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Isolation and identification of turmeric rhizome endophytic bacteria (*Curcuma longa*. L.) using the 16s rRNA method

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ABSTRACT

Turmeric (*Curcuma longa*. L.) is one of the components of traditional medicine. Oleoresin, curcumin, resin, essential oils, desmetoxycurcumin and bidesmetoxic curcumin are secondary metabolites found in turmeric rhizomes. The aim of this research is to obtain pure isolates and identify specific bacterial species using Gram staining and 16S rRNA methods, which are expected to be used as raw materials for new drugs. The hypothesis of this research is that isolates and species of endophytic bacteria have been obtained from turmeric rhizomes (*Curcuma longa*. L.). The methods used included material preparation, sample preparation and sterilisation, bacterial isolation, DNA isolation and sequencing. Seven isolates of turmeric rhizome bacteria (*Curcuma longa*. L.) were obtained and the results of molecular identification were carried out on one isolate using the 16S rRNA method with code BE.K.5, which is homologous/shows 94% similarity to Lysinibacilus sphaericus.

Keywords: bacterial Isolation; endophytic bacteria; turmeric rhizome; 16S rRNA

INTRODUCTION

Traditional medicinal plants are plants that are very popular as raw materials for traditional medicine. Consuming or using medicinal plants is said to increase the body's endurance (improve the immune system). The Ministry of Agriculture, in this case the Department General of Horticulture, as the state institution that manages the production of medicinal plants, states that medicinal plants are plants that are useful for medicine, cosmetics and health and are consumed or utilized from plant parts such as leaves, stems, fruit, tubers (rhizomes) or roots (Yani & Susilawati, 2023). Most of Indonesian people choose traditional treatment with medicinal plants (herbs). There is a tendency for recently tren "back to nature" where people prefer to use herbal medicine. Herbal medicine has several advantages over synthetic drugs, including having minor side effects and being safer. So the state and related agencies must pay attention to facilities and regulations.

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Turmeric (*Curcuma longa.* L.) is one of the components of traditional medicine. *Curcuma longa.* L, an annual medicinal plant, produces turmeric rhizomes. The word "turmeric" comes from the Arabic word kurkum refers to the yellow hue. Curcuminoids are what give turmeric its distinctive orange-yellow color. Turmeric (*Curcuma longa.* L) contains a large number of bioactive compounds which have the potential to be developed into antibiotics (Adamczak et al., 2020). This biologically active component is mainly found in turmeric. Turmeric also contains secondary metabolites such as saponins, alkaloids, triterpenoids, flavonoids, tannins and polyphenols in addition to the bioactive compounds that make up its main components. Oleoresin, curcumin, resin, essential oils, desmetoxycurcumin, and bidesmetoxic curcumin are secondary metabolite compounds found in turmeric rhizomes (Jyotirmayee et al., 2023).

Research efforts on turmeric rhizomes can be assisted by optimizing the utilization of various secondary metabolites (Elita et al., 2019). Some varieties of turmeric take a long time to cultivate, as a raw material for medicine, it requires quite a lot of simplicia, so one alternative is to use endophytic bacteria. The diversity of endophytic bacteria in medicinal plants in the Zingiberaceae family is very high. Up to 17 genera of bacteria have been identified from crosses (Curcuma heyneana) (Prayoga et al., 2021). Vinarayani and Prakash (2018) reported that 20 species of endophytic bacteria belonging to 12 different genera had been isolated from turmeric plants (*C. longa*)(Widowati et al., 2020). Endophytic bacterial microorganisms are not only economical, but also contain bioactive compounds and are easy to culture. On host plants, bacterial microorganisms have a very short life cycle (Yahya et al., 2017). Besides that, endophytic bacteria are able to produce compounds produced by their host plants (Hanif, 2017). Moreover, it has similar to its host. Therefore, this study isolated endophytic bacteria from turmeric rhizomes to determine the type of bacteria that grow in them and test their biological activity. One of the antibacterial tests is that turmeric contains active antibacterial compounds.

METHODOLOGY

This research consists of several stages, namely, preparation of materials (collection of materials and determination), preparation, sterilization of raw materials, isolation of endophytic bacteria, purification of endophytic bacteria, gram staining, isolation of chromosomal DNA, sequencing, and species determination.

The material preparation process starts from collecting materials taken from the Kasomalang Lembang area, for determination carried out at the

Bandungense Zoological Herbarium SITH ITB Jatinangor Sumedang Campus. At the preparation and sterilization stage, the sample is sterilized by immersing the surface of the sample. The sterilization method commonly used is the surface sterilization method. The bacterial isolation stage uses the streak plate method. The sample is aseptically inoculated into the medium and incubated. The purification stage of endophytic bacteria using the scratch technique until pure isolates are obtained. Then, characterization of the bacteria was observed macroscopically by observing the colony shape, colony surface and colony edges of each endophytic bacterial isolate. The chromosomal DNA isolation stage was carried out after obtaining the characteristics of the endophytic bacteria and it was known that the bacteria were gram positive or gram negative. Next, the PCR process (Polymerase Chain Reaction) is carried out. The band is multiplied using 4 processes of predenaturation; denaturation; annealing and elongation; and the last is sequencing and homology analysis. The sample is examined and data processed to determine whether the sample is included in the what species according to the data contained in NCBI GenBank.

1. Materials

The materials used in this research include: Turmeric rhizome, Merck Nutrient Agar (NA), Merck Nutrient Broth (NB), sterile tissue, sterile cotton, sterile gauze, plastic wrap, aluminum foil, filter paper, disc paper, 96% alcohol, physiological NaCl 0.9%, DNA isolation kit, PCR kit, Gram stain kit, sterile aquadest, aqua dm, nystatin, ethanol 70%, sodium hypochlorite 5%, crystal violet and Iodine. Preparation of ingredients consists of collecting ingredients, namely yellow turmeric (*Curcuma longa*. L.). Materials are taken and carried and closed using plastic bags. The plant part selected and used in the research was turmeric rhizome (*Curcuma longa*. L.) obtained from Kasomalang Subang.

2. Preparation and Sterilization of Raw Materials

One gram of *Curcuma longa*. L. sample was taken and washed thoroughly with running water and then dried. After that, sample was sterilized on its surface by immersing it for 60 seconds in 70% ethanol. The final stage was rinsing using sterile distilled water and drying using sterile tissue.

3. Control Media Nutrient Agar (NA) media

Dissolving 0.74 grams in 20 ml of sterile distilled water in an Erlenmeyer flask. Next, heat it on a hot plate at a stable temperature for 10-15 minutes. The Erlenmeyer containing NA is sterilized in an autoclave at 121°C for 15 minutes. Next, the sterile media is put into a petri dish with aseptic treatment

and put into an incubator and then kept for 2 x 24 hours at a temperature of 37°C.

4. Isolation of *Curcuma longa*. Bacterial Endhopytic

The outer skin of samples that has been previously sterilized, was cleaned and thinly sliced vertically and horizontally using a sterilized knife. Samples then were planted using the Plan Peace method (Direct Planting) and incubated for 2 x 24 hours at a temperature of 37°C.

Purification of endophytic bacteria was carried out by inoculating the isolate on new Nutrient Agar media with a toothpick and incubating 1 x 24 hours in an incubator at a temperature of 37°C until a pure isolate is obtained. Then the purified endophytic bacteria were taken by inoculating the isolate in Nutrient Broth liquid media in a test tube using the dipping technique with a toothpick and incubating for 18 hours in a shaker Incubator at a temperature of 37°C with a speed of 180 rpm (Ayunda, 2015).

5. Identification with 16s RNA Method

As much as 1 ml of bacterial isolate from each test tubes (samples that have been incubated in a shaker incubator) were transferred to a microtube, then 125 µL of sterile glycerol and 350 µL of Physiological NaCl were added and homogenized. Samples then covered with wrap and stored in the freezer (refrigerator).

6. Gram staining

Purified endophytic bacterial isolates (isolate stock) were taken from one of them. Gram staining was carried out using crystal violet, iodine, 96% alcohol and fuhsin. The gram staining procedure consists of the following steps. 1) A small amount of pure isolate is taken using a loop needle aseptically and spread slowly over a glass object. 2) The preparation is fused over Bunsen until dry. 3) The preparation is dripped with purple crystal solution and left for 1 minute then rinse with running water and dried 4) The preparation is dripped with iodine solution. Leave it for 1 minute then rinse with running water and dry. 5) The preparation is dripped with 96% alcohol until the purple color disappears. 6) The preparation is dripped with safranin solution. Leave it for 30 seconds then rinse with running water and dry. 7) The preparation is dripped with fuhsin solution for 2 minutes, rinse with running water and dry. 8) Rinse the preparation again and dry it, then observe it under a microscope. To observe the preparations, use a microscope with 10x, 40x and 100x magnification.

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7. Chromosomal DNA Isolation

Isolating chromosomal DNA were done by growing bacteria in Luria Bertani (LB) liquid medium and the Geneaid Kit. Bacterial isolates that have more turbidity than other isolates in the test tube were taken using a micro pipette and put into a microtube and centrifuged for 1 minute at a speed of 14000 rpm. The next process was carried out in several stages with DNA kit for isolation including: 1. Lysis of cell walls and membranes 2. DNA Extraction 3. DNA Purification 4. DNA Precipitation.

Chromosome 15 DNA Amplification PCR was used to amplify genomic DNA using primers BactF and UniB1(Fatmawati et al., 2016). The process consists of 3 stages including denaturation, annealing, and extension.

Agarose Gel Electrophoresis was used as a tool to see the results of DNA amplification under UV light (360 nm). The electrophoresis process began with making agarose gel with 1% agarose concentration by dissolving 1 gram of agarose in 100 ml of 10X TAE. After that, heated it until boiling and completely dissolved using microwave soup. Next, add 1 mikroliter ethium bromide then poured it into the casting tray mold with the comb installed. After the gel has solidified for ±30 minutes, added ampicon samples of the PCR product. To determine the size of the PCR amplification product, 1kbp DNA ladders and loading dyes were inserted into the first gel well, then the amplified sample DNA into the next gel well. In the last well, positive controls were added. After that, the electrophoresis tool was turned off, then the gel from the tool was taken and transferred into a UV-transilluminator for visualization and the results were observed and documented.

8. Sequencing Determination of the nucleotide

Sequence using the dye-end terminator method was carried out at MACROGEN South Korea. For one determination, 100 µl of DNA template and 10 pmol of each of the primers BactF, UniB1, ComF and ComR are required. Homology Analysis The nucleotide sequence of the 16S rRNA gene that has been obtained is then identified for grouping. Groupings were processed using the BLAST program (www.blast.ncbi.nml.gov/blast.egi) . This program is carried out to compare sequences and look for matches with nucleotide sequences contained in GenBank data (www.ncbi.nlm.gov/genbank/) (Fatmawati et al., 2016). Meanwhile, data processing from sequencing results was carried out using the MEGA11 application to obtain the sequence of 16

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nucleotide bases and construct a pylogenic tree.

RESULTS AND DISCUSSION

1. Material preparation and determination

The sample used was turmeric rhizome (*Curcuma longa.* L.) taken from the Manoko Experimental Garden, Lembang District, West Bandung Regency, West Java Province. The sample was cleaned and sorted before used. Then a determination was carried out to determine the validity of the species and names of the plants used which was carried out at the Bandungense Zoological Herbarium SITH ITB Jatinangor Sumedang Campus with identification number 8606/ITI.C11.2/TA.00/2022 as one of the accuracy processes that the plant is a valid plant, the same as the plants used as research material. From this process, the species name *Curcuma longa.* L was obtained from the Zingiberaceae family.

2. Preparation and Sterilization of Raw Materials

The raw materials that have been sterilization aims to remove dirt that is visible macroscopically such as soil, etc. as well as microorganisms that stick to the outside or surface of the sample, which is expected by this process to isolate colonies. is an endophytic bacterium.

3. Control Media

This stage is for confirmed that there are no visible spots or foreign objects growing on the control media, which indicates that the media is not contaminated. Figure 1 shows that the media is clear from contaminatination.

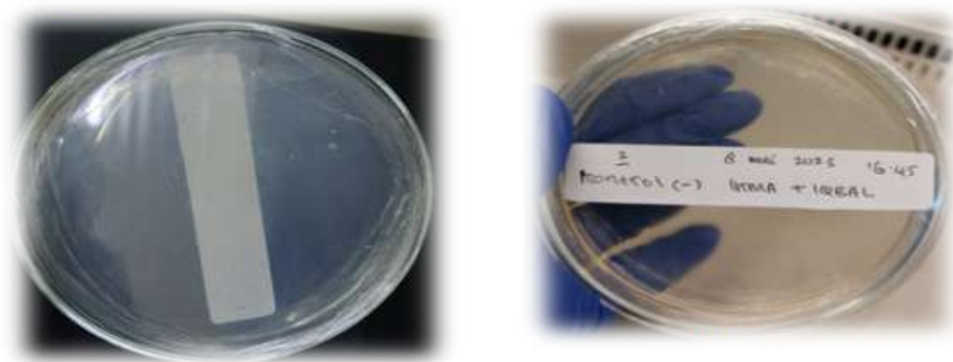

Figure 1. Control media

4. Isolation of Endophytic Bacteria

Endophytic bacteria can be isolated from several parts of plants. One of them is taken from the rhizome. The plant used is turmeric rhizome. Based on research conducted by Asep Irfan Muttaqin (2021), who isolated endophytic bacteria from turmeric plants (*Curcuma longa*. L.), he obtained 3 isolates that had quite good scores. Therefore, isolation was carried out again so that 13 isolates were obtained. Then the isolates were purified to obtain 7 pure isolates. Those with the best value are identified based on morphological and biochemical characters. In order to get more specific results, molecular identification is carried out so that the morphological and biochemical data can be used as supporting data to determine the species specifications of endophytic bacterial isolates.

Samples that have previously been sterilized are then taken from one segment and cut thinly in a round and slightly slanted shape. The sample is placed on a medium, namely Nutrient Agar, which is useful as a supporting material or medium in which there is a source of vitamins and energy for bacterial growth. The nutrient medium was incubated in an incubator in an inverted position. This is so that the sample attached to the medium does not fall during the incubation process. The agar nutrient medium is placed in an incubator at a temperature of 37 ℃, where this temperature is the temperature usually used in the incubation process so that the bacteria grow well. The time used in this process is ±2×24 hours because endophytic bacteria require a longer incubation process to ensure that the bacteria are truly endophytic bacteria. Apart from that, according to Ifnawati (2013), bacteria are likely to grow in this time period which is in the logarithmic and extinential phases, in these phases constant division occurs in bacteria and the number of cells increases. Figure 2 showing the results of the isolation of endophytic bacteria.

a) BE C1 Isolate b) BE C 2 Isolate c) BE C3 isolate

Figure 2. Isolate of endophytic bacteria from turmeric rhizomes for 2 x 24 hours

Table 1.
Colony Characteristics of Turmeric Rhizome Endophyte Bacterial Isolates

Isolate Code	Morphology		
	Color	Form	Edge
BE C1	White bone	Round	Irregular
BE C2	White bone	Round	Irregular
BE C3	White bone	Round	Irregular

Samples were incubated for 2 x 24 hours at 37°C. From samples grown on 3 Nutrient Agar media, it can be seen that the bacteria grow well. The results of macroscopic identification of endophytic bacteria from turmeric (*Curcuma longa.* L.) rhizomes are provide in Table 1.

5. Single Colony

After the isolate is obtained, incubation is carried out in new media to obtain a pure isolate. From this process, 7 isolates were selected to be inoculated into new media again aseptically. This is useful for obtaining pure cultures of these endophytic bacteria. After that, the medium containing the bacteria was incubated again in the incubator for 1 x 24 hours at a temperature of 37°C. This is useful for prolonging the life of bacteria in the Nutrient Agar medium. After the process is complete and there is no visible contamination, the Nutrient Agar medium containing bacteria is placed in a cooler to be used in the next process, namely cultivating bacteria in Nutrien Broth medium (Mikdarullah & Nugraha, 2017).

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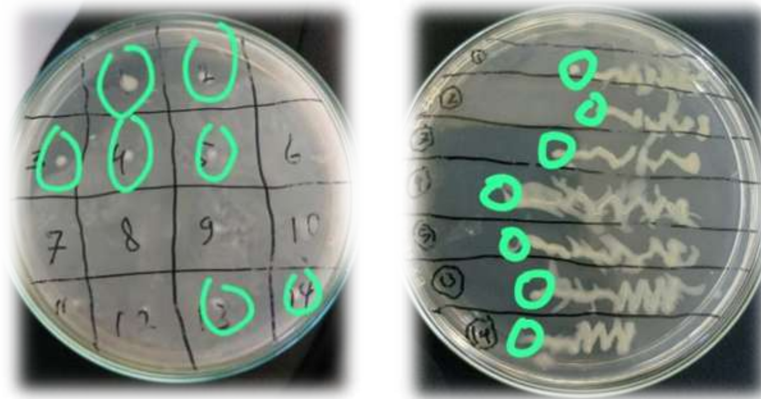


Purification Results (a) Inoculation results (b)

Figure 3. Results of inoculation into new media and pure isolate

The green marker in Figure 3 shows the selected isolate use for the next process. There were 7 pure isolates taken which were named with the codes BE.K.FI.1, BE.K.FI.2, BE.K.FI.3, BE.K.FI.4, BE.K.FI.5, BE.K.FI.13, BE.K.FI.14.

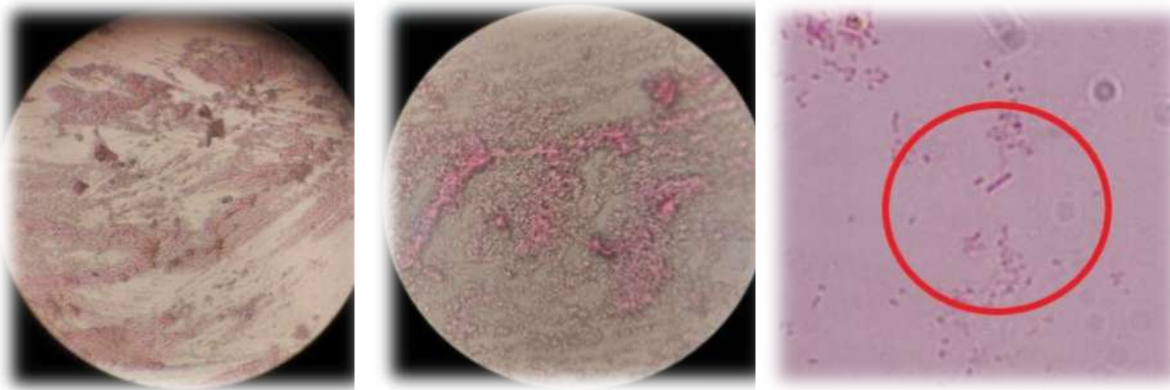
6. Isolate Stock

In the process of identifying bacteria, it cannot be denied that there is a very high risk that isolates will be contaminated either from the work area or from human error itself. So the isolation stock is made to minimize this. One ml of the harvested isolate was taken, then glycerol and 0.9% Physiological NaCl were added and put into a microtube using a micro pipette aseptically. Glycerol is useful as a cryoprotective agent which aims to extend shelf life by protecting microbial cell membranes. Meanwhile, 0.9% Physiological NaCl is useful for maintaining the survival of isolates by balancing microbial cell ions. One by one the isolates were transferred into a 1.5 ml microtub and stored in the refrigerator (freezer). This is useful so that the isolate that has been made as stock is not degraded. Each microtube is named according to the code carried out in the previous process

7. Gram stain

The gram staining process is carried out before the molecular identification process. This aims to find out what types of bacteria are contained in the sample so as to make it easier to select the appropriate Gen KIT in the molecular identification process. The results of the gram staining carried out showed that the sample belonged to the gram positive type of bacteria. This is characterized by the appearance of a purple color in the colony which is because most of the cell wall layers are composed of thick

peptideglycans so that they are able to bind the dye and are not damaged when washed with alcohol. Apart from that, the structure and composition of the cell wall also looks simple and thick compared to gram-negative bacteria. The following are the results of gram bacterial staining.



10x magnification (a) 40x magnification (b) 100x magnification (c)

Figure 4. Gram Staining Results

Gram staining was carried out by taking one of the 7 isolates. The color of the 3 magnifications seen from the microscope is purple (gram +).

8. Chromosomal DNA Isolation

In the molecular identification process, a DNA isolation process is carried out to obtain DNA and RNA cell tissue extracts. The isolate taken was the isolate with the code BE.K.FI.5. In this process, the extract needed is RNA, so the cells are purified by centrifugation. This process will produce pellets which will later be used for the identification process using the PCR (Polymerase Chain Reaction) technique through sequencing the 16S rRNA coding gene. The sequence of nitrogen bases obtained will later determine the specific species of the sample. This extraction process works by breaking down the cell walls so that the DNA is separated from the main cell.

Bacterial cultivation is carried out by taking isolates from Nutrient Agar medium using a sterile toothpick. This is done to make it easier to collect bacteria so that they do not become contaminated and mix with other bacteria. After that, the toothpicks are dipped into the Nutrient Broth media which is in the test tube one by one under aseptic conditions.

After all the isolates had been transferred, the test tube containing the medium and isolates was incubated in a Shaker Incubator for 18 hours at 180 rpm at a temperature of 37°C. The working principle is to incubate using rotation. Isolates grown in Nutrient Broth medium changed color to become more cloudy, after previously being clear. This indicates that the isolate grown in Nutrien Broth medium grows well and is ready to be harvested.

Molecular identification was carried out on one of the 7 isolates that had been purified. This is taken based on the highest turbidity index, which indicates the growth of bacteria in the liquid medium. So 7 isolates were obtained with the codes BE.K.Fl.1, BE.K.Fl.2, BE.K.Fl.3, BE.K.Fl.4, BE.K.Fl.5, BE.K. Fl.13, BE.K.Fl.14. Of the 7 isolates, the isolate with the code BE.K.Fl.5 was taken for molecular identification.

The procedures used in DNA isolation/extraction were carried out according to the instructions stated in the Geneid PrestoTM Mini gDNA Bacteria Kit manual. Where in these guidelines there are several main processes that must be carried out, namely sample preparation, cell lysis, DNA binding, washing and elution.

The commercial PrestoTM Mini gDNA Bacteria Kit uses DNA filtration or the mini column principle. For gram-positive bacteria, use gram (+) buffer with lysozyme, while gram-negative bacteria must use gram (-) buffer and proteinase K to dissolve the cell wall first. Then using lysis buffer (GB buffer), the cells are lysed. Absolute ethanol was used to precipitate DNA, which was then filtered and cleaned with W1 and wash buffer. Finally, elution buffer to dissolve the DNA.

The components used in this research have several purposes. The cell walls of gram-positive bacteria are specifically destroyed by gram-positive buffer (Geneaid), which has been added to lysozyme. The purpose of adding a buffer is to maintain pH stability so that DNA is not damaged during processing. Proteinase K, which breaks down cell components (mainly proteins), and Gram(-) buffer (Geneaid), which has the unique ability to break down the cell walls of gram-negative bacteria, are two examples. The purpose of GB Buffer (Geneaid) is to lyse bacterial cells. DNA is concentrated or precipitated using absolute ethanol. Wash buffer is used to remove additional contaminants from the DNA. Using this method is a method that is commonly used, apart from the affordable price, the processing process is also fast (Handoyo & Rudiretna, 2001).

9. **PCR (Polymerase Chain Reaction) for 16s Gene Amplification**

The next stage is Polymerase Chain Reaction, where this stage aims to duplicate the DNA band in vitro. The processes that occur in the PCR process include pre-denaturation, annealing and elongation. The materials used in the PCR process which was carried out in 35 cycles for ± 2 hours were NFW (Nuclease Free Water), Master Mix, forward primer (27F), reverse primer (1492R), and DNA template. The purpose of this commercial PCR master mix is as a component or template DNA mixture for PCR amplification. The reverse primer acts to start DNA strand synthesis from the 3' -------- 5' end, while the forward primer starts from the 5' -------- 3' end. The DNA template functions as a template for creating new identical DNA molecules during the PCR process, and NFW (Nuclease Free Water) as a DNA solvent (Green & Sambrook, 2018).

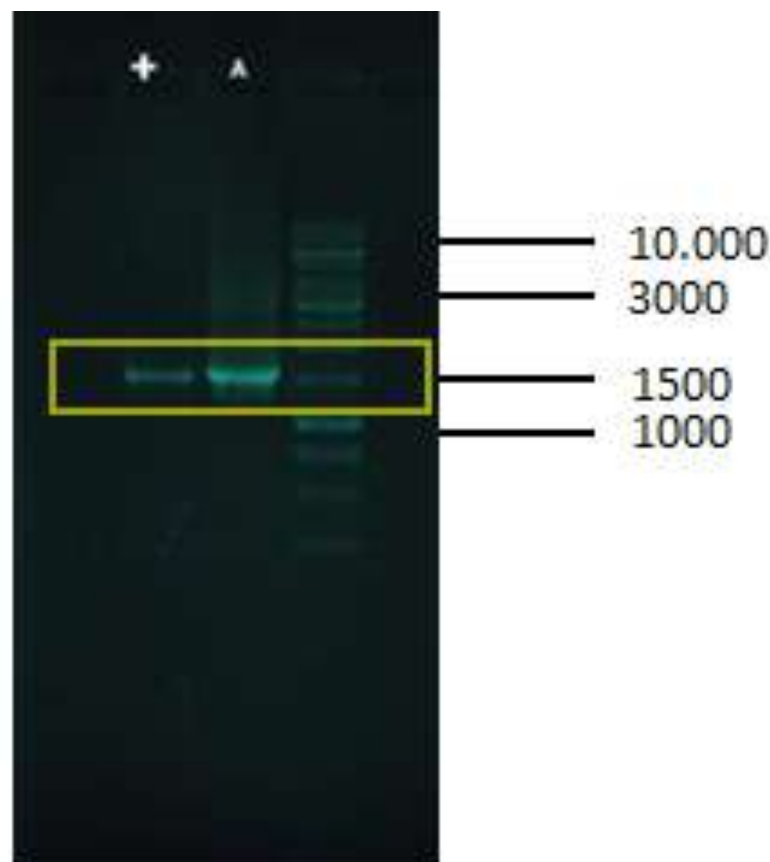


Figure 5. Results of 16S gene amplification

Electrophoresis results from amplification of the 16S rRNA gene of isolates of the Endophytic Bacteria of Turmeric Rhizome (*Curcuma longa.* L.) with a DNA band size of ±1500 bp. M (DNA Marker) 1kb DNA ladder. The results obtained from the PCR process were electrophoresed for 35 minutes at a voltage of 100 with a concentration of 1% agarose gel. The electrophoresis results showed that there were bands that were separated and parallel to the 1500 bp marker. This validates that the amplified gene fragment has a size of ± 1500 bp, where this size corresponds to the nucleotide size of the 16S rRNA gene, namely 1500 bp (Noer, 2021).

10. Homology Analysis

BLAST analysis was carried out with the aim of comparing the results obtained with DNA sequence results from around the world stored in the Gen Bank database. BLAST analysis produces information about which bacteria are similar to the DNA sequence of the sample so that it can be used to identify bacteria (Wangiyana, 2016). The information produced from BLAST analysis is in the form of Query Coverage and Maximum Identity. BLAST analysis is carried out online on the National Center for Biotechnology Information (NCBI, 2016). The characteristics that are most similar to the DNA sequence obtained can be seen from the Max Score value, which is the value of similarity (identical) base pairs with the same Total Score. The higher the score, the higher the level of homology of the two sequences. and Query Coverage which is the percentage of samples used in BLAST analysis that is close to 100%, E-value is close to 0, while Max Identity is the percentage of identification accuracy that is close to 100%. Of the four parameters, the Query Coverage value is the most important because it shows the database covered by the query. If the E-value is increasingly an estimated value that provides a statistically significant measure of the two sequences, the value is closer to 0, which means it is more reliable and if the value is 1, then it should not be used (Narita, 2012). According to Akihary (2020), if the 16S rRNA sequence homology shows <97.5% it can be said to be a different species or a novel species and if it is within the 99% range then it is said to be one species.

After the data results from the BLAST process are carried out, the data is processed again in the MEGA11 application to obtain data in the form of a pylogenic tree (Wangiyana, 2016). This aims to construct precisely the relationships between organisms and estimate the differences that occur from one ancestor to its pylogenic tree was formed using the neighbour-joining method which is included in the distance method with the principle of grouping taxa based on the

evolutionary distance value of pairs of operational taxonomic units where each branch in the phylogenic tree evolves at a different rate (Hartl, 2000).

The results of phylogenic analysis using the 16S rRNA coding DNA sequence are shown in Figure 5.11.2 where it can be seen that isolate BE.K.5 is close to the species Lysinibacillus sphaericus.

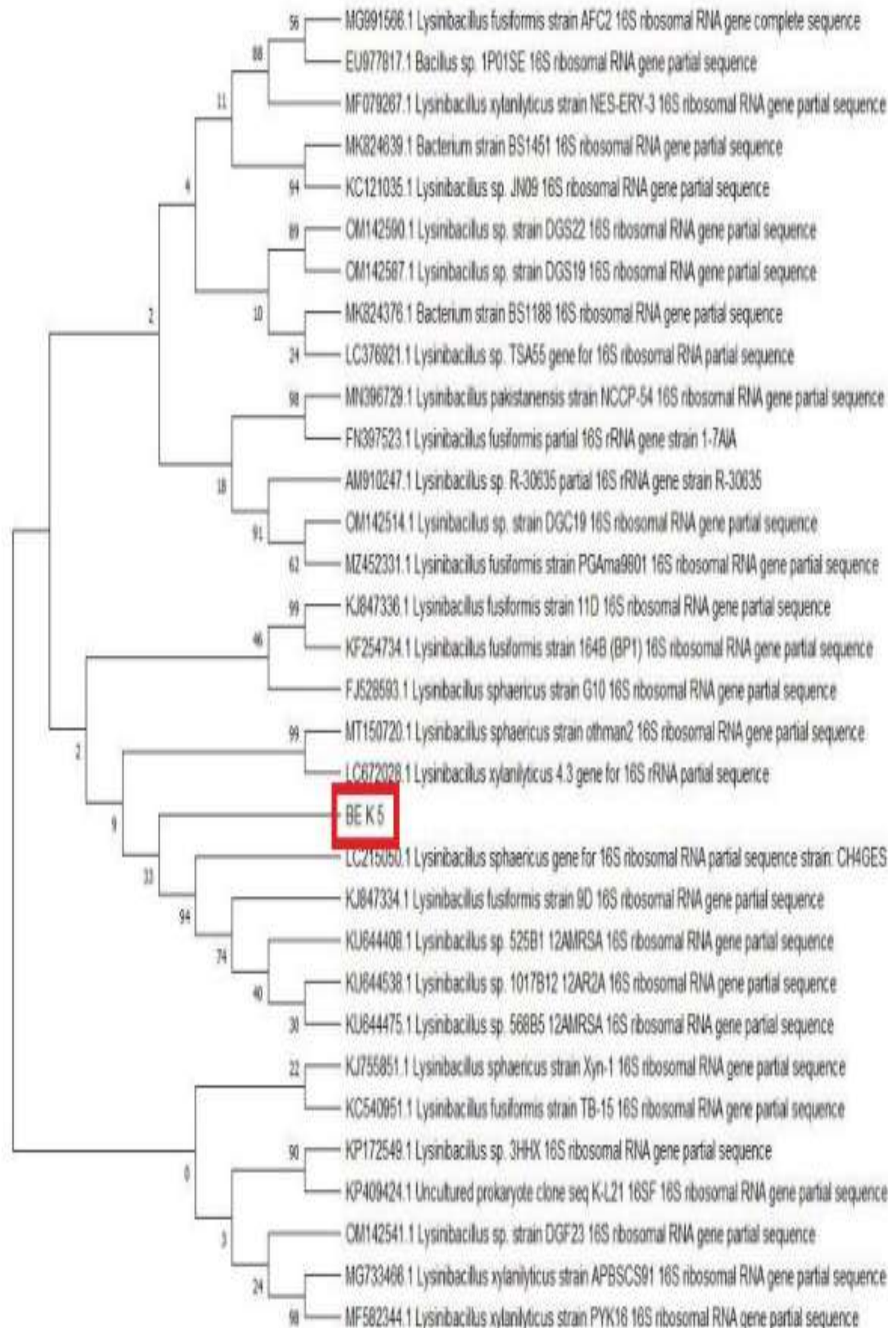


Figure 6. Visualization results using the MEGA11 application in the form of a PYLOGENIC TREE using Heuristic Model

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CONCLUSION

Isolation process of Turmeric Rhizome Endophytic Bacteria (*Curcuma longa*. L) obtained 7 isolates with the code BE. K.FI.1, BE. K.FI.2, BE. K.FI.3, BE. K.FI.4, BE. K.FI.5, BE. K.FI.13, BE. K.FI.14. Isolate with the code BE. K.F1.5 is taken as a sample used in the molecular identification process.

Identification of Turmeric Rhizome Endophytic Bacteria (*Curcuma longa*. L) in the form of gram-positive bacteria visualized, while for molecular identification with the 16S rRNA method, there was a similarity in percentage 94% with Lysinibacilus sphaericus.

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