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Ice Nucleation Active bacteria in Mount Lawu forest, Indonesia: 1. Isolation and estimation of bacterial populations on lichens

KHUSNUL FU'ADAH, SITI LUSI ARUM SARI, ARI SUSILOWATI♥
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: arisusilowati@staff.uns.ac.id

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Abstract. *Fu'adah K, Sari SL, Susilowati A. 2017. Ice Nucleation Active bacteria in Mount Lawu forest, Indonesia: 1. Isolation and estimation of bacterial populations on lichens. Asian J For 1: 83-91.* Ice Nucleation Active (INA) bacterias cause frost injury in plants. These bacteria are also predicted to play an important role in bioprecipitation, cloud formation, and rain. Most INA bacteria studies are conducted in subtropical areas. Therefore, studies on INA bacteria from tropical areas, especially Indonesia, need to be conducted. This study aimed to isolate and determine the number of INA bacteria in lichenes. samples of lichenes were taken from the hiking pathway of Cemoro Sewu, Mount Lawu forest, Java, Indonesia, at ±2.200, ±2.400, and ±2.600 m asl. INA bacteria were isolated by the spread plate method on the NA medium with 2.5 % glycerol and King's B medium. A tube nucleation test determined ice nucleation activity. A multiple-tube nucleation test estimated the number of INA bacteria. The data were analyzed descriptively based on colony morphology, cell morphology, biochemical tests, and analysis of the numbers of INA bacteria. The result showed that 7 isolates from *Parmelia* sp. at an altitude of 2.532 m asl (station 2) have been known as INA bacterias. The number of INA bacteria in lichenes was 5 x 104/g, which was very low.

Keywords: Bioprecipitation, Ice Nucleation Active bacteria, lichens, Mount Lawu, number of bacteria

INTRODUCTION

Ice Nucleation Active (INA) bacteria is one of the bacteria that can be found on plant surfaces. The population of INA bacteria reached 106 cells/g in plant tissue (Lindow 1993). INA bacterial species are generally epiphytic in plants, and their presence on plant surfaces increases the likelihood of frost injury at temperatures above -5°C (Lindow et al. 1982). Most frost-sensitive plants will experience frost damage between -2 and -5ºC. When the water cools, it turns to ice between and within the cells, causing freeze damage.

INA bacteria can generally initiate ice core formation at temperatures above -10ºC. Some of these species can even form ice cores at -1.5ºC. INA bacteria can naturally initiate ice core formation due to the presence of ice core-forming substances (Lindow 1983). *Pseudomonas syringae* is the most widely distributed and studied INA bacterial species (Lindow 1983; Lindow 1990). The *P. syringae* expresses a certain type of protein on the cell surface, namely ice nucleation protein (INP), which increases the freezing temperature of the water. If there is no ice core, the cold water can become supercooled, and freezing will not occur until the temperature is low enough for the most active ice core to initiate cold water crystallization. Although ice-core activity in bacteria is limited to Gram-negative bacterial species, the presence of these species in plants and other natural habitats is an interesting general phenomenon (Gurian-Sherman and Lindow 1993).

According to Wahyudi (1995), ice cores in bacteria that are active at relatively warm temperatures (≥ -5ºC) have the potential to play an important practical role in making snow, changing weather, freezing certain types of food, and enabling their use to make artificial rain. Therefore, especially in freezing food, it is also necessary to consider bacteria carrying the ice+ gene to be classified as a safe group (Generally Recognized As Safe = GRAS).

In its current development, INA bacteria play a role in bioprecipitation, which can affect cloud and rain formation and climate. As aerosol particles, bacterial cells can act as cloud condensation nuclei to form raindrops. On a larger scale, INA bacteria have been studied in the atmosphere. According to research by Amato et al. (2007) and Morris et al. (2008), INA bacteria have been detected in the rain, snow, and the atmosphere. It suggests that INA bacteria can be propagated through the global water cycle and is an important part of precipitation initiation research.

The hiking pathway of Cemoro Sewu, Mount Lawu, is located on the southern slope of Mount Lawu, Java, Indonesia. According to Setyawan (2000), this location is the most fertile area in the Mount Lawu area because it is a rainwater catchment area. A cloudy southeast wind containing drops of water hits the mountain and is lifted up, causing condensation and drops of water to fall as rain. As a result, the south (southeast) slope gets relatively more rainfall throughout the year than the other slopes. Rainwater is a dominant factor for the growth of epiphytes because these plants generally live far from the soil surface, so the arrival of rain generally fulfills that water need.

The results of Samsali's research in 2008 showed 12 types of epiphytes found along the hiking pathway of Cemoro Sewu. These epiphytes consist of 4 from the

Lichenes division, 1 from the Bryophyta division, 5 from the Pteridophyta division, and 2 from the Spermathophyta division. A study by Kieft (1988) concluded that INA bacteria are found in various lichens in the southwestern United States. This condition allows a great opportunity to find INA bacteria and test their nucleation activity.

Most of the studies on INA bacteria were carried out in the subtropics, while there were very few reports of studies on INA bacteria in the tropics. Given the important role of INA bacteria and the practical use of these bacteria, as well as scientific applications, it is necessary to conduct a study on INA bacteria in tropical mountain areas, one of which is the hiking pathway of Cemoro Sewu, Mount Lawu.

The objectives of this study were: (i) obtaining isolates of INA bacteria from lichenes on the hiking pathway of Cemoro Sewu, Mount Lawu; (ii) determining the number of INA bacteria in lichenes on the hiking pathway of Cemoro Sewu, Mount Lawu.

MATERIALS AND METHODS

Materials

Lichens originated from the hiking pathway of Cemoro Sewu, Mount Lawu, Java, Indonesia. Lichens sampling was carried out on the hiking pathway of Cemoro Sewu. The sampling point is set at as many as three stations based on height differences, namely at an altitude of ± 2,200 m asl (Montana), ± 2,400 m asl (sub-alpine), and ± 2,600 m asl (sub-alpine). There were two sampling points at each station. Lichens are put into a sterile paper coated with plastic bags and immediately carried to the laboratory (<12 hours) for insulation or stored in a refrigerator at 5°C to be isolated for the next few days. Figure 1 shows the location of each station. Lichens identification is carried out using a reference book by van Steenis (1978).

Measurement of abiotic environmental factors

At each sampling point, several environmental factors are measured, namely:

Light intensity. Measurement of light intensity is done with a lux meter. The measurement distance was 50 cm above the ground surface. After 60 seconds of waiting, the size of the light intensity is recorded.

Air temperature and humidity. Measurement of air temperature and humidity is carried out with a hygrometer. The measurement distance was 50 cm above the ground surface. After 60 seconds of waiting, the air temperature and humidity were recorded.

Sterilization of tools and materials

Equipment and materials to be used to isolate must be sterilized in advance to prevent contamination. Equipment that must be sterilized includes petri dishes, test tubes, Erlenmeyer, tips, and Eppendorf tubes. The sterilized materials are aquades, King's B media, NAG media, and phosphate buffer. Wet sterilization is done using an autoclave at 121°C, with a pressure of 1 atm for 20 minutes.

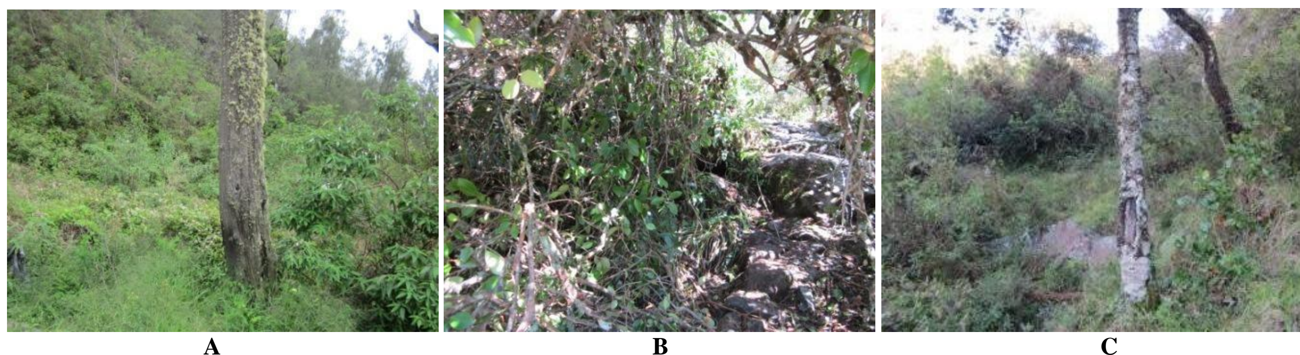
The making of media

King's B (KB)

King's B Media was made by dissolving 20 g of protease peptone, 15 mL of glycerol, 1.5 g of K_2HPO_4; 1.5 g of $MgSO_4.7H_2O$; 20 g of agar in 1000 mL of distilled water. First, Erlenmeyer containing KB media was heated on a hotplate stirrer with a temperature of 350°C for 20 minutes. The clear media was then covered with cotton and coated with aluminum foil, and then it was sterilized using an autoclave at 121°C, with a pressure of 1 atm for 20 minutes. Next, sterile media was poured into each aseptic petri dish for as much as ± 15 mL. After the media was cold, then it was wrapped in paper and placed at room temperature for ± 2 days or until it was dry in an upside-down position with the above position for the media.

Nutrient agar and 2.5 % glycerol (NAG)

Six (6) g of Nutrient Agar was dissolved in 200 mL of distilled water and added 5 mL of glycerol. Erlenmeyer containing Na and glycerol was heated on the hotplate stirrer with a temperature setting of 350°C for 20 minutes. The clear media was then covered with cotton and coated with aluminum foil, and then it was sterilized using an autoclave at 121°C with a pressure of 1 atm for 20 minutes. Sterile media was poured as much as ± 15 mL in each aseptic petri dish. When it was cold, the media was wrapped in paper and placed at room temperature for ± 2 days, or until it was dry in an upside-down position, the media was above.



A B C

Figure 1. Lichens sampling location in Cemoro Sewu, Mount Lawu, Indonesia. A. Montane (sta. 1), B. Sub-alpine (sta. 2), C. Alpine (sta. 3)

To make tilted agar media, after the media became clear, 4 mL of it was poured into a test tube and covered with cotton, then sterilized using an autoclave at 121°C with a pressure of 1 atm for 20 minutes. Sterile media was tilted up to half of the tube. The media was placed at room temperature for ± 2 days or until it was dry. If not contaminated, the media was ready to use.

Phosphate buffer 0.1 M pH 7 and 0.1% peptone meat

Phosphate buffer was made with the composition of 0.6 g of $NaH_2PO_4.2H_2O$ and 1.6 g of $Na_2HPO_4.2H_2O$ dissolved in 1000 mL of distilled water. Then, it was added with 1 g of peptone meat. Furthermore, the phosphate buffer medium was sterilized using an autoclave at a temperature of 121°C with a pressure of 1 atm for 20 minutes.

Isolation and pure culture of INA bacteria

Each 5 g of lichens sample was cut into pieces with a size of about 5 cm^2. Then it was inserted into a 500 mL Erlenmeyer tube containing 200 mL of 0.1 M phosphate buffer with a pH of 7.0 with 0.1% peptone (Difco) (Lindow et al. 1978a). The Erlenmeyer tube containing the buffer solution and the Lichens sample was shaken on a rotary shaker at 150 rpm for 2 hours. Next, 1 mL of sample was taken and put in a test tube containing 9 mL of sterile distilled water. Furthermore, 3 series of dilutions were carried out, namely 10^{-1}, 10^{-2}, and 10^{-3}. Finally, 0.1 mL was taken from each dilution and then spread on NA media containing 2.5% glycerol (NAG) and King's B (KB) media using a spread plate technique. Each dilution series was spread over two petri dishes. After the surface of the media was dry, it was wrapped in paper and incubated at 18º-24ºC in an inverted position for 2 days (Lindow et al. 1982b). Colonies with different morphological appearances were then taken, purified on new media, and stored in a tilted media at 4ºC.

INA test

Ice nucleation activity was determined by the tube nucleation test method (Stephanie and Waturangi 2011). Bacterial colonies aged 4 to 6 days were transferred with an ose loop, suspended in 400 µL sterile phosphate buffer, and tested for ice nucleation activity at -10°C in a circulating alcohol bath for 5 minutes. The freezing of the phosphate buffer indicated a positive test result due to the presence of INA bacteria (Lindow et al. 1978a).

INA protein classification based on freezing temperature

One ose isolates positive for INA bacteria was suspended in 400 µL sterile phosphate buffer and tested for ice nucleation activity at -2°C to -10°C in a circulating alcohol bath (Lindow et al. 1978a).

Characterization of INA bacterial isolates

INA bacterial isolates were characterized based on colony morphology, cell morphology observations, and biochemical tests.

Colony morphological observations

Morphological observations were carried out, including observations of colony color, shape, edge, elevation, and size.

Cell morphological observations

Microscopic observation of bacterial cell morphology was carried out by Gram staining. Gram staining was carried out by taking the INA bacterial isolate aseptically, placing them on a sterile glass object, and then making them into suspension with a drop of sterile distilled water. Preparations fixed over the flame were stained with crystal violet for 1 minute and rinsed with water. Staining was continued with iodine for 2 minutes and rinsed again with water. Next, bleaching was carried out with 95% alcohol and rinsed with water. Then it was stained with safranin for 30 seconds. After washing and drying, observations were made with a microscope to see the type of gram and cell shape (Hadioetomo 1993).

Biochemical test

Biochemical tests carried out include the catalase test and oxidase test.

The catalase test was carried out to detect the presence of the catalase enzyme, which can convert hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). The INA bacterial isolate fixed on a glass object was added with one drop of 3% H_2O_2 solution. The gas formation was observed. Furthermore, it gave positive results if air bubbles appeared (Hadioetomo 1993).

An oxidase test was used to determine the oxidation ability in bacteria that produce oxidase enzymes. A total of one ose of the bacterial isolate was streaked on Oxidase Test Strips. The color changes on the trip test paper were observed. A positive test was indicated by the formation of black or blue-violet color.

Estimation of the number of INA bacteria

The number of INA bacteria was estimated using the multiple tube nucleation method (Cazorla et al. 1995). First, the test tube containing 9 mL of sterile phosphate buffer was cooled at -10°C for 30 minutes. Then the tubes were shaken, and all the frozen tubes were separated. Next, the tube containing the unfrozen phosphate buffer was heated to 5°C. Two (2) g of Lichens sample was homogenized in 20 mL of phosphate buffer medium and 0.1% peptone meat. Then 1 mL was taken and put in a test tube containing 9 mL of sterile phosphate buffer.

Furthermore, 3 series of dilutions were carried out, 10^{-1}, 10^{-2}, 10^{-3}, in the tube containing unfrozen phosphate buffer. Each dilution series was carried out in three replications to obtain a series of 3 tubes. Each tube containing unfrozen phosphate buffer and a homogenized sample of Lichens was put into a circulating alcohol bath for 10 minutes so that the temperature reached -3°C to -9°C. The number of INA bacteria per gram of fresh weight of the sample was estimated based on the MPN method. First, the number of frozen test tubes was calculated for each dilution. Then it was compared with the numbers in the MPN series 3 tube table to obtain the table values. Finally, the value obtained

is multiplied by the dilution factor of the medium tube to obtain the number of microbial MPNs in the sample (Fardiaz 1993). The results of the microbial MPN calculation were multiplied by 100 because the Lichens sample was classified as a solid sample.

Microbial MPN = MPN value x 1/ Dilution of the middle tube

Data analysis

The obtained data were analyzed descriptively based on the observations of colony morphology, cell morphology, biochemical tests, and analysis of the number of INA bacteria in Lichens living in the hiking pathway of Cemoro Sewu.

RESULTS AND DISCUSSION

Lichens sample

Based on the results of field observations and the identification of Lichens living in the hiking pathway of Cemoro Sewu, it is known that the most common species found at an altitude of 2,200-2,600 m asl are *Usnea* sp. and *Parmelia* sp. (Figure 2). Therefore, in this study, 2 types of Lichens, namely *Usnea* sp. and *Parmelia* sp., were determined to be used as the main source for isolating INA bacteria. The sampling stations and the types of lichenes are presented in Table 1.

Station 1 is located at coordinates 07°.39.067'LS and 111°.11.727'BT with an altitude of 2,207 m asl. The sampling location is in front of post 1, a field with sparse tree vegetation. The light intensity is 7510 Lux because the canopy density is low enough so that most of the sunlight can reach the ground surface.

Station 2 is located at coordinates 07°.39.043'LS and 111°.11.660'BT with an altitude of 2,532 m asl. It is dominated by shrubs and large trees with a dense enough canopy so that sunlight is blocked enough to reach plant vegetation. The air temperature is around 20ºC. It can be attributed to the low light intensity of 3676 Lux.

Station 3 is located at coordinates 07°.38.524'LS and 111°.11.714'E with an altitude of 2,601 m asl. The sampling location is near post 2. There is a lot of tree vegetation, but the plant canopy does not block sunlight from reaching the vegetation.

Isolation of INA bacteria from lichenes samples on the hiking pathway of Cemoro Sewu

Isolation of INA bacteria from lichenes was carried out using selective media for INA bacteria isolation, namely King's B (KB) media containing 1.5% glycerol. Another isolation medium used was Nutrient Agar media which was enriched with the addition of 2.5% glycerol (NAG). The content of glycerol was used as a carbon source for bacterial growth.

Lindow (1990) stated that KB media containing glycerol is a common medium used to isolate ice-core-forming bacteria. On the other hand, Lindow et al. (1982a) explained that culture growth in media containing polyalcohols such as glycerol, mannitol, sorbitol, etc., can increase the frequency of ice core formation. Waturangi and Tjhen (2009) also reported that family planning media is a selective medium expected to have almost the same nutritional content as the nutrients in the leaves. Meanwhile, NA media which has been enriched with the addition of 2.5% glycerol (NAG), is also expected to grow INA bacteria.

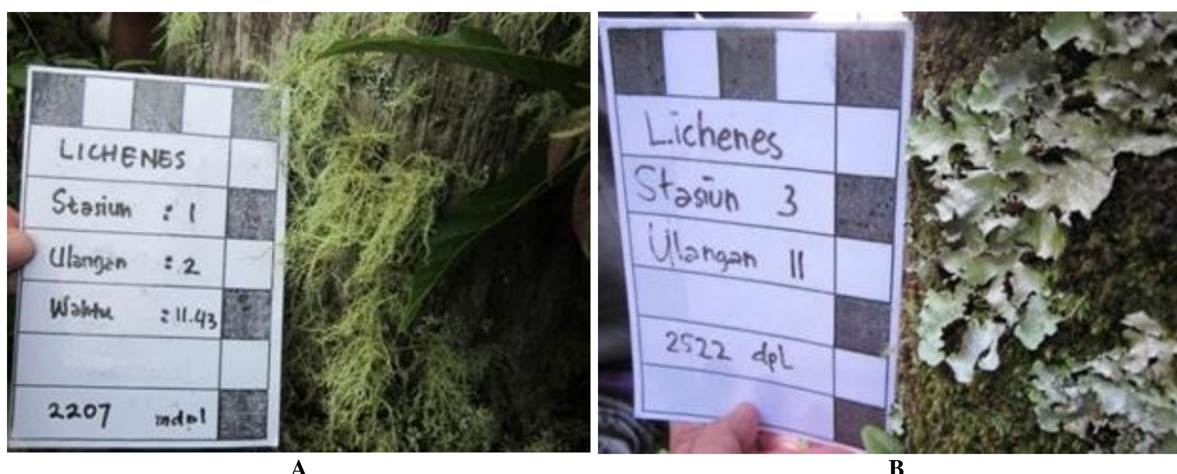


Figure 2. Lichens living on the hiking pathway of Cemoro Sewu, Mount Lawu, Indonesia. A. *Usnea* sp., B. *Parmelia* sp.

Table 1. Lichenes Sampling Station on the hiking pathway of Cemoro Sewu, Mount Lawu, Indonesia

Sampling station	Altitude (m asl.)	Light Intensity (Lux)	Air temperature (°C)	Air humidity (%)	Lichen types
1	2,207	7510	21	71	*Usnea* sp.
2	2,532	3676	20	85	*Parmelia* sp.
3	2,601	4260	20	86	*Parmelia* sp.

The isolation results obtained 37 different isolates based on the appearance of morphology and pigmentation produced. All colonies with different morphological appearances were purified before being tested for ice nucleation activity. All isolates were tested for ice nucleation activity at a temperature of -10°C in a circulating alcohol bath for 5 minutes. In line with Lindow's (1990) opinion, INA bacteria can initiate ice core formation at temperatures above -10ºC. On the other hand, Kieft and Ruscetti (1990) also stated that the formation of an ice core indicated a positive test for INA bacteria after an incubation period of 5 minutes.

The ice nucleation activity test resulted in 7 positive isolates of INA bacteria. They were from *Parmelia* sp., located at an altitude of 2,532 m asl (station 2). The number of bacterial isolates obtained from each station and the results of the ice nucleation activation test is presented in Table 2. Tubes with positive results are shown in Figure 3.

Based on their activity, INA proteins are divided into three main classes, namely class A, B, and C. Class A is active in forming ice cores at temperatures > -2°C to -5°C, class B is active at temperatures > -5°C to -7°C, and class C is active at temperatures -> 7°C to -10°C (Ruggles et al. 1993). Based on the test results, 2 isolates were classified into Class B. The five isolates were classified into the Ice core formation temperature class for each isolate as presented in Table 3.

Characterization of the obtained isolates

The observations result of colony morphology showed differences in the colonies of each isolate, which indicated that the seven isolates were different. Furthermore, this difference was seen in almost all parts of the colony, such as color, shape, margins, and size (Table 4 and Figure 4). Since colony morphology alone was insufficient, cell characterization of each isolate, including microscopic observations and biochemical tests, was needed.

The morphological characterization of bacterial cells was carried out by observing the shape of the cells and the type of gram bacteria. Table 5 shows the morphological characteristics of cells, indicating that there are differences in cell shape, namely, five isolates are rod-shaped, and 2 other isolates are coccobacillus (short stem). The similarity of the morphological characteristics of the cells was indicated by the reaction of the bacterial cells to the same gram staining of the seven isolates, namely Gram-negative, which was indicated by the appearance of pink-colored bacterial cells (Figure 5). According to Hadioetomo (1993), Gram-negative bacteria will be stained with safranin counter-dye at the end of the staining process because they cannot withstand the primary purple dye complex with iodine crystals.

Table 2. Number of bacterial isolates obtained by isolation from lichens on the hiking pathway of Cemoro Sewu, Mount Lawu, Indonesia

Station	Lichen species	Number of obtained isolates	Number of positive isolates of INA bacteria
1	*Usnea* sp.	7	-
2	*Parmelia* sp.	17	7
3	*Parmelia* sp.	13	-

Table 3. Classification of INA proteins based on freezing temperatures isolated from *Parmelia* sp. at Station 2 of Cemoro Sewu, Mount Lawu, Indonesia

Isolation code	Freezing temperature	INA protein class
K21B-5	-10°C	C
N21B-13	-5°C	B
N21B-15	-8°C	C
K22B-2	-5°C	B
K22B-4	-9°C	C
N22B-6	-10°C	C
N22B-7	-10°C	C

Note: K: Growth medium (K: King's B; N: NAG), 2: Sampling Station Number, 1: Repetition Number, B: Lichens Species Names (A: *Usnea*; B: *Parmelia*), 5: Number of isolates

Table 5. Cell morphological characteristics and biochemical test of INA bacteria isolates

Isolate	Morphology of cell		Biochemical test	
	Gram	Shape	Catalase	Oxidase
K21B-5	-	Coccobacillus	+	+
N21B-13	-	Coccobacillus	+	+
N21B-15	-	rod-shaped	-	-
K22B-2	-	rod-shaped	+	+
K22B-4	-	rod-shaped	+	+
N22B-6	-	rod-shaped	+	+
N22B-7	-	rod-shaped	+	+

Note: (+) positive, (-) negative. K: Growth medium (K: King's B; N: NAG), 2: Sampling Station Number, 1: Repetition Number, B: Lichens Species Names (A: *Usnea*; B: *Parmelia*), 5: Number of isolates

Table 4. Morphological characteristics of INA bacterial isolate colony

Isolate code	Colony morphological character				
	Color	Shape	Edge	Elevation	Size (mm)
K21B-5	Cloudy white	Circular	Entire	Convex	2
N21B-13	White	Irregular	Undulate	Convex	2.5-3.0
N21B-15	Clear yellow	Circular	Entire	Convex	2
K22B-2	Cloudy white	Circular	Undulate	Convex	1
K22B-4	Flash yellow	Circular	Entire	Convex	1
N22B-6	Flash yellow	Circular	Undulate	Convex	2
N22B-7	Flash yellow	Circular	Undulate	Convex	2

Note: Circular (round), Irregular, Entire (slick), Lobate (curvy), Undulate (wavy), K: Growth medium (K: King's B; N: NAG), 2: Sampling Station Number, 1: Repetition Number, B: Lichens Species Names (A: *Usnea*; B: *Parmelia*), 5: Number of isolates

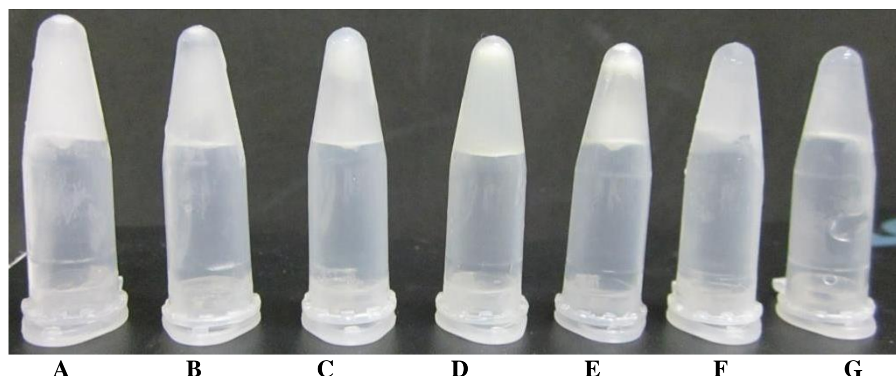


Figure 3. Ice nucleation activity isolated from *Parmelia* sp. Note: isolate code: A. K22B-5, B. N21B-13, C. N21B-15, D. K22B-2, E. K22B-4, F. N22B-6, G. N22B-7

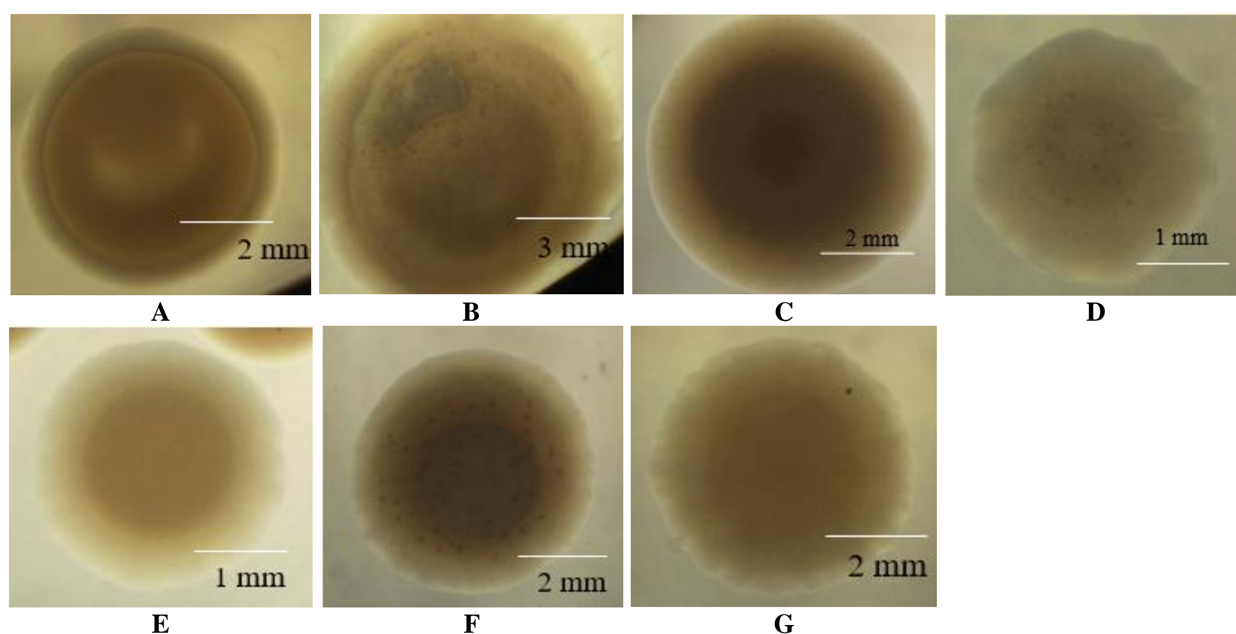


Figure 4. Morphology of colony of INA bacteria isolate from *Parmelia* sp. (40X Magnification). Note: isolate code: A. K22B-5, B. N21B-13, C. N21B-15, D. K22B-2, E.K22B-4, F. N22B-6, G. N22B-7

The morphological characters of bacterial cells isolated from *Parmelia* sp. generally showed Gram-negative characteristics with rod-shaped cells. It is in line with the opinion of Gurian-Sherman and Lindow (1993), which stated that ice core formation activity in bacteria is limited to Gram-negative bacterial species. Still, the presence of these species in plants and other natural habitats is an interesting general phenomenon. The most commonly found ice-core bacteria associated with plants include five species, namely: *P. syringae*, *P. viridiflava*, *P. fluorescens*, *Erwinia herbicola*, and *Xanthomonas campestris* pv translucens. The five species are Gram-negative bacteria. It is supported by the opinion of Lindow et al. (1978b) that *P. syringae* is a Gram-negative bacterium, rod-shaped, does not produce spores, and is oxidase negative. The *E. herbicola* species are Gram-negative bacteria, yellowish in color, motile, can use citrate, and produce H_2S. Members of the *Erwinia* genus are major plant pathogens that cause damage, wilt, and several other plant diseases.

Biochemical tests were carried out to determine the metabolism of isolated bacteria, which strengthened the different characteristics of each bacterium. The biochemical tests were: the catalase test and oxidase test. The different reactions of 7 bacterial isolates to biochemical tests can be seen in Table 5, which shows physiological differences between the isolates obtained. Each different microorganism has its biochemical characteristics called biochemical fingerprints. This device is controlled by the enzymatic activity of the cell and is responsible for biosynthesis and biodegradation (Cappucino and Sherman 1987).

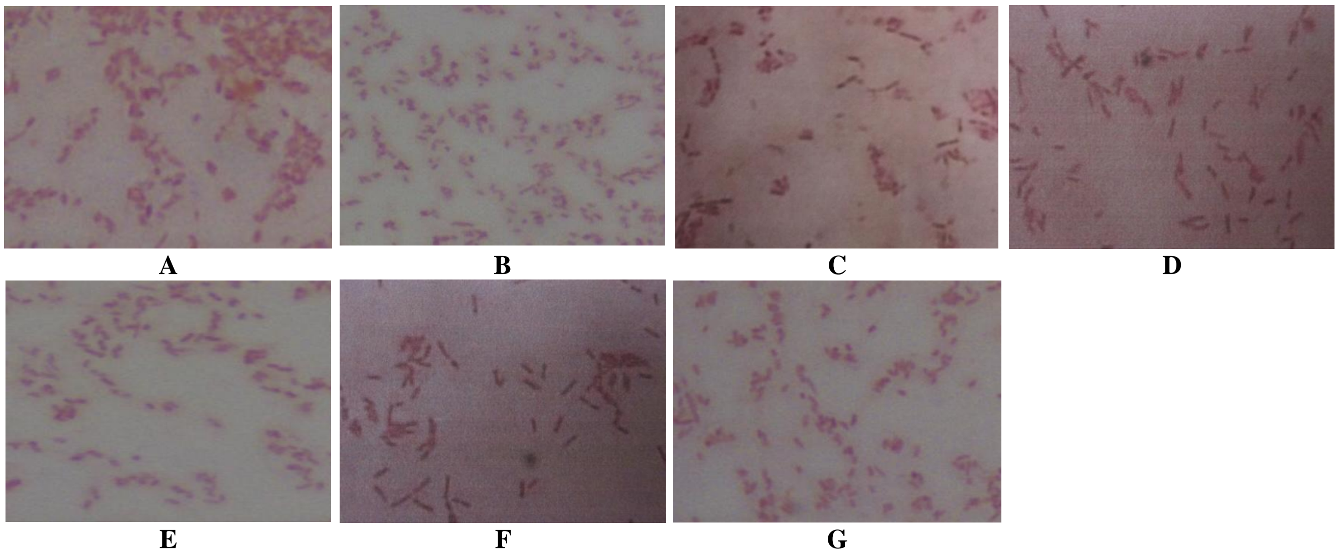


Figure 5. Gram staining of INA bacterial isolates isolated from *Parmelia* sp. (1000X Magnification). Note: Isolate code: A. K22B-5, B. N21B-13, C. N21B-15, D. K22B-2, E. K22B-4, F. N22B-6, G. N22B-7

Differences in the character of each obtained bacterial isolate can be seen in colony morphology, cell morphology and bacterial physiology characteristics. The characteristics of each isolate are as follows:

Isolate K22B-5 had the following characteristics: cloudy white colonies, circular shape (round), entire edge (slippery), convex elevation, 2 mm colony size, and coccobacillus cell shape (short rods). In addition, bacterial cells showed a negative reaction to Gram staining, i.e., cells looked pink and reacted positively to the catalase enzyme indicating that the bacteria were aerobic or facultative anaerobes. Aerobic bacteria and facultative anaerobes can produce the enzyme catalase, which converts hydrogen peroxide into water and oxygen. In the oxidase test, isolate K22B-5 showed a positive reaction indicating that the bacteria were able to produce oxidase enzymes.

Isolate N21B-13 had white colony characteristics, irregular shape (irregular), undulate edges (wavy), convex elevation, colony size of 2.5-3.0 mm, coccobacillus cell shape (short rods), and reacted negatively to Gram staining. Bacteria were aerobic or facultative anaerobes indicated by the formation of bubbles in the catalase test and the positive reaction in the oxidase test.

Isolate N21B-15 had the following characteristics: clear yellow colonies, circular shape (round), entire edge (slippery), convex elevation, colony size of 2 mm, Gram-negative, rod-shaped cell. The biochemical test results showed a negative reaction to the catalase enzyme, indicated by the absence of air bubbles. In addition, the K22B-5 isolate showed a negative reaction in the oxidase test, indicating that the bacteria could not produce the oxidase enzyme.

Isolate K22B-2 with colony character of cloudy white color, circular shape, undulate edge (wavy), convex elevation, 1 mm colony size, rod-shaped cells reacted negatively to Gram staining, i.e., cells looked pink. Positive reactions to the catalase test and the oxidase test indicated that the bacteria were aerobic or facultative anaerobes and the bacteria were able to produce oxidase enzymes.

The K22B-4 isolate had the following characteristics: bright yellow colonies, circular shape, entire edge (slick), convex elevation, 1 mm colony size, reacted negatively to Gram staining, and rod-shaped cells. In addition, K22B-4 isolate produced catalase enzyme, which converts hydrogen peroxide into water and oxygen. In addition, it could oxidize, which produces the enzyme oxidase.

Isolate N22B-6 had morphological characteristics of bright yellow colonies, circular shape (round), undulate edges (wavy), convex elevation, 2 mm colony size, Gram-negative, and rod-shaped cells. In addition, it reacted positively for the catalase test, as indicated by the formation of air bubbles and positive oxidase.

Isolate N22B-7 had a bright yellow colony appearance, circular shape (round), undulate edge (wavy), convex elevation, colony size 2 mm, Gram-negative and rod-shaped cell. It had a positive reaction to the catalase and oxidase tests.

Estimation of INA bacteria

The number of INA bacteria was estimated by the multiple tube nucleation method, which is a method for estimating the number of ice nuclei from bacterial suspensions and the population of INA bacteria on plant parts (Cazorla et al. 1995).

According to Anon (1975), the tube nucleation test is used to calculate the number of INA bacteria associated with plants based on and developed from the multiple-tube or MPN (Most Propable Number) method is a method to determine the number of coliforms in water. According to Montesinos and Viraldell (1991), the use of a tube assay to determine the number of ice cores is based on the number of tubes that freeze in each dilution series. The MPN method assumes that a frozen tube contains at least one ice core (Govindarajan and Lindow 1988).

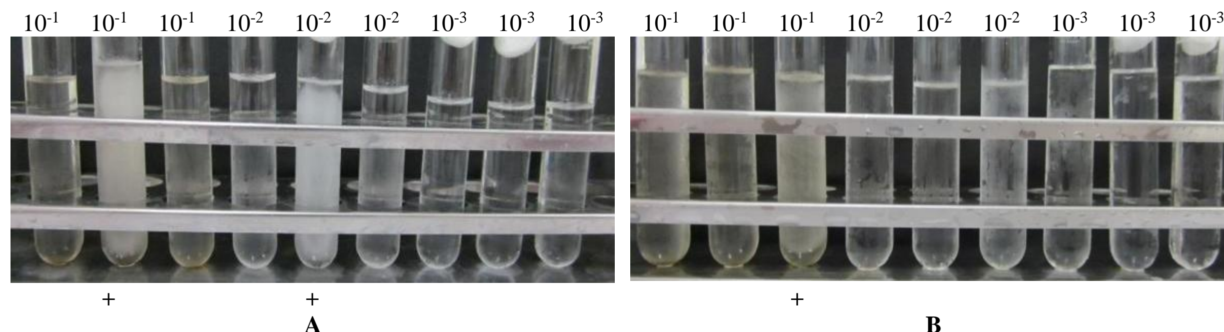


Figure 6. Positive tube (frozen) on the MPN test. A. Tube combination 1-1-0; B. Tube combination 1-0-0

Table 6. Average MPN value based on the 3-tube series MPN table

Station	Lichen types	MPN tabel value (MPN/g)
1	*Usnea* sp.	<3
2	*Parmelia* sp.	5
3	*Parmelia* sp.	<3

Fardiaz (1993) stated that the MPN method is usually used to count the number of microbes in a liquid sample, although it can also be used for solid samples by first making a 1:10 suspension of the sample. The output of the MPN method is the MPN value. The MPN value estimates the number of growth units or colony-forming units in the sample. However, in general, the MPN value is also interpreted as an estimate of the number of individual bacteria.

The number of bacteria was estimated using the multiple tube nucleation test method at a temperature of -5°C for 10 minutes in a circulating alcohol bath. In a study conducted by Cazorla et al. (1995), the optimum temperature for the estimation test is -5ºC. Furthermore, it is supported by the opinion of Hirano et al. (1985) and Baertlein et al. (1992) that a temperature of -5°C is sensitive enough to detect the presence of INA bacteria. On the other hand, Lindow et al. (1982a), Hirano and Upper (1986), Olive and McCarter (1988) used a temperature of -5°C for routine testing to determine INA bacteria.

Based on the test results, the frozen positive tube was only found in sample station 2 (2,532 m asl). In replicate 1, the number of frozen positive tubes with a combination of 1-1-0 was matched with the MPN table, which shows a value of 7 MPN/g. In the second sample, the number of frozen positive tubes was obtained with a combination of 1-0-0, then matched with the MPN table, which shows a value of 4 MPN/g. At stations 1 and 3, the combination of frozen positive tubes is 0-0-0, which indicates a value of <3 MPN/g. Of the three stations, the highest table MPN value came from the sample *Parmelia* sp. at station 2 (2532 m asl.) with an average value of 5 MPN/g (Table 6). The combination of frozen positive tubes can be seen in Figure 6.

Based on the calculation results, the number of INA bacteria in Lichens on the hiking pathway of Cemoro Sewu is 5 x 104/g. This value indicates a low number of bacteria compared to several previous studies. Several studies have been carried out, including by Lindow (1993), which states that the population of INA bacteria reaches 106 cells/g in plant tissue. On the other hand, Kieft (1988) reported that the highest density of ice-core bacteria population in lichenes was between 2.3 x 106 to more than 1 x 108 cells/g at -5ºC. Lindow et al. (1978a,b) also reported that the lowest bacterial population ranged from 102 to 2.4 x 106 cells/g. The low population in plant tissue is between 102 to >104 cells/g. Differences in environmental conditions may be one of the factors that cause the high and the low number of INA bacteria on the plant surface.

This study provides the following conclusions: (i) 7 isolates of INA bacteria can be isolated from lichenes on the hiking pathway of Cemoro Sewu, which were isolated from lichenes type *Parmelia* sp. at an altitude of 2,532 m asl; (ii) the number of INA bacteria in Lichens on the hiking pathway of Cemoro Sewu is 5 x 104/g, indicating a low number of bacteria.

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