Jurnal Pembelajaran Dan Biologi Nukleus p-ISSN: 2442-9481
Vol 11 (1): 40 - 54, March 2025 e-ISSN: 2685-7332

Isolation and Identification of Endophytic Bacteria from *Euphorbia hirta* L. (Patikan Kebo)

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Submitted December 09 Th 2024 and Accepted February 25 Th 2025

Abstract

Patikan Kebo (Euphorbia hirta L.) is a plant that lives scattered in the yard, has potential as an antibacterial, one way to extract plants is by isolating endophytic bacteria. Endophytes are bacteria found in plant tissue. The purpose of this study was to isolate and identify the morphology of endophytic bacteria from plant Patikan Kebo (E. hirta L.). The method used is the Total Plate Count (TPC) method by taking samples, isolating bacteria, pure culture, morphological identification, and Gram staining. Endophytic bacteria were obtained by isolating parts of the plant Patikan Kebo (E. hirta L.), namely the roots of patikan kebo (PRE), Patikan kebo stem endophyte (PSE) and Patikan kebo leaves endophyte (PLE). The results of the study found 22 isolates, endophytic bacterial isolates have diverse morphological characteristics including appearance, elevation, edges and color. There are 20 isolates of endophytic bacteria that belong to the Gram positive group and 2 isolates of endophytic bacteria that belong to the Gram negative group. It can be concluded that endophytic bacterial isolates from Patikan Kebo (E. hirta L.) plants have different morphologies

Keywords: *Endophytes Bacteria; Euphorbia hirta; Identification; Isolation*

https://doi.org/10.36987/jpbn.v11i1.6688

INTRODUCTION

Patikan Kebo (*Euphorbia hirta* L.) is a plant that can survive in a place that has a tropical climate which includes herbaceous plants (Ardiansyah et al., 2018). This plant is a plant that lives in the soil in an environment with low humidity. Patikan Kebo

(*E. hirta* L.) is a type of plant that is found scattered in Indonesia and is used as a medicine (Najib & Ahmad, 2020). This plant lives scattered on empty land such as home yards (Karim et al., 2015). This plant is not cultivated and is usually considered a weed by the surrounding community. *E. hirta* L. has a small size belonging to the Euphorbiaceae family. It has morphological characteristics of single-leaved crossed before with monoecious type flowers, small stems and hairy with monopodial branching patterns (Fathiya & Yulisma, 2023). This plant contains various chemical substances such as *Myricyl alcohol, Tirukalol, Friedlin, β-sitosterol, β-eufol, Euphorbol, triterpenoid eufol, taraxerol, euposterol, hentriacontane, flavonoids, tannins, β-amyrin and Ellagic acid* (Naki et al., 2023). In addition to its diuretic properties, this plant also has properties that are slightly toxic, anti-inflammatory and antipruritic properties. These pharmacological effects are obtained by using all parts of the plant, either fresh or dried (Permadi, 2008).

Every plant has secondary metabolites in the form of antibacterial substances. Antibacterial substances produced by endophytic bacteria in colonies. Since many of these active ingredients are obtained through extraction from plants, especially medicinal plants, the ability of endophytic bacteria to produce these active ingredients can be developed. Obtaining active compounds directly from plants requires longer time and more complex procedures than extracting compounds from endophytic bacteria. Endophytic bacteria are found in the tissues of plants (Pratiwi, 2019).

Endophytes are bacteria that can spend their life without causing certain effects on their host plants in the plant body (Rori et al., 2020). Endophytic bacteria are living microorganisms that cannot be seen directly. These bacteria are spread throughout all parts of the host plant. Then the bacteria are able to stimulate and improve plant growth by obtaining nutrients and tolerating plant stress to soil conditions (Abd-Allah et al., 2018). Endophytic bacteria can be found in all plants. Endophytic bacteria are able to live in plant tissues from roots, stems, and leaves (Christina et al., 2013). Endophytic bacteria utilize plant nutrients obtained from plant metabolism. In addition, endophytic bacterial groups are beneficial to their host plants such as protecting plants in controlling insects or pathogens that can stimulate plant growth (Harmileni et al., 2023). Given the important contribution of the bacterial community, research on the identification of endophytic bacteria of Patikan Kebo (*E. hirta* L.) is still small. Therefore, this study aims to isolate and identify the morphological characteristics of endophytic bacteria from patikan kebo plants (*E. hirta* L.).

METHODS

Sample Collection

This research was conducted from Mei 2023. Samples *E. hirta* L. were collected from a residential garden in Bentiring Permai, Muara Bangkahulu, Bengkulu City. The data processing was performed in the Microbiology Laboratory, Basic Science building, Department of Biology, Faculty of Mathematics and Natural Sciences, Bengkulu University.

Materials and Tools

The equipment used in this study included an Petri dishes 100 x 15 mm, test tubes 10 ml, beaker glass 100 ml, Erlenmeyer 250 ml, volumetric pipette 10 ml, mortar and pastle, analytical balance, binocular microscope, refrigerator, Bunsen burner, bulb, Pasteur pipette, object glass, an incubator, a laminar airflow, an oven, an autoclave dan colony counter. The materials used were *E. hirta* L. plant (patikan kebo), alcohol 70 % and 96 %, distilled water (aquadest), Nutrient Agar (NA), crystal violet, safranin, lugol, immersion oil, cling warp, alumunium foil, tissue, methylated spirit, label text, nystatin, bayclin and cotton wool.

Procedures

Plant samples were selected in their young growth stage with fresh green leaves. Data processing was conducted in the Microbiology Laboratory, Basic Science Building, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Bengkulu. Parts of the plant, including roots, stems, and leaves, were collected, each weighing 100 grams. To prevent fungal growth and bacterial contamination, samples were stored in plastic bags to maintain dryness.

Isolation was performed using serial dilution from concentrations of 10^{-1} to 10^{-5} with two repetitions. Plant parts (roots, stems, and leaves) were cut into 1–2 cm pieces, washed under running water, and sterilized sequentially with alcohol, 3 % Bayclin, and sterile distilled water. The sterilized samples were ground, mixed with 10 ml sterile distilled water, and added to a test tube containing 9 ml sterile water, then vortexed to create suspensions with concentrations from 10^{-1} to 10^{-5}. A 0.1 ml aliquot of each suspension was spread onto Nutrient Agar (NA) plates with nystatin and incubated at 37 °C for 48 hours. Colony morphology was observed and counted using a colony counter (Lay, 1994). After initial isolation, each bacterial colony was transferred individually to fresh NA media in Petri dishes to obtain pure isolates. Re-isolation was conducted to ensure macroscopic uniformity of colonies (Rustini et al., 2022).

Morphological identification included observing colony characteristics such as color, edge, elevation, appearance, shape, and surface texture (Prihanto et al., 2018). Gram staining was performed on bacteria incubated for 48 hours. A bacterial smear was prepared on a glass slide, stained with crystal violet 1 minute, iodine 2 minutes, decolorized with alcohol 96 %, and counterstained with saffranin 30 seconds. The smear was examined microscopically at 1000 x magnification with immersion oil. Gram positive bacteria are purplish and Gram negative are reddish (Lay, 1994).

Data analysis

Data analysis of research on Isolation And Identification Of Endophytic Bacteria From Patikan Kebo (*E. hirta* L.) using descriptive qualitative, identification of endophytic bacterial isolates with reference to Bergey's Manual of Determinative Bacteriology 9th Edition (Holt et al., 1994).

RESULT AND DISCUSSION

E. *hirta* L. (Patikan kebo) Plant

E. hirta L. (Patikan kebo) were collected from a residential garden in Bentiring Permai, Muara Bangkahulu, Bengkulu City. The morphology of *E. hirta* L. (Patikan kebo) plants can be see in Figure 1.

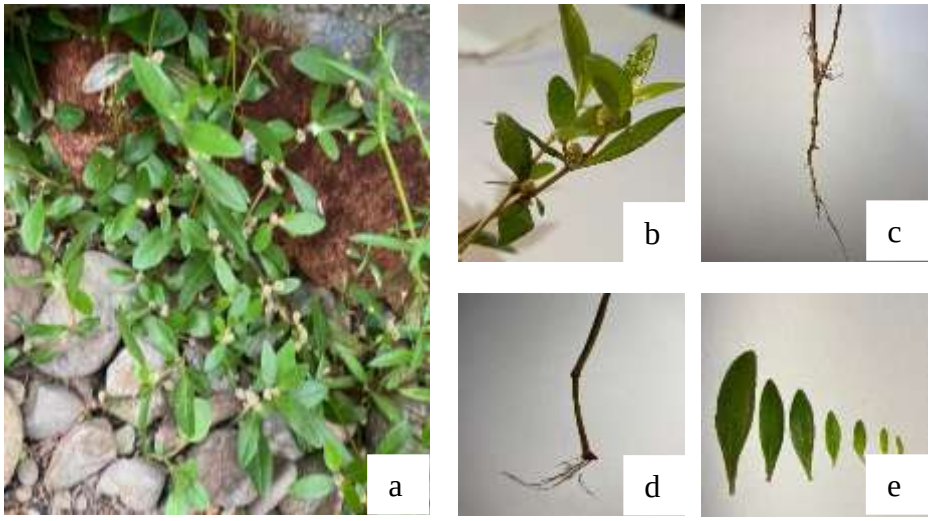


Figure 1. Plant morphology *E. hirta* L. a= *E. hirta* L.; b= flower; c= root; d= stem; and e= leaf (photo source: private documentation).

Endophytic Bacteria (*E. hirta* L.)

Based on the results of the research that has been done, the calculation of the total number of bacterial colonies isolated from plants Patikan Kebo (*E. hirta* L.) is shown in Figure 2. Based on the results of the research that has been done, the total calculation of bacterial colonies isolated from plants Patikan Kebo (*E. hirta* L.) is listed in Table 1.

Table 1. Calculation of total bacterial colonies isolated from plants Patikan Kebo (*E. hirta* L.)

Isolate Codes	Colony Numbers			
	10^{-3}		10^{-5}	
	U1	U2	U1	U2
PRE	30	15	3	55
PSE	28	23	20	65
PLE	18	11	5	27

Remarks: Bacterial colonies from E. *hirta* L. plant; PRE= Patikan kebo Root Endophyte; PSE= Patikan kebo Stem Endophyte; PLE= Patikan kebo Leaves Endophyte; U1 = Repetition 1; and U2 = Repetitions 2.

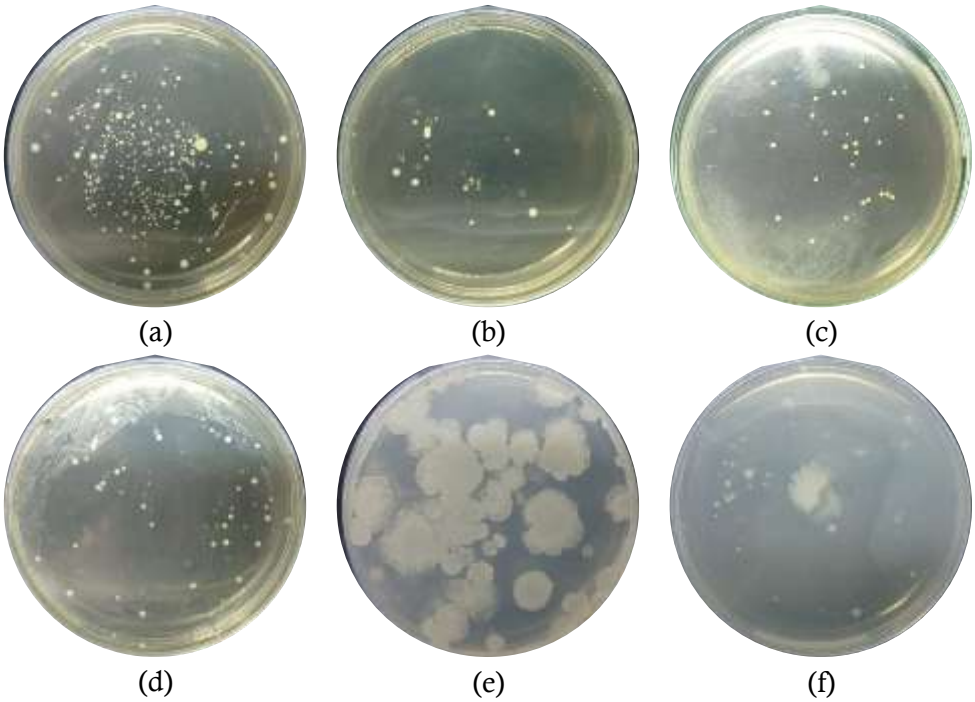
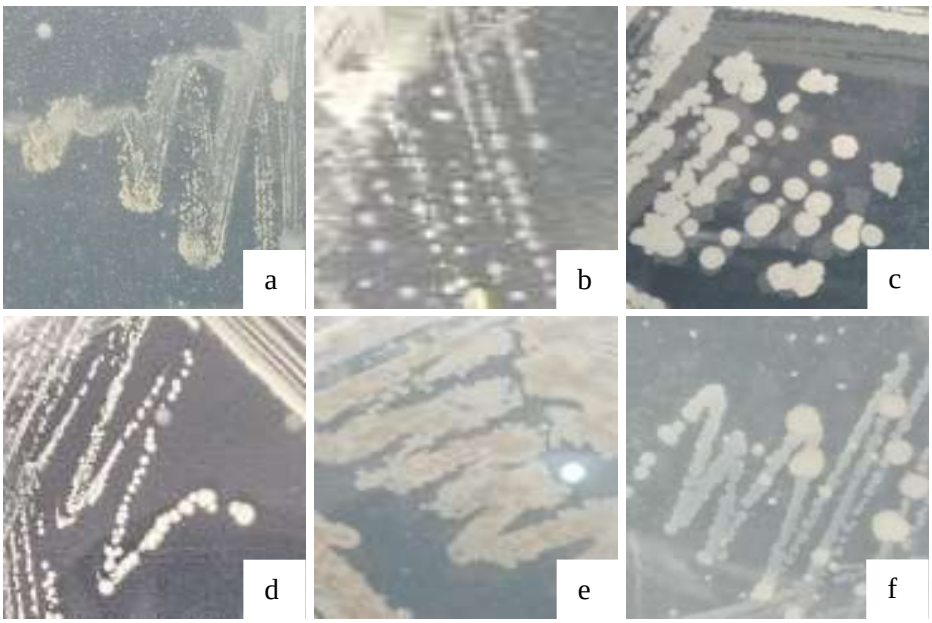


Figure 2. Bacterial colonies from plant E. *hirta* L. on Nutrient Agar media after incubation for 2 x 24 hours at 37 ºC

Remarks: (a) PSE= Patikan kebo Stem Endophytic 10^{-3} U2; (b) PSE= Patikan kebo Stem Endophytic 10^{-5} U1; (c) PLE= Patikan kebo leaves Endophytic 10^{-3} U1; (d) PLE= Patikan kebo leaves Endophytic 10^{-5} U2; (e) PRE = Patikan kebo Root Endophytic 10^{-3} U1; and (f) PRE = Patikan kebo Root Endophytic 10^{-3} U1.

Based on the research, the results of purification (purification) of endophytic bacteria of Patikan Kebo (*E. hirta* L.) plants are shown in Figure 3.



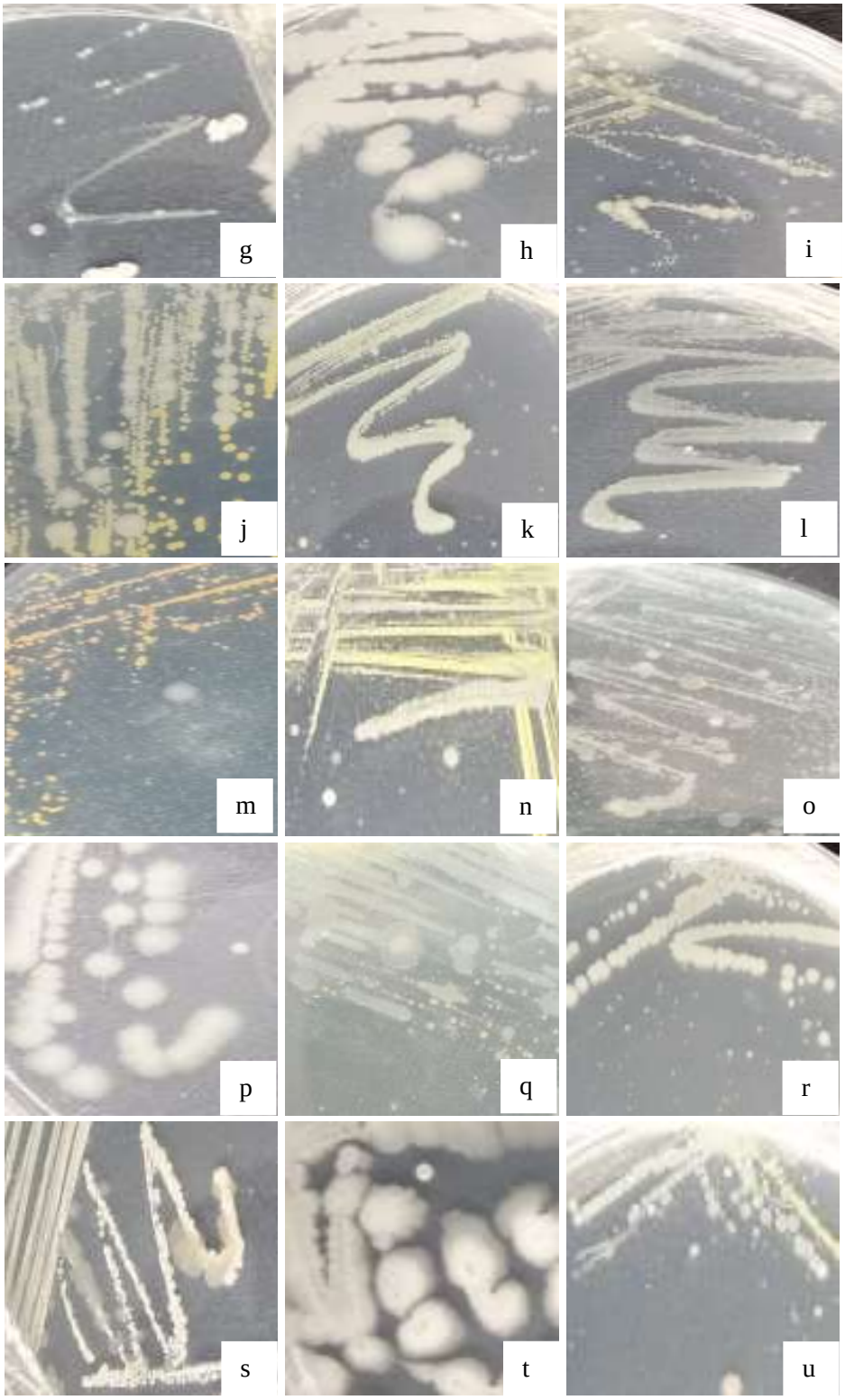

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Figure 3. Morphological observation of colonies of bacterial isolated from Patikan Kebo (*E. hirta* L.) plants, PRE = figure a-e; PSE = figure f-n; PLE = figure o-v.

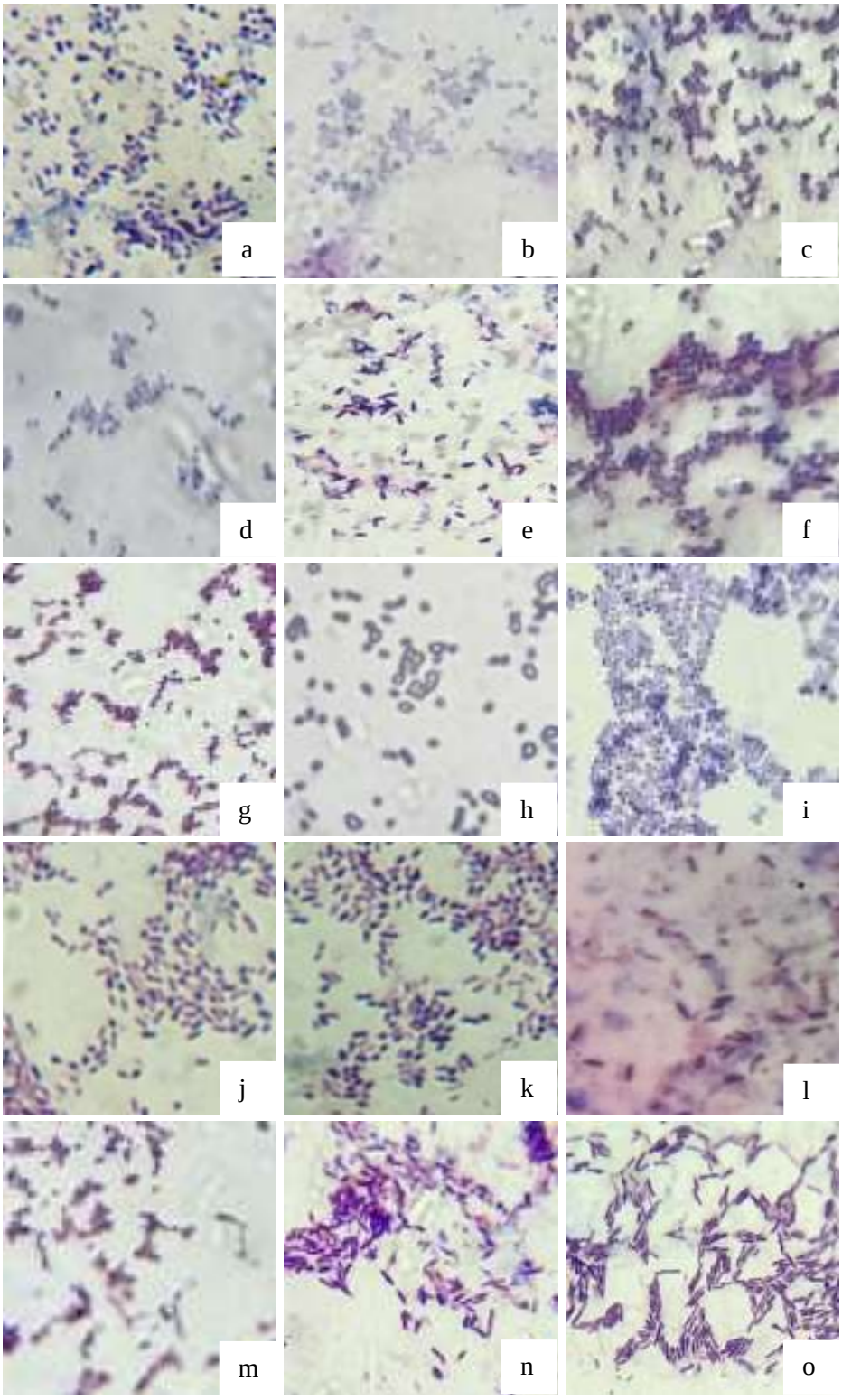
Based on the results of the research that has been done, the morphological characteristics of the colonies of bacterial isolates from plants E. *hirta* L. are listed in Table 2.

Table 2. Colony morphology characteristics of bacterial isolates from *E. hirta* L.

Isolate Code	Species	Shape	Elevation	Edge	Color
PRE	1	*Circular*	*Flat*	*Entire*	Creamy white
	2	*Circular*	*Flat*	*Entire*	White
	3	*Circular*	*Flat*	*Undulate*	White
	4	*Circular*	*Convex*	*Entire*	Creamy white
	5	*Irregular*	*Flat*	*Undulate*	Creamy white
PSE	1	*Circular*	*Flat*	*Entire*	Creamy white
	2	*Circular*	*Convex*	*Entire*	White
	3	*Irregular*	*Flat*	*Undulate*	Creamy white
	4	*Circular*	*Flat*	*Entire*	White
	5	*Circular*	*Flat*	*Entire*	White
	6	*Circular*	*Flat*	*Entire*	White
	7	*Circular*	*Convex*	*Entire*	Orange
	8	*Circular*	*Flat*	*Entire*	Creamy white
	9	*Circular*	*Flat*	*Entire*	White
PLE	1	*Circular*	*Flat*	*Entire*	Creamy white
	2	*Irregular*	*Flat*	*Undulate*	White
	3	*Circular*	*Flat*	*Entire*	Yellowish
	4	*Circular*	*Flat*	*Entire*	White
	5	*Circular*	*Convex*	*Entire*	White
	6	*Irregular*	*Flat*	*Undulate*	White
	7	*Circular*	*Convex*	*Entire*	White
	8	*Circular*	*Convex*	*Entire*	Yellow white

Remarks: PRE= Patikan kebo Root Endophyte; PSE = Patikan kebo Stem Endophyte; PLE = Patikan kebo Leaves Endophyte.

Based on the results of the research that has been done, the coloring results of bacterial isolates isolated from plants (*E. hirta* L.) are shown in Figure 4.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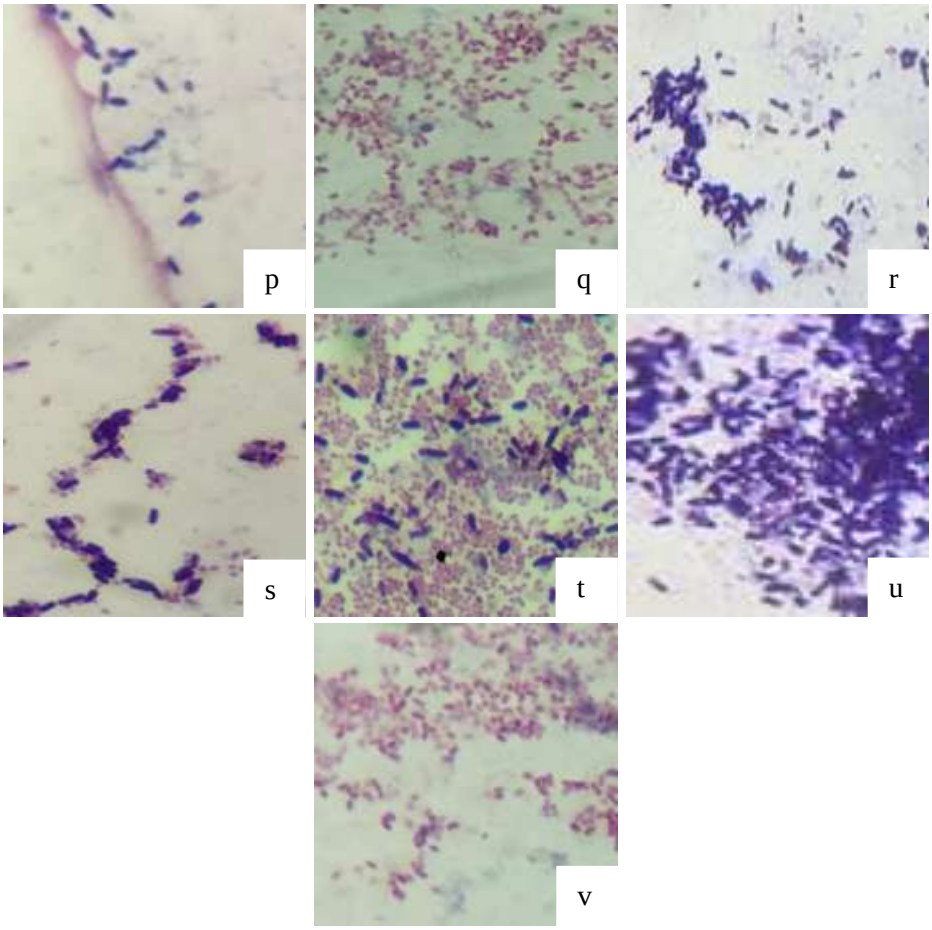

Figure 4. Gram staining of endophytic bacterial isolates isolated from plants *Patikan Kebo* (*E. hirta L.*) observed under a binocular microscope with a magnification of 10 x 100, PRE = figure a-e; PSE = figure f-n; PLE = figure o-v.

Based on the results of the research that has been done, obtained data on the coloring results of bacterial isolates isolated from plants Patikan Kebo (*E. hirta L.*) listed in Table 3.

Table 3. Calculation of total bacterial colonies isolated from plants Patikan Kebo (*E. hirta* L.)

Isolate Code	Species	Gram Staining		Cell Shape	Cell Arrangement
		+	-		
PRE	sp1	√		*Basil*	*Bacillus*
	sp2	√		*Coccus*	*Staphylococcus*
	sp3	√		*Coccus*	*Staphylococcus*
	sp4	√		*Coccus*	*Streptococcus*
	sp5	√		*Basil*	*Diplobacilus*
PSE	sp1	√		*Coccus*	*Staphylococcus*
	sp2	√		*Coccus*	*Diplococcus*
	sp3	√		*Coccus*	*Staphylococcus*
	sp4	√		*Coccus*	*Diplococcus*
	sp5	√		*Basil*	*Diplobacillus*
	sp6	√		*Basil*	*Diplobacillus*

Isolate Code	Species	Gram Staining +	Gram Staining -	Cell Shape	Cell Arrangement
	sp7	√		*Basil*	*Bacillus*
	sp8	√		*Coccus*	*Streptococcus*
	sp9	√		*Basil*	*Streptobacillus*
PLE	sp1	√		*Basil*	*Streptobacillus*
	sp2	√		*Basil*	*Bacillus*
	sp3		√	*Basil*	*Diplobacillus*
	sp4	√		*Basil*	*Diplobacillus*
	sp5	√		*Basil*	*Streptobacillus*
	sp6	√		*Basil*	*Bacillus*
	sp7	√		*Basil*	*Diplobacillus*
	sp8		√	*Basil*	*Diplobacillus*

Remarks: PRE= Patikan kebo Root Endophyte; PSE= Patikan kebo Stem Endophyte; PLE= Patikan kebo Leaves Endophyte; (+) = positive, (-) = negative.

Based on research that has been done by isolating bacteria from plants Patikan Kebo (*E. hirta L.)* in 3 parts, namely Patikan kebo root endophyte with code (PRE), Patikan kebo stem endophyte with code (PSE) and Patikan kebo leaves endophyte with code (PLE) at dilutions of 10^{-3} and 10^{-5} using the total plate count (TPC) method, the total number of colony counts obtained shows good isolation results because it does not exceed the total bacterial colonies which are between 30-300 colonies. Based on these results according to Sukmawati and Hardianti (2018), which states that the number of colonies in samples with more than 300 colonies is reported as TBUD (Too Many to Count).

Each encapsulation medium produces different survival rates, the high survival rate is obtained from pure culture media with a colony count of 30 to 300 (Kurniawan et al., 2023). This is because it is influenced by the composition of the media used. The amount of nutrients available in pure culture media can affect the growth of the bacteria themselves. TBUD colony counts are performed when the number of colonies on the plate exceeds 300, which makes the colonies too close to each other to be distinguished as colony forming units (CFU) (Marini et al., 2014). In this situation, each bacterial cell is separated from each other and will grow into a separate single colony (CFU). Marini et al. (2014) stated that when the number of CFU exceeds 300, counting becomes very difficult due to the high density of colonies.

Isolation of endophytic bacteria from the stems of patikan kebo (PSE) produced colonies with a higher total number compared to other plant parts. In line with Suryani & Ayun (2022), that endophytic bacteria are obtained more in the upper tissues (stem and leaf parts) than the lower parts (root parts). The stem is the part that provides a conducive environment for the growth of endophytic bacteria. Plant stems are used as one of the parts directly exposed to air, acting as an entry point for endophytic bacteria through stomata or other openings. After entering, these bacteria can grow in one area or spread throughout the plant tissue (Purwanto et al., 2014). Stems can be utilized. The stem can be utilized as a conductor of food and as a storage place for the results that have become food, thus affecting the total colonies of endophytic bacteria contained in the stem. According to Kusumawati et al. (2014), which states that the stem in plants has a function as a carrier of photosynthetic

products such as water and nutrients needed by plant cells, these conditions create a suitable environment for the growth of endophytic bacteria. According to Sepriana et al. (2017), explained that endophytic bacteria are bacillus-shaped cells and capable of forming spores. This ability makes the bacteria more resistant to extreme environmental pressures, because their cell metabolism stops or enters a dormancy phase when in unfavorable conditions.

Based on the research that has been done, the morphological characteristics of endophytic bacterial isolates from plants Patikan Kebo (*E. hirta* L.) are obtained by looking at the parameters carried out, namely appearance, elevation, edges and color. It has a varied appearance, elevation, edge and color as can be seen in (Table 2). Isolates show differences in colony morphological characteristics. The appearance of isolates with isolate codes PRE sp1, PSE sp8, and PLE sp1, has a Circular-shaped morphological character with flat elevations or surfaces, the edges of the isolate form Entire with a creamy white color. The appearance of isolates with isolate codes PRE sp2, PSE sp4, and PLE sp4, is circular with flat elevations or surfaces, the edges of the isolate form an Entire with a white color. The appearance of isolates with isolate code PRE sp3, circular in shape with flat elevation or surface, the edge of the isolate shape is Undulate with white color. The appearance of isolates with isolate code PRE sp3, Circular with elevation or Convex surface, the edge of the isolate shape is Entire with creamy white color.

The appearance of isolates with isolate codes PRE sp5 and PSE sp3, Irregular shaped with Flat elevations or surfaces, the edges of the isolate form Undulate with a creamy white color. The appearance of isolates with isolate code PSE sp1, Circular in shape with flat elevations or surfaces, the edges of the isolate form Entire with a creamy white color. Appearance of isolates with isolate code PSE sp2 and PLE sp7, Circular in shape with Convex elevation or surface, the edge of the isolate shape is Entire with white color. white color. The appearance of isolates with isolate codes PSE sp5, PSE sp6, and PSE sp9, Circular-shaped with flat elevation or surface, the edge of the isolate is Entire with white color of the isolate form is Entire with white color. The appearance of the isolate with the isolate code PSE sp7, Circular with elevation or Convex surface, the edge of the isolate form is Entire with orange color.

The appearance of isolates with isolate codes PLE sp2 and PLE sp6, Irregular shaped with Flat elevation or surface, the edge of the isolate shape is Entire with white color. Undulate with white color. The appearance of isolates with isolate codes PLE sp3 and PLE sp8, shaped Circular with elevation or surface Flat, the edge of the isolate shape is Entire with a yellowish white color. The appearance of isolates with isolate code PLE sp5, Circular with elevation or Convex surface, the edge of the isolate shape is Undulate with white color. According to Cappucino & Sherman (2001), which states that the factors that trigger variations in the number of bacteria obtained are caused by the isolation medium used as a type of bacterial growth medium. The composition of nutrients in the medium can affect the growth of bacteria.

Based on the results of the above research, the coloring results of bacterial isolates isolated from patikan kebo (*E. hirta* L.) were obtained, which resulted in 22 isolates of endophytic bacteria, including Gram-positive and Gram-negative bacteria. Where 20 isolates of endophytic bacteria are included in the group of Gram-positive bacteria and 2 other isolates are included in the group of Gram negative bacteria. Gram

negative bacteria. With morphological characteristics of cell shape in the form of Basil and Coccus. Isolates isolated from patikan kebo (*E. hirta* L.) are Gram positive bacteria on average are Gram positive bacteria, Gram negative bacteria are only found in isolates with the code PLE spp. in isolates with code PLE sp3 and PLE sp8. Purple colored cells mark Gram positive bacteria and red-colored cells to mark Gram negative bacteria (Cappucino & Sherman, 2001).

These Gram negative bacteria were suspected is *Pseudomonas aeruginosa* bacteria. This is in line with research conducted by Purwaningsih & Wulandari (2021), that *Pseudomonas aeruginosa* shows colonies with morphological characteristics that have a round, smooth shape and have a green color. *P. auriginosa* bacteria produce a green color which is *pyocianin* pigment. Based on gram staining results show the arrangement of tesebar cells and have a bacillus shape, and the cells show a change in color to red this indicates that the identified bacteria are *P. auriginosa* bacteria. Gram-negative bacteria become transparent because they cannot iodine dye complex, but a red substance (safranin) can help in coloring, thus causing gram-negative bacteria to look red. According to Susanto (2016) gram staining is one of the most important and widespread staining techniques used to identify bacteria. In this process, bacterial smears that have been fixed are subjected to solutions, namely crystal violet dye, iodine solution, alcohol solution (bleach) and a counter dye in the form of safranin or fuchsin water. Stained bacteria if they are gram-positive will retain the crystal violet dye, while gram-negative bacteria will lose the crystal violet dye after washing with fuchsin or safranin water dye.

Gram staining technique is based on the structure and composition of the bacterial cell wall. Gram negative bacteria have more fat or fatty substances than Gram positive bacteria. A thinner peptidoglycan layer exists in the cell wall of Gram negative bacteria and is covered by the outer membrane, which makes it more easily affected by alcohol treatment during the staining process. This alcohol treatment can trigger lipid reactions that increase the permeability of the Gram negative bacterial cell wall and allow the extraction of crystal violet complexes that have penetrated the cell wall in the first step of this decolorization staining process (Pelczar & Chan, 1986). Rini & Rochmah (2020) mentioned that Gram positive bacteria cell wall is composed of Peptidoglycan (PG) there is a compound called teichoic acid. Gram negative bacteria contain much less PG, but on the outside of PG there is an outer membrane composed of lipoproteins and phospholipids, and contains lipopolysaccharides. The difference in cell wall composition is that Gram positive bacteria and gram-negative bacteria have different resistance. Gram positive bacteria are more susceptible to penicillin antibiotics, because these antibiotics can damage PG.

The purple color indicates the results of Gram positive bacteria staining that can be observed under a microscope, cell shape in the form of Basil and Coccus, with cell arrangement in the form of Bacillus, Diplobacillus, Streptobacilus, Diplococcus, Staphylococcus, and Streptococcus. Further identification was not carried out due to several obstacles so that the genus identification of these Gram positive bacteria could not be known. Identification of bacteria is at least done up to biochemical tests, but in this research it was not done. According to Lay (1994), that the process of identifying bacterial species can be done based on morphological characteristics of bacterial colonies and pure cultures, but Gram staining and biochemical testing must be

continued to obtain complete identification results. One way to classify bacteria is by gram staining, where bacteria are divided into two groups, namely Gram positive bacteria and gram-negative bacteria. Gram negative bacteria are red, while gram-positive bacteria are purple (NauE et al., 2022).

CONCLUSION

Based on the results of the research that has been done, it is concluded that endophytic bacteria are obtained by isolating parts of the Patikan Kebo (*E. hirta* L.), namely the roots of kebo (PRE), Patikan kebo stem endophyte (PSE) and Patikan kebo leaves endophyte (PLE) obtained 22 isolates of endophytic bacteria by observing morphological characters by looking at parameters such as appearance, elevation, edges and color. the 22 endophytic bacterial isolates, there were 20 endophytic bacterial isolates that belonged to the Gram-positive group, and 2 endophytic bacterial isolates that belonged to the Gram-negative group. So it can be concluded that the endophytic bacterial isolates obtained vary so that future prospects can be utilized to create products such as medicines.

ACKNOWLEDGMENTS

The authors would like to thank the Directorate of Learning and Student Affairs (Belmawa), Ministry of Education, Culture, Research and Technology (Kemdikbudristek) through the Scientific Article Student Creativity Program (PKM-AI) incentive in 2024 with number 4335/E2/DT.01.00/2024. The author would also like to thank the University (Bengkulu University) and the Department of Biology for helping to accommodate and support the implementation of the research that we have done.

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How To Cite This Article, with *APA style* :

Rindiyani, R., Anugrah, R.I., Nabila, B., Salsabilla, S., Susanti, E., Rofiani, D., Puspitha, Y., Wibowo, R.H., Sipriyadi, S., & Darwis, W. (2025). Isolation and Identification of Endophytic Bacteria from *Euphorbia hirta* L. (Patikan Kebo). *Jurnal Pembelajaran dan Biologi Nukleus*, *11*(1), 40-54. https://doi.org/10.36987/jpbn.v11i1.6688

Conflict of interest : The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions : All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by all authors. The first draft of the manuscript was submited by [**Rindiyani**]. All authors contributed on previous version and revisions process of the manuscript. All authors read and approved the final manuscript.