *Pseudomonas putida* RTI2 bacteria,

which is known as a cellulolytic bacterium. The bacterial isolates that have been found can be developed to produce cellulase. The discovery of bacterial cellulolytic activity can be developed for biomass degradation, bioethanol production, and agricultural biotechnology.

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Conflict of interest

The authors whose names are listed immediately below certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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