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Ice Nucleation Active bacteria in Mount Lawu forest, Indonesia: 3. Isolation and estimation of bacterial populations on bryophyte

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Abstract. *Latifah NH, Susilowati A, Suratman. 2018. Ice Nucleation Active bacteria in Mount Lawu forest, Indonesia: 3. Isolation and estimation of bacterial populations on bryophyte. Asian J For 2: 67-75.* This study aimed to isolate and estimate the Ice Nucleation Active (INA) bacteria population on bryophyte from the trekking route of Cemoro Sewu, Mount Lawu, Indonesia. Bryophyte samples were taken at three different altitudes, i.e., 2,000, 2,300, and 2,500 m above sea level (asl). Isolation of INA bacteria was carried out by the method of spread plate on King's B and nutrient agar plus 2.5% glycerol (NAG) medium. Bryophyte was identified by referring to the literature of Sutama (1995), Hasan and Ariyanti (2004), Ignatova and Samkova (2006), and Damayanti (2006). Ice nucleation activity was determined by the multiple-tube test method. The bacterial suspension tube was put into the circulating alcohol bath at a temperature of -10°C for 5 minutes. The number of INA bacteria on the bryophyte was estimated with the nucleation multiple tube method. The number (cell/g) of INA bacteria from the fresh weight of bryophyte was estimated based on the Most Probable Number (MPN) tables according to the formula of Thomas's 333 series. INA bacteria were identified through morphological and biochemical characters. Independent Sample T-Test analyzed the population of INA bacteria on terrestrial and epiphytes bryophytes with a significance of 5%. The results showed that 7 INA bacteria were isolated from the bryophyte *Campylopus umbellatus* (Arn.) Paris (Leucobryaceae, Musci). The population of INA bacteria in terrestrial bryophytes at each altitude were greater with 346 cell/g, 86 cell/g, and 396 cell/g, respectively, than that in epiphytes bryophyte with 50 cell/g, 50 cell/g, 176 cell/g, respectively.

Keywords: Bryophyte, Ice Nucleation Active, INA, isolation, Lawu, population

INTRODUCTION

The number of bacteria in nature is very large and diverse in terms of morphology, physiology, and genetics. Even in different habitats, the diversity of growing bacteria is also high. The bacteria that inhabit the leaf surface vary according to the plant species because each plant produces a certain exudate suitable for certain bacteria. The height of a place also affects the species of bacteria that grow on the leaves. Altitude affects the low air temperature, so only certain bacteria can adapt to this extreme environment (Morris et al. 2004). One bacterial species that grow on the leaf surface of upland plants with low air temperature is the Ice Nucleation Active (INA) bacteria (Lindow et al. 1978). INA bacteria that have been found are *Pseudomonas syringae*, *Pseudomonas viridiflava*, *Pseudomonas fluorescens*, *Erwinia herbicola*, and *Xanthomonas campestris* pathovar *translucens* (Edwards et al. 1994). These five bacterial species can catalyze ice formation at temperatures of -1.5°C to -10°C, even at temperatures above -5°C. These species could cause frost injury on the leaf surface. It was caused by changes in the water between and inside the leaf cells into ice at a temperature of -5°C (Gurian-Sherman and Lindow 1993).

INA bacteria are bacteria capable of catalyzing ice formation at temperatures above -10°C. INA bacteria can express Ice nucleation proteins on the cell surface, lowering the water temperature and freezing it. If there is no ice nucleation, pure cold water (H_2O) can only be supercooled and will not spontaneously freeze until the temperature reaches -40°C. INA bacteria function to accelerate the ice freezing process (Stephani and Waturangi 2011).

Ice cores in INA bacteria, active at relatively warm temperatures (≥-5°C), can play an important role in climatology by helping the process of condensation and forming ice nuclei in clouds. Ice core formation in troposphere clouds is required for snow formation and most precipitation (Christner et al. 2008a,b). Ice nucleating bacteria participate in the bioprecipitation cycle, by which the bacteria move to the cloud from the surface of plant leaves carried by the wind cycle and stimulate rain. On the other hand, rain provides favorable conditions for the growth of these ice-nucleating bacteria on plant leaf surfaces (Stephanie and Waturangi 2011). Moss plants are a group of plants that have a high enough water requirement. The greatest need for water depends on rainfall and humidity in the surrounding air (Sutama 1995). Since their high water requirement, it is estimated that INA bacteria can also be found in mosses. Besides having an important role in bioprecipitation, INA bacteria can also be used to make artificial rain and snow by seeding clouds with INA bacteria as a substitute for salt sowing, which is now widely used to make artificial rain (Wahyudi 1995).

Lindow et al. (1982) research showed that INA bacteria live in low-temperature areas with an optimum temperature of around 18-24°C. Mount Lawu, Indonesia, which has an altitude of 3,265 m above sea level (asl), is a tropical

mountain that is rich in moss plants, namely, those that grow terrestrial such as *Marchantia polymorpha*, *Polytrichum* sp., *Riccia* sp., or those that grow epiphytes such as *Cyathophorum bulbosum*, *Dicranoloma robustum*, and *Leptostomum inclinans*, and those that can grow terrestrial and epiphytes such as *Thuidium furfurosum* and *Dicranoloma dicarpium* (Setyawan and Sugiyarto 2001). In addition to having a variety of moss plants, Mount Lawu also has a relatively low air temperature, thus allowing a great opportunity to find ice crystal-forming phyllosphere bacteria.

Given the important role of INA bacteria in bioprecipitation and there is still little information about INA bacteria in the tropics, it is necessary to research the isolation and population number of INA bacteria from mosses in Mount Lawu. This research data can be used to understand the role of INA bacteria in bioprecipitation, which can affect cloud and rain formation.

The aims of this study were: (i) isolating INA bacteria from mosses on the trekking route of Cemoro Sewu, Mount Lawu. (ii) determining the number of INA bacteria in mosses on the trekking route of Cemoro Sewu, Mount Lawu.

MATERIALS AND METHODS

Materials

The materials to obtain INA bacteria were moss plants from the trekking route of Cemoro Sewu, Mount Lawu, East Java, Indonesia. Bacterial isolation and mosses were identified at the Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia.

Procedure

Moss plant sampling

The moss plant sample consisted of one epiphytic species and one terrestrial species taken from the trekking route of Cemoro Sewu, Mount Lawu. The sampling technique used in this research is the purposive sampling method by prioritizing places that are relatively overgrown with mosses and paying attention to abiotic factors (humid environmental conditions) with no plots (Windadri 2007). The survey resulted in three sampling points with different altitudes: 2,000 m asl, 2,300 m asl, and 2,500 m asl.

The plant material was 5-10 g of moss leaves. They were put in a paper bag and labeled with information on altitude, air, soil humidity, soil pH, light intensity, and air temperature as environmental factors affecting the number of INA bacteria populations on moss plant samples. In addition, the height of the place was measured by GPS, soil pH and soil moisture were measured by a pH meter plugged into the soil where the sample was taken, a hygrometer measured humidity and air temperature, and light intensity was measured by a lux meter. During the trip to the laboratory, samples of moss plants were put in an ice box. Immediately, they were brought to the laboratory and stored in a refrigerator at a temperature of 5°C.

Moss plant identification

The identification of mosses is performed by comparing and matching the characters of mosses with the literature/references from Sutama (1995), Hasan and Ariyanti (2004), Ignatova and Samkova (2006), and Damayanti (2006).

Sterilization of tools and materials

Petri dishes, test tubes, Erlenmeyer flask, NA agar media, and other tools are sterilized first. Then, the sterilization process was carried out by wet sterilization using an autoclave with a pressure of 1 atm and a temperature of 121°C for 20 minutes.

Preparation of NA + 2.5% Glycerol (NAG), King's B, and Slant Agar Media

NA medium was prepared by mixing 6 g of NA powder with 5 mL of glycerol in a 200 mL Erlenmeyer flask and added with distilled water to a limit of 200 mL. The composition for King's B media is 20 g Proteose peptone, 10 mL glycerol, 1.5 g K_2HPO_4, 1.5 g $MgSO_4.7H_2O$ and 15 g agar put into an Erlenmeyer flask, then added with 1 L aquades (Kartika 2009). Erlenmeyer flask was placed on a hot plate and let to boil until the solution was clear. The hole in the Erlenmeyer flask was covered with cotton and aluminum foil and sterilized using an autoclave at 121°C for 20 minutes. Each NA and King's B media was poured into a petri dish and allowed to dry at room temperature (28°C).

To make the slant agar, after the solution boiled and turned clear, the NA medium was immediately poured into 4 mL test tubes and autoclaved at 121°C for 20 minutes. After sterilization, the tube was slanted at 45° until it hardened.

Isolation of INA bacteria

Each 5 g of moss leaf sample was cut into small pieces and put in a 500 mL Erlenmeyer flask containing 200 mL of 0.1 M phosphate buffer with pH 7.0 and 0.1% peptone (Difco) (Waturangi and Amelia 2009). Phosphate buffer pH 7.0 was made with 0.6 g Monosodium Phosphate (NaH_2 $PO_4.2H_2O$) and 1.6 g Disodium Phosphate Hepta Hydrate ($Na_2HPO_4.7H_2O$) for 1 L of distilled water. The Erlenmeyer flask was shaken on a rotary shaker for 2 hours at 150 rpm. After shaking, serial dilutions were carried out to reach 10^{-3} with 9 mL of sterile distilled water. A 0.1 mL of each series of dilutions was taken for spread-plating on NA + 2.5% glycerol media and King's B media and then incubated at room temperature for 24 hours (King et al. 1954).

Pure culture collection

Each colony of different bacteria that grew on NA + 2.5% glycerol media and King's B media was taken with ose and strained on NA + 2.5% glycerol media and King's B media. Then, it was incubated at room temperature for 24 hours. Next, each separate bacterial colony was streaked onto the slant agar. They were grown for 4-6 days at room temperature and then stored in the freezer at 4-5°C.

Ice nucleation activity test

Each pure culture was tested for its nucleation activity. A bacterial suspension was prepared by taking 1 ose of pure culture and diluting it in 400 µL phosphate buffer. Ice nucleation activity was determined by the multiple-tube test method by inserting a microtube containing the bacterial suspension into a circulating alcohol bath at a temperature of -10°C for 5 minutes. Frozen microtubes were colonies containing ice cores (Lindow et al. 1978).

Population number estimation of INA bacteria

Estimation of the number of INA bacteria in moss leaves was carried out by the multiple tube nucleation method. The test tube containing 9 ml of sterile phosphate buffer was cooled in an alcohol bath at -10°C for 30 minutes. The tubes were shaken, and all the frozen tubes were separated. The unfrozen tube is warmed at 5°C. Leaf moss samples in phosphate buffer solution with a ratio of 10:100, which have been shaken, are diluted 10^{-1} to 10^{-3} in series 3.3.3 tubes. After the tube was dripped, each bacterial suspension resulting from the dilution was put back in the alcohol bath at a temperature of -5°C for 10 minutes (Cazorla et al. 1995). The number of frozen test tubes was counted for each dilution. The total population of INA bacteria/g fresh weight of the sample was estimated by counting the number of frozen tubes in each dilution (Cazorla et al. 1995), then matched to the MPN (Most Probable Number) table according to the Thomas series 333 formula (APHA 1975).

Characterization and identification of INA bacteria

Morphological observation of INA bacterial isolate colonies

Macroscopic morphological observations of the colonies included observations of the shape of the colony, the edge of the colony, the texture of the colony (the condition of the surface of the colony), the size of the colony, and the resulting pigmentation.

Observation of isolates was carried out using Gram stain to determine the type and shape of the cells. Gram staining was carried out by taking a pure culture of INA bacteria aseptically, placing it on a sterile glass object, and then making a suspension with a drop of sterile distilled water. Preparations that had been fixed on a flame were then stained with crystal violet for 1 minute and rinsed with water. Staining was continued with iodine for 2 minutes and rinsed again with water. Bleaching was done with 95% alcohol after rinsing with water, then painted with safranin for 30 seconds. After washing and drying, observations were made with a microscope to see the type of Gram and the shape of the cell (Bangun 1989).

Biochemical test

Catalase test. The INA bacterial isolate that had been fixed on a glass object was added with one drop of 3% H_2O_2 solution, then shaken. The gas formation was observed. The results were positive when bubbles of O_2 gas appeared (Bangun 1989).

Oxidase test. The filter paper was moistened with a 1% *tetramethyl p-phenylenediamine dihydrochloride* reagent. Then the bacterial culture was taken with a sterile straight ose and smeared on the filter paper. When pink, dark red, and black appeared on the paper in the area smeared with the bacterial culture, the test was positive (Hadioetomo 1993).

Indole test. The indole test used the Arnold and Weaver method. It was done by inoculating 2-day-old INA bacteria into the media and then incubating at 37°C. This test used Kovac's reagent, which dripped on the culture after incubation. The reaction was considered positive (+) if a red color was formed on the top layer of the culture. The red color indicated the presence of indole (Cowan 1985).

Data analysis

Observation resulted in quantitative and qualitative data. Qualitative data was morphological and biochemical characteristics of isolates, including observations of color, shape, edges, texture, colony size, Gram stain, catalase test, oxidase test, and indole test, which were analyzed descriptively comparatively by comparing one isolate with other isolates. Quantitative data was the number of INA bacteria analyzed by the Independent-Sample T-Test with a significance of 5% (α= 0.05).

RESULTS AND DISCUSSION

Moss plant sample

Samples of epiphytic and terrestrial mosses were taken from the trekking route of Cemoro Sewu, Mount Lawu, by purposive sampling. Sampling was carried out at three different altitudes, namely 2,026 m asl, 2,331 m asl, and 2,509 m asl, relatively, overgrown with moss. The sample of this moss plant was taken in the rainy season at the end of January 2013. The average results of measurements of abiotic factors at each point of elevation of sampling showed different results with not too much difference in intensity, as shown in Table 1.

Table 1. Abiotic factors at each altitude of moss sampling

Station	Altitude (m asl)	Light intensity (Lux)	Temperature (°C)	Humidity (%)	Soil pH	Soil moisture (%)
1	2,026	589 x 10	21	90	7.5	2
2	2,331	789 x 10	23	77	7	2
3	2,509	652 x 10	22	73	7	1.2

The diversity of abiotic factors is influenced by conditions at different altitudes at the time of sampling. The first station at an altitude of 2,026 m asl and the third at an altitude of 2,509 m asl tend to be wetter due to rain. The second station at an altitude of 2,331 m asl tends to be foggy. Samples of terrestrial mosses and epiphytes from the Cemoro Sewu hiking trail can be seen in Figure 1. Samples of terrestrial mosses growing on rocks are much wetter than epiphytic mosses growing at the base of Pinus merkusii stems. It is because terrestrial mosses are more susceptible to rainwater than more-shaded epiphytic mosses.

Based on observations, the epiphytic mosses mostly grow at the base of the pine trunk. One of the reasons is that there is a lot of humus or soil at the base of the stem so that the rhizoid of the moss is more easily attached to the tree bark. Pine bark as a substrate for epiphytic moss is dry, so the water requirement depends on the rainfall and humidity of the surrounding air. The moss can absorb and retain rainwater, reaching 5-25 times its dry weight. It is the important role of mosses in ecology (Kirmaci and Agcagil 2009).

The comparison results of the morphology of mosses samples showed that epiphytic mosses and terrestrial mosses were of the same species, namely *Campylopus umbellatus* (Arn.) Paris (Leucobryaceae, Musci) (Figure 2) (Sutama 1995; Hasan and Ariyanti 2004; Damayanti 2006; Ignatova and Samkova 2006). Some mosses not only grow epiphytic or terrestrial but can also grow in both places, such as *C. umbellatus*. These results also indicate that this moss was not found in the Mount Lawu area in a previous study conducted by Setyawan and Sugiyarto (2001).

The *C. umbellatus* has several synonyms: *Campylopus coreensis* Cardot., and *Campylopus ferriei* Broth (Jean Edouard Gabriel Narcisse Paris, 1894). This moss is one of the most common species of *Campylopus* found in humid forests. During the rainy season, this moss will grow upright and bright green, while in winter, the color of the leaves of this moss will be black and curved to one side. Talus is like a small tree, 3-5 cm high. Stems grow erect, unbranched, and are covered with fine reddish-brown hairs. The leaves are arranged radially symmetrical, covering the stem. The leaves are needle-shaped with a pointed tip and flat base. The leaf edges are slightly rolled up. Single rib, 1/4-1/3 leaf width, extending to leaf tip. The capsules are brown with short seta (Sutama 1995; Hasan and Ariyanti 2004; Damayanti 2006; Ignatova and Samkova 2006).

Isolation of INA bacteria from moss plants *Campylopus umbellatus*

Isolation of INA bacteria from samples of moss *C. umbellatus* using King's B and NAG media. King's B media is a selective medium for *Pseudomonas* bacteria. It causes *Pseudomonas* colonies to appear fluorescent in King's B media (Arwiyanto et al. 2007). All different colonies found in King's B and NAG media from all samples were tested for ice nucleation activity using a circulating alcohol bath until 7 isolates were positive for INA bacteria, which can be seen in Table 2.

The total number of different isolates obtained from both terrestrial and epiphytic mosses was 76 isolates. All isolates were tested for nucleation activity in a circulating alcohol bath with a temperature of -10°C for 5 minutes, and isolates that were positive for INA bacteria would freeze, as shown in Figure 3.

Colonies that can be categorized as positive for INA bacteria are colonies that have ice nucleation activity. The ice nucleation activity will be seen when the colony suspension in the microtube froze after being put into a circulating alcohol bath with a temperature of -10°C for 5 minutes. The freezing suspension indicates the presence of INA bacteria, which have a single protein as an initiator of ice core formation (Morris et al. 2008). On the other hand, when the suspension of the colony did not freeze, the colony was categorized as negative for INA bacteria.

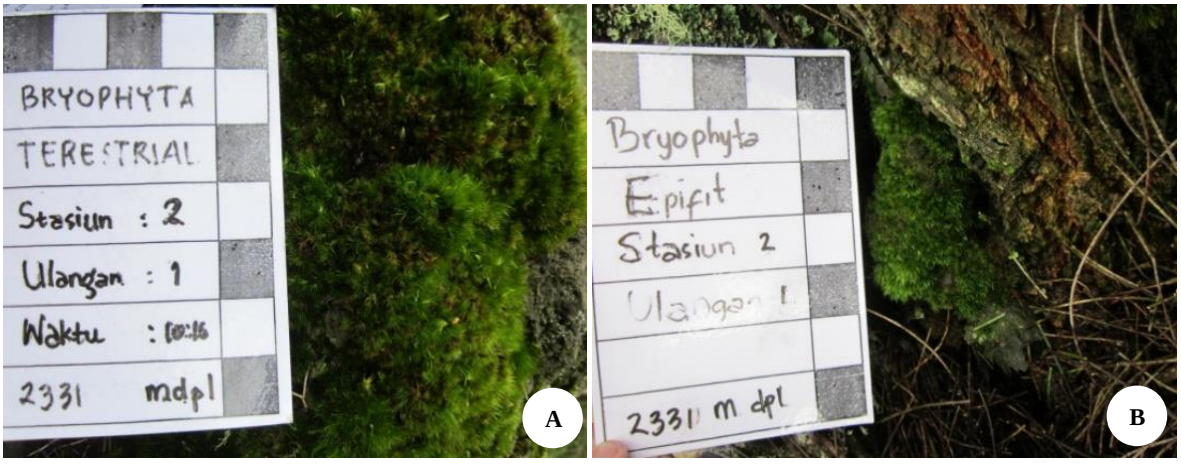

Figure 1. Moss plant samples. A. Terrestrial moss growing on rock surfaces, B. Epiphytic moss growing at the base of *Pinus merkusii* stems

Based on the results above, it can be seen that the most isolates positive for INA bacteria were from station 3, with an altitude of 2,509 m asl in terrestrial mosses. It is because INA bacteria are more commonly found in samples that absorb a lot of rainwater, and terrestrial *C.umbellatus* mosses attached to rocks are more susceptible to rainwater than *C.umbellatus* mosses grow epiphytes on *P.merkusii*. Therefore, considering the distribution of INA bacteria is very closely related to rainwater.

The seven isolates were observed for their morphological characteristics to determine whether they showed different colonies of each isolate, indicating that they were different isolates. The observed colony characteristics included color, shape, edge, elevation, diameter, and structure in the colony, as shown in Table 3 and Figure 4.

Table 3 and Figure 4 show the differences in each isolate's colonies, indicating that the seven isolates are different. For example, isolates 1 and 2 had yellow colony color, circular shape, entire edges, and convex elevation. But the size and structure in the colonies of isolates 1 and 2 were different. Isolate 1 had a colony size of 1500-2000 m, and the internal structure was coarsely granular, while isolate 2 had a colony size of 1000-1500 m and the internal structure was smooth.

Figure 2. Habitus and morphology of moss *Campylopus umbellatus* (40X magnification) A. Habitus of talus of *C. umbellatus*; B. Leaf base; C. Leaf edge; and D. Leaf tip. Description: S: Sporophyte; R1: Flat leaf base; M: Rolled Leaf edge; R2: Pointed leaf tip

Figure 3. Colonies that are positive for INA bacteria: A. Isolate 1, 2, 3; B. Isolate 4, 5, 6; C. Isolate 7; and Control (left to right)

Table 2. Seven isolates that are positive for INA bacteria

Altitude	Number of isolates in terrestrial mosses	Number of isolates in epiphytic mosses	Number of isolates positive INA	Code
2,026	9	12	2 (1 Epiphytic Mosses and 1 Terrestrial Moss)	Isolate 1 (E) Isolate 2 (T)
2,331	10	8	1 (Epiphytic Moss)	Isolate 3 (E)
2,509	17	20	4 (3 Terrestrial Mosses and 1 Epiphytic Moss)	Isolate 4 (T) Isolate 5 (T) Isolate 6 (T) Isolate 7 (E)

Note: T: Terrestrial mosses, and E: Epiphytic mosses

Table 3. Colony morphology isolated from moss plants

Isolate	Color	Shape	Edge	Elevation	Size (µm)	Inner Structure
1	Yellow	*Circular*	*Entire*	*Convex*	1500-2000	*Coarsely granuler*
2	Yellow	*Circular*	*Entire*	*Convex*	1000-1500	*Smooth*
3	White	*Circular*	*Undulate*	*Convex*	1000-1500	*Smooth*
4	White	*Circular*	*Undulate*	*Convex*	1500-2000	*Coarsely granuler*
5	White	*Circular*	*Entire*	*Convex*	1500-2000	*Coarsely granuler*
6	White	*Circular*	*Undulate*	*Convex*	1000-1500	*Smooth*
7	White	*Circular*	*Undulate*	*Convex*	1000-1500	*Smooth*

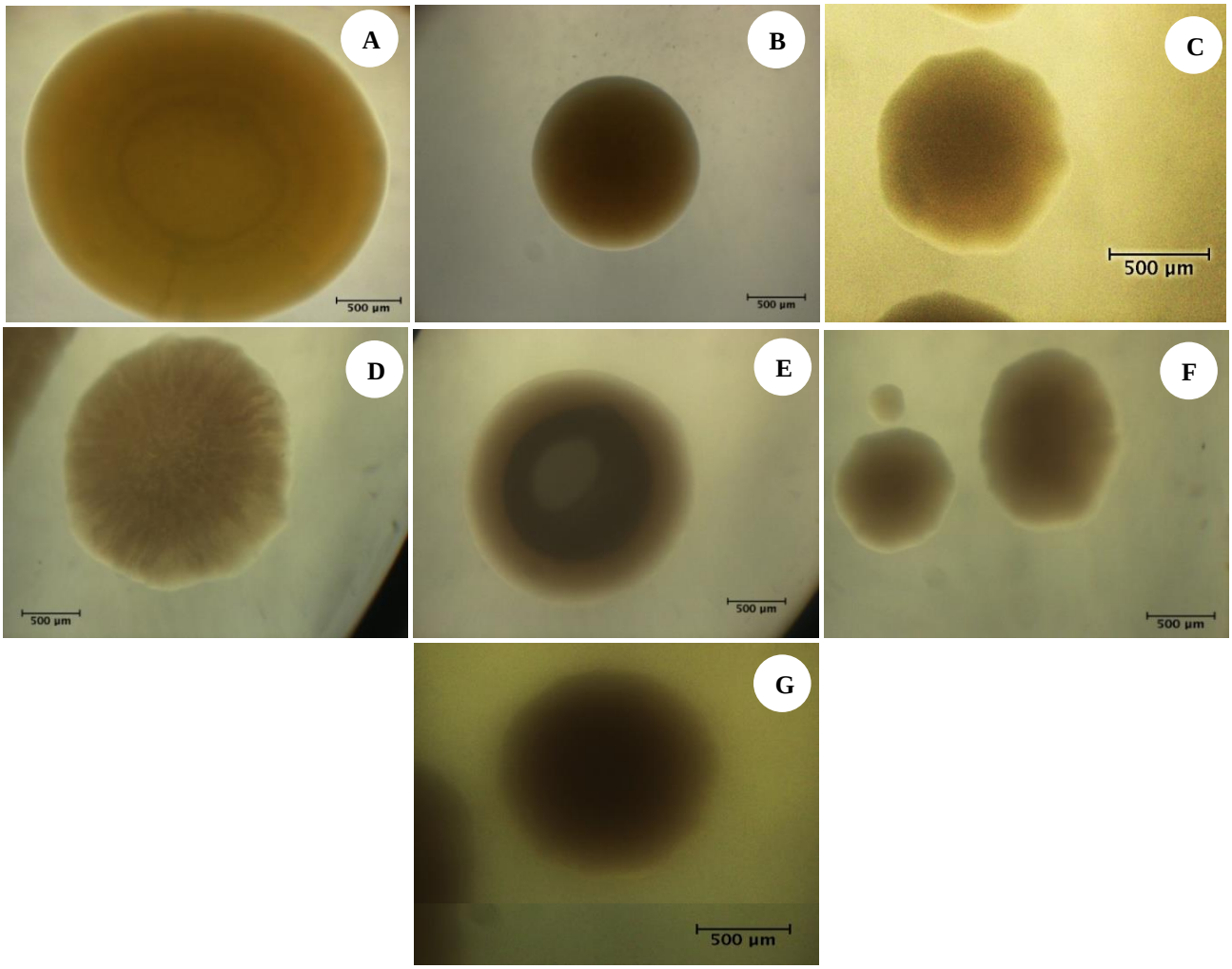

Figure 4. Colony morphology of INA bacterial isolates isolated from moss *C. umbellatus* (40X magnification) A. Isolate 1; B. Isolate 2; C. Isolate 3; D. Isolate 4; E. Isolate 5; F. Isolate 6; G. Isolate 7

Isolates 3, 4, 5, 6, and 7 had white colonies, circular in shape, convex elevation, and all edges were undulate except for isolate 5 with the entire edge. Colony size and internal structure for isolates 3, 6, and 7 were the same, namely 1000-1500 m and smooth, but based on the morphology seen in Figure 4, the three of them looked different. However, colony morphology alone was insufficient to determine the differences between the seven isolates. Therefore, it is necessary to characterize the isolates' cells, including microscopic observations of bacterial isolates and biochemical tests.

Microscopic observation is the most important step in the characterization of bacteria. Microscopic observations of bacterial isolates in the form of cell morphology observations, namely the shape and type of Gram, can be seen from the Gram staining results. Of the seven isolates, all bacteria obtained were rod-shaped. In addition, the Gram staining results on seven bacterial isolates showed that all of them were Gram-negative bacteria which was indicated by the formation of red on the bacterial cells (Figure 5).

Gram-negative bacteria have a cell wall composition mostly composed of a lipid layer, so during staining, they cannot retain the main dye, especially when washed with alcohol, because the lipids are damaged. As a result, this group of bacteria gives a red appearance (the color of the safranin dye) at the end of the Gram staining activity (Hadioetomo 1993).

After microscopic observation of the seven bacterial isolates, it was followed by biochemical tests to determine the physiological properties of bacteria. The biochemical tests included the catalase, oxidase, and indole tests.

The catalase test was carried out to detect the presence of the catalase enzyme, which can convert hydrogen peroxide into water and oxygen. This test is important to determine the nature of bacteria to the need for oxygen. The test results showed that the seven isolates could break down H_2O_2 into H_2O and O_2, as evidenced by the air bubbles on the bacterial preparations after dripping with a 3% H_2O_2 solution. So it can be concluded that the seven isolates were aerobic (Bangun 1989).

The Oxidase test determines the presence of cytochrome oxidase enzymes in certain microorganisms. Cytochromes are respiratory or cellular pigments, which are hemoproteins similar to hemoglobin. These cytochromes are divided into 3 groups, namely cytochromes a, b, and c. Cytochrome c is more abundant in nature than other types of cytochromes. All these cytochromes can undergo oxidation and reduction, and almost all act as hydrogen carriers. This cytochrome is shared by all aerobic bacteria (Salle 1961).

Cytochrome oxidase enzyme is an enzyme that catalyzes the direct transfer of hydrogen by cytochrome c to molecular oxygen and will cause the formation of water. This enzyme is active under aerobic conditions. The results of the oxidase test showed that the seven bacterial isolates produced cytochrome oxidase enzymes. It can be seen in the change in the color of the colony to pink, then dark red, dark red, and finally black (Hadioetomo 1993).

The indole test was used to determine the presence of the tryptophanase enzyme in bacteria. This enzyme can hydrolyze the amino acid tryptophan into indole compounds and pyruvic acid. Indole compounds are spoilage components produced by some bacteria from the amino acid tryptophan. Tryptophan is the only amino acid that naturally contains an indole ring (Salle 1961). The results of the indole test showed that the seven bacterial isolates did not have the tryptophanase enzyme that could hydrolyze the amino acid tryptophan into indole and pyruvic acid compounds. It can be seen from the absence of red color on the agar media after being dropped with Kovacs reagent (Cowan 1985).

The overall characterization results based on morphological and biochemical characteristics in Table 4 show that the seven bacterial isolates could be categorized into 5 different groups. Isolates 3, 6, and 7 were categorized in the same group, while isolates 1, 2, 4, and 5 were in different groups.

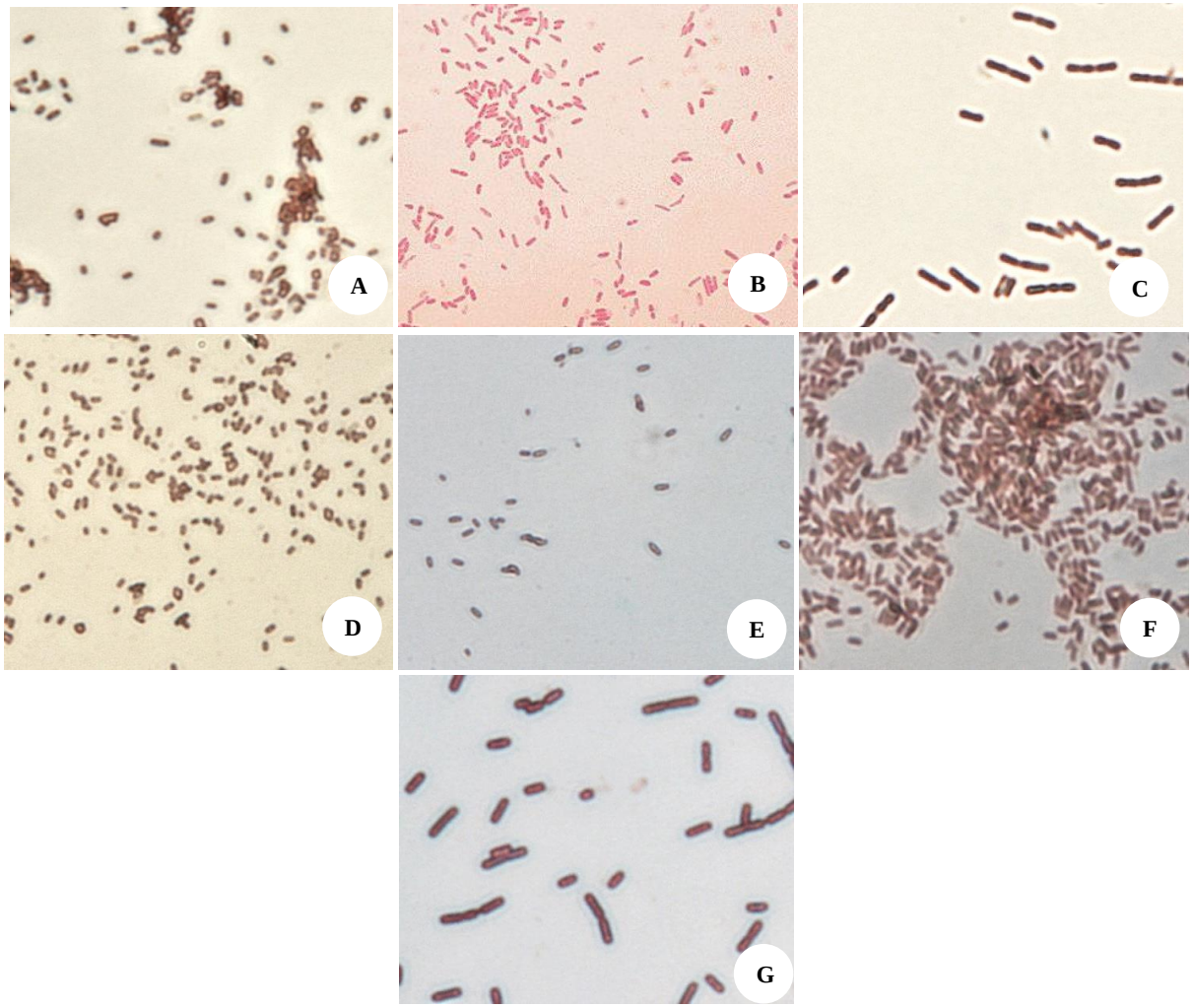

Figure 5. Gram staining of INA bacterial isolates: A. Isolate 1; B. Isolate 2; C. Isolate 3; D. Isolate 4; E. Isolate 5; F. Isolate 6, G. Isolate 7

Table 4. Characterization of 7 Isolates of INA bacteria isolated from moss *C. umbellatus* based on morphological and biochemical characters

Character	Isolate						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Colony color	Yellow	Yellow	White	White	White	White	White
Colony form	*Circular*	*Circular*	*Circular*	*Circular*	*Circular*	*Circular*	*Circular*
Colony edge	*Entire*	*Entire*	*Undulate*	*Undulate*	*Entire*	*Undulate*	*Undulate*
Colony elevation	*Convex*	*Convex*	*Convex*	*Convex*	*Convex*	*Convex*	*Convex*
Colony size (µm)	1500-2000	1000-1500	1000-1500	1500-2000	1500-2000	1000-1500	1000-1500
Inner structure	*Coarsely granuler*	*Smooth*	*Smooth*	*Coarsely granuler*	*Coarsely granuler*	*Smooth*	*Smooth*
Cell shape	Rod-shaped	Rod-shaped	Rod-shaped	Rod-shaped	Rod-shaped	Rod-shaped	Rod-shaped
Gram stain	-	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	-	-

Note: + (test result: positive), - (test result: negative)

Table 5. Total population of INA cells/g in epiphytic and terrestrial moss of *C. Umbellatus*

Altitude	Average population of INA	
	Epiphytic moss (cells/g)	**Terrestrial moss (cells/g)**
2,026	50	346
2,331	50	86
2,509	176	396

Estimation of INA bacterial population

INA bacteria are phyllosphere bacteria that grow non-specifically on certain plant species. Its distribution is more influenced by rainwater, humidity, and low air temperature. Therefore, it is necessary to test the estimated population of *C. umbellatus* moss samples to determine the total population at each moss sampling station, terrestrial and epiphytic.

The estimation of the INA population in epiphytic and terrestrial moss samples was obtained using the MPN method of dilution of 3 series of tubes. Each frozen tube in each dilution was counted and matched with the MPN table to obtain the total INA population of cells/g of the moss sample. These results showed that the highest INA population was 930 cells/g, and the lowest was <30 cells/g. The INA population <30 cells/g was not sufficient to freeze 10 ml of phosphate buffer, indicated by the absence of a frozen tube in each dilution.

Based on the results of MPN in each replication, the average population of INA bacteria cells/g of epiphytic and terrestrial *C. umbellatus* mosses at each station was calculated and presented in Table 5.

The data shows the population of INA (cells/g) of epiphytic moss *C. umbellatus* growing at stations 1 and 2 is the same, namely 50 cells/g, and the highest is at station 3, 176 cells/g. While the number of INA population (cells/g) of terrestrial moss *C. umbellatus* was lowest at station 2, namely 86 cells/g, and the highest was at station 3, namely 396 cells/g. The diversity of these results is influenced by abiotic factors around the habitat environment, which vary from one habitat to another. In addition, INA bacteria do not have a specific leaf habitat. Therefore, the activity and number of INA bacteria populations in several locations are more influenced by the season and the amount of rainfall (Morris et al. 2004).

Based on the results of the Independent-Sample T-Test, the comparison of the results of the INA bacterial population between terrestrial and epiphytic *C. umbellatus* mosses showed the number 0.017 in its significance value (Appendix 2). It means that since the significance value ≤ 0.05, then Ho is rejected. The average INA (cells/g) population of terrestrial mosses is greater than that of epiphytic mosses. The average population of INA bacteria in terrestrial mosses, which was 276 ± 377.9 cells/g, was greater than that of epiphytic mosses, which was only 92 ± 129.4 cells/g. It was because terrestrial mosses which lived in open areas received more rainwater than epiphytic mosses which lived in more shaded areas.

Previous research stated that INA bacteria are taken from rainwater, and air between March-May 2008 from Jakarta, Bogor, Bekasi, Tangerang, and Depok obtained the percentage of INA population in each area of 19.4%, 18.7%, 5, 3%, 2.2%, and 6.4% and 9.5%, 6.5%, 0%, 2.7%, and 1.8%, respectively. It shows that the percentage of the population of INA bacteria in samples from rainwater in all these areas is greater than that from the air. The presence of INA bacteria in rainwater and air may play an important role in the nucleation process required for rain induction (Stephanie and Waturangi 2011).

From this research, it can be concluded as follows: (i) 7 isolates of INA bacteria were isolated from the moss *C. umbellatus* on the trekking route of Cemoro Sewu, Mount Lawu; (ii) the average number of INA bacteria in the moss *C. umbellatus* terrestrial mosses, namely 346 cells/g, 86 cells/g, and 396 cells/g, were larger than the moss *C. umbellatus* epiphytes, namely 50 cells/g, 50 cells/g, and 176 cells/g, at stations 1, 2, and 3 on the trekking route of Cemoro Sewu, Mount Lawu.

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