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Isolation and identification of Ice Nucleation Active (INA) bacteria causing *embun upas* (frost injury) on leaves of potato plant in the Dieng Plateau, Central Java, Indonesia

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Abstract. *Rajasa AK, Susilowati A, Suranto. 2020. Isolation and identification of Ice Nucleation Active (INA) bacteria causing embun upas (frost injury) on potato plant leaves in the Dieng Plateau, Central Java, Indonesia. Bioteknologi 17: 67-74. Embun upas* (frost injury) is one of the symptoms of ice crystal formation that occurs on the leaves of potato plants (*Solanum tuberosum* L.) in the Dieng area, Central Java, Indonesia. Symptoms of *embun upas* occur on a large scale and cause a decrease in potato yields of up to 6 tons per hectare. The cause of the symptoms of *embun upas* on potato plants is suspected to be the activity of ice-core-forming bacteria. This study aimed (i) to obtain isolates of Ice Nucleation Active (INA) bacteria from potato plants in Batur District, Dieng, (ii) to determine the INA bacterial species which can be identified using the gene encoding 16S rRNA, and (iii) to determine the population density of INA bacteria in one gram of plant leaves. Sampling used the purposive sampling technique by taking potato leaves at three different altitudes: ±1,800 m above sea level (Bakal Village), ±2,000 m above sea level (Diengkulon Village), and ±2,200 m above sea level (Sembungan Village) with two repetitions. Bacterial isolation was carried out using the spread plate method on Nutrien Agar media containing 2.5% glycerol (NAG). Colonies with different morphology were taken and purified on slanted agar media. Ice nucleation activity was determined by the tube nucleation test method. INA bacteria were estimated using the multiple-tube nucleation test 3,3,3 method. Identification of INA bacteria was carried out by amplifying the gene encoding 16S rRNA by the PCR method. The gene encoding 16S rRNA amplicon was purified using PCR/DNA Fragments Extraction Kit (Genaid) and continued with determining the concentration and purity of DNA using a biophotometer. Sequencing was carried out by sending samples to 1st BASE Singapore, and the data from the sequencing results were analyzed using the BLAST Nucleotide device on the NCBI website. Analysis of the data used is descriptive analysis. The isolation results obtained in this study were six isolates of INA bacteria in all sampling villages. The bacterial species obtained were *Arthrobacter sulfonivorans* (98%), *Curtobacterium luteum* (98%), and *Pseudomonas azotoformans* (99%). The total bacterial population on potato leaves was 1.44 x 102 MPN INA/gram in Bakal Village, 3 x 103 MPN INA/gram in Diengkulon Village, and 1.824 x 105 MPN INA/gram in Sembungan Village.

Keywords: 16S rRNA, Dieng, *embun upas*, INA bacteria, potato

INTRODUCTION

The Dieng Plateau is an area located in the province of Central Java, Indonesia. Dieng is mainly located between Banjarnegara District and Wonosobo District and Temanggung District and Kendal District. Most of the Dieng area is fertile land with cold temperatures. Dieng Plateau is 1,500-2,500 meters above sea level (m asl). Dieng has an average rainfall of 4,000-7,000 mm in October-May, but in June-September, the rainfall decreases by 2,000-3,500 mm (Nijman and Van Balen 1998).

The air temperature in the Dieng area with an altitude below 1,500 m asl is different from the area with an altitude above 1,500 m asl. At an altitude below 1,500 m asl, the air temperature ranges from 22-34°C with an average of 26°C. In areas above 1,500 m asl, the average air temperature is 14°C. In the Dieng Plateau, an area above 1,500 m asl, frost can form, especially during the dry season (Arwiyanto 1996).

Potato (*Solanum tuberosum* L.) is one of the primary commodities cultivated in the Dieng highlands. Many people have been cultivating this plant since 1974. The productivity of potato plants from 1975 to 1990 has increased very significantly, where yields can reach 20-25 tons per hectare. In the year 2000, potato production experienced a drastic decline. The yield obtained is only 16 tons per hectare (Sularso 2009). It is due to the many land problems in the Dieng Plateau. These land management problems do not apply soil and water conservation principles only; potato plants are very susceptible to environmental and temperature balance in the Dieng Plateau and plant diseases that attack cultivated plants (Bondansari et al. 2011).

The main problem with potato plantations in the Dieng area is the phenomenon of frost injury or *embun upas* called by local people. It has a detrimental impact on potato farmers. According to Arwiyanto (1996), *embun upas* occurs during the dry season, and it is recorded that more than 35 ha of potato plantations are affected by the *embun upas* phenomenon. The loss value of potato farmers reaches hundreds of millions of rupiah.

Embun upas not only occurs in Indonesia but also in several countries in the world. In the United States, it occurs on maize, tomatoes, wheat, and some lignosus crops

such as mangoes, oranges, and apples. *Embun upas* is also found in Japan, namely cabbage, broccoli, and mulberry plants (Goto et al. 1988). Lindow (1983a) proved that *embun upas* that occurred in some of these plants resulted from the activity of epiphytic bacteria on the leaf surface. These bacteria are harmful to plants and can cause plant death.

According to Gurian-Sherman and Lindow (1993), bacteria that can cause *embun upas* can be Ice Nucleation Active (INA) bacteria. INA bacteria have active proteins located on the cell surface, which function to increase the temperature of ice core formation. The protein in INA bacteria is called INP (Ice Nucleation Protein). Several species of bacteria that are INA bacteria are *Pseudomonas syringae, Pseudomonas fluorescens, Pseudomonas viridiflava, Xanthomonas campestris, Erwinia herbicola* (Lindow 1983b), and *Erwinia ananas* (Goto et al. 1989).

In the Americas, INA strains of *P. syringae* and *E. herbicola* bacteria have been detected on the leaf surface of corn plants. INA bacteria can cause *embun upas* at -2°C to -4°C. Leaf surfaces that do not detect the presence of INA bacteria will experience *embun upas* at a temperature of around -20°C. Detection and identification of INA bacterial species can be made by comparing the biochemical activity between isolates of one bacterial species and another (Arwiyanto 1996). However, along with advances in science and technology, researchers use molecular methods, namely identification with the gene encoding 16S rRNA. This molecular method has a higher accuracy and validity level than the comparison of biochemical activity between bacterial species. The time required for molecular identification is faster and shorter, with a lower error rate than other methods (Lindow et al. 1978; Goto et al. 1989; Chakravorti et al. 2007). Therefore, research related to INA bacteria suspected as the cause of *embun upas* on potato plants in the Dieng Plateau and identification using the 16S rRNA encoding gene needs to be carried out.

In addition to the activity of INA bacteria on the leaf surface, leaf damage is also influenced by the large population of INA bacteria on the leaf surface. The more the people of INA bacteria on the leaf surface, the faster the plant damage will occur. The calculation of the bacterial population on the plant surface can be done using the Most Probable Number (MPN) method. The MPN method determined the bacterial population per gram of plant leaves (APHA 1975).

Based on these problems, the research objectives are (i) obtaining INA bacterial isolates from potato plants in Batur District, Dieng, (ii) knowing the INA bacterial species from potato plants in Batur District, Dieng, to be identified using the gene encoding 16S rRNA, (iii) knowing the population density of INA bacteria in one gram of potato plant leaves.

MATERIALS AND METHODS

Research site

This research was carried out from November 2014 to February 2015 at the laboratory of the Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Central Java, Indonesia, and the Biotechnology Laboratory of Universitas Katolik Atmajaya (Unika), Jakarta, Indonesia. A sampling of potato plants (*S. tuberosum*) was carried out in November 2014 at the potato plantation, Dieng Plateau, Central Java, Indonesia.

Ingredient

The leaf samples were potato plant leaves originating from Dieng Plateau and growing above 1,500 m asl. The leaves taken were old leaves of the third order from the shoots, which had brownish spots on the leaf surface and leaf with a slightly dry texture. Materials for culture media include nutrient agar (NA) weighing 6 grams, 1 L aquadest, and 25 mL glycerol.

Materials for bacterial isolation and pure bacterial culture include potato leaves weighing 5 grams that have been cut into small pieces, phosphate buffer pH 7 0.1 m, pure bacterial isolate, and 0.1% peptone meat. DNA extraction materials and amplification of the 16s rRNA encoding gene include DNA templates, presto™ mini gDNA bacteria kit, Kapa 2g fast ready mix, reverse primer, forward primer, PCR mix, tae buffer, and ddh2o.

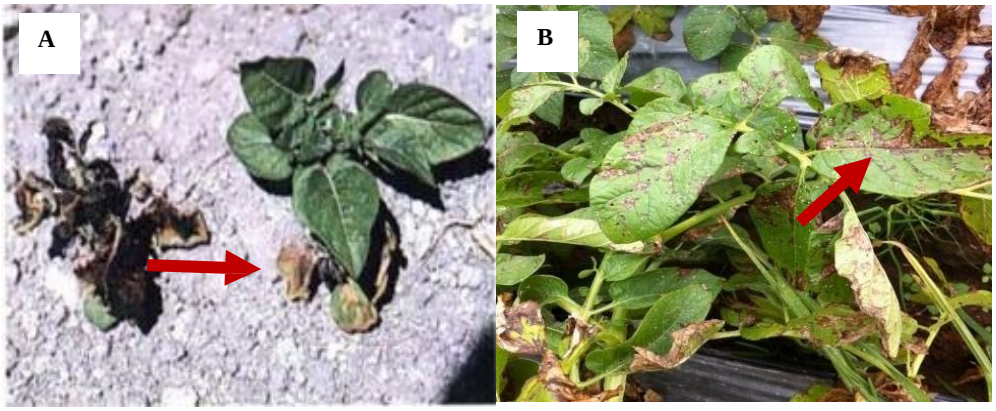


Figure 1. A. Damage to potato leaves infected with INA bacteria based on research by Lindow (1983a). B. Potato leaves suspected of being infected with INA bacteria in the Dieng Plateau, Central Java, Indonesia

Procedure

*Potato leaf sampling (*Solanum tuberosum *L.)*

A sampling of potato leaves was carried out in November 2014 in potato plantations in Batur District, Dieng Plateau, with a purposive sampling method based on altitude. Potato leaf samples were taken at an altitude above 1,500 m asl. Sampling was carried out in three villages with different altitude ranges, namely Bakal Village with a height of ± 1,800 m asl, Dieng Kulon Village with an altitude of ± 2,000 m asl, and Sembungan Village with an altitude of ± 2,200 m asl. Potato leaves are cut using a cutter at an angle to the stem, then put in a plastic clip. Leaf samples that have been taken must be immediately brought to the laboratory using a cooler box for isolation at <24 hours. While in the laboratory, the sample was put in a refrigerator at a temperature of about 5°C so that it could be isolated for the next few days.

Sterilization of tools and materials

Petri dishes, test tubes, Erlenmeyer, NA, and other tools that will be used are sterilized first. Petri dishes are sterilized first before being packaged using old newsprint. The sterilization process was carried out by wet sterilization using an autoclave at a pressure of 1 atm and a temperature of 121°C for 20 minutes.

Preparation of nutrient agar with 2.5% glycerol (NAG) media and inclined media

NA weighing 6 grams was put into a 250 mL Erlenmeyer tube, then added 200 mL of distilled water into the Erlenmeyer. Stir until homogeneously mixed. The homogeneously mixed solution is added with 5 mL of glycerol, then stirring is done again until blended. Erlenmeyer covered with cotton and aluminum foil, then heated using a microwave for 2 minutes. Erlenmeyer was then sterilized by autoclave at 121°C, 1 atm, for 20 minutes. The sterilized media was then poured into a petri dish aseptically in a laminar airflow cabinet (LAFC). The dry and solid nag media cup was then wrapped in old newsprint and stored upside-down at room temperature or in an incubator. If the media is not contaminated, then the media is ready for use (Kartika 2009).

The first process carried out in the manufacture of inclined media is that nag is inserted into the test tube up to a quarter of the tube. Then the test tube was closed using cotton and aluminum foil and put in an autoclave for sterilization. The nag in a sterile test tube is then placed at an angle of 35° or tilted until the nag medium reaches half of the tube. The slanted media is left at room temperature, and if there is no contamination and it has solidified, it can be used (Kartika 2009).

Preparation of 0.1 m phosphate buffer pH 7 with 0.1% peptone meat

Phosphate buffer pH 7.0 was made with 0.6 grams of monosodium phosphate ($NaH_2PO_4.2H_2O$) and 1.6 grams of disodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$) dissolved in 1 L of sterile distilled water and added with 1 gram of peptone meat. Stirring is done so that the solution is homogeneously mixed. Furthermore, the phosphate buffer medium was sterilized using an autoclave at a temperature of 121°C and a pressure of 1 atm for 20 minutes.

Estimation of bacterial count INA

The number of INA bacteria was estimated using the multiple tube nucleation method (Cazorla et al. 1995). 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. The tube containing the unfrozen phosphate buffer is heated to 5°C. Potato plant samples weighing 2 grams were homogenized in 20 mL phosphate buffer media and 0.1% peptone meat, then 1 mL was taken and put in a test tube containing 9 mL sterile phosphate buffer. Furthermore, three dilutions series were carried out, namely 10^{-1}, 10^{-2}, 10^{-3}, into a tube containing unfrozen phosphate buffer. Each dilution series was carried out in three replications so that a series dilution of 3.3.3 tubes was obtained. The diluted bacterial suspension was put in an alcohol bath at a temperature of -5°C for 10 minutes (Cazorla et al. 1995).

The number of frozen test tubes was counted at each dilution. The total population of INA bacteria/gram fresh weight of the sample was estimated by measuring the number of frozen tubes in each dilution (Cazorla et al. 1995), then matched to the Most Probable Number (MPN) table according to the Thomas formula series 3.3.3 so that the table value was obtained (APHA 1975).

Amplification of 16S rRNA encoding gene using PCR method

Genomic DNA was extracted with the Presto™ Mini gDNA Bacteria Kit. Then the gene encoding the bacterial 16S rRNA was amplified using Polymerase Chain Reaction (PCR). The PCR reaction was carried out by mixing 25 µL *Kapa2G Fast Ready Mix*, one µL 63 forward primers (63f: 5'-CAGGCCTAACACATGCAAGTC-3') with a concentration of 10 pmol, one µL 1387 reverse primer (1387r: 5'-GGGCGGAWGTGTACAAGGC-3') with a concentration of 10 pmol, one µL DNA template, and 9.5 µL ddH_2O. Before entering the PCR cycle, pre-denaturation was conducted at 94°C for 2 minutes. One cycle of PCR carried out consisted of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute, and finalizing at 72°C for 20 minutes, and then stopped for storing at 4°C. The sample can then be electrophoresed. The denaturation, annealing, and elongation processes consist of 25 cycles (Marchesi et al. 1998).

16S rRNA coding gene sequencing

The results of the PCR gene encoding 16S rRNA for INA bacteria were purified using the PCR/DNA Fragments Extraction Kit (Genaid) and continued with DNA concentration treatment using a Biophotometer. The DNA was then sent to 1st Base Singapore for sequencing using the ABI PRISM 310 Genetic Analyzer. The DNA sequences were then analyzed using bioinformatics techniques, namely, the BLAST Nucleotide device on the

NCBI website (www.blast.ncbi.nlm.nih.gov/blast.cgi) (Waturangi et al. 2008; Arifin 2014).

Data analysis

Analysis of the data used for the results of the isolation of INA bacteria is descriptive data analysis. Bacterial population data per gram of leaf was analyzed descriptively. INA bacterial DNA sequences obtained from DNA extraction and 16S rRNA gene amplification were analyzed using the BLAST Nucleotide device on the NCBI website (www.blast.ncbi.nlm.nih.gov/blast.cgi) (Arifin 2014).

RESULTS AND DISCUSSION

Sampling location

Based on the results of surveys and field observations conducted in the Dieng Plateau, it is known that potato plants are mostly found at an altitude between 1,800-2,200 m asl. Therefore, in this study, samples of potato plants used as the main source for isolating INA bacteria that cause *embun upas* were taken between 1,800-2,200 m asl. Potato crop sampling stations are presented in Table 1.

Sampling station one is in Bakal Village (sampling code: BK) with an altitude of 1,800 m asl. The location for the collection is a potato plantation located in the vicinity of residents' housing. The potato planting system in Bakal Village is intercropping, where potato plants are planted with several other crops such as chili, cabbage, and shallots. Not many cases of *embun upas* were found at station one because the air temperature tends to be warm and does not change rapidly and significantly.

Sampling station two is located in Diengkulon Village (sample code: DK) with an altitude of 2,000 m asl. The collection location is a potato plantation in the Mount Prau hiking trail area. The potato planting system in Diengkulon village is single planting, i.e., only potato plants are grown in one garden plot. There are several cases of *embun upas* that occurred in Diengkulon Village. *Embun upas* occurred at 04.00 West Indonesian Time (WIB), when the air temperature was lowest.

Sampling station three is located in Sembungan Village (Sampling code: SB) with an altitude of 2,200 m asl. Station three is the sampling station with the highest altitude. The sampling location is a potato plantation located around the PT. Geodipa Energi, more precisely, in front of the Dieng gas station. The case of *embun upas* in Sembungan Village occurred during the dry season, namely July-October. *Embun upas* occurred between 24.00-05.00 WIB. The air temperature in Sembungan village can reach 2°C at night at 23.00 WIB.

Bacterial isolates of Ice Nucleation Active (INA) from potato plant leaves in Dieng Plateau

INA bacteria were isolated using NA media with 2.5% glycerol (NAG). The addition of glycerol to NA media is beneficial for the growth and development of cultured bacteria. Lindow (1990) stated that media containing glycerol is a common medium used to isolate ice-core bacteria because it can optimize the growth of the culture. On the other hand, Lindow et al. (1982) explained that the growth of INA bacteria culture on media containing polyalcohols such as glycerol, sorbitol, mannitol, and the like could increase the frequency of ice core formation. Waturangi and Tjhen (2009) explained that NA media enriched with 2.5% glycerol was also expected to grow INA bacteria and increase ice core formation activity.

Bacterial colonies obtained from the plates were then used as pure cultures on inclined NAG media. Pure cultures were incubated at room temperature at 25-27°C for 4-6 days. According to Kieft and Ruscetti (1990), the INA bacterial culture with the highest ice core formation activity is cultured at 4-6 days of age. Pure cultures that are either too young or old do not have high ice core-forming activity. Samples were taken at each station with two repetitions, and each repetition was carried out three times with dilutions so that 18 cultures were obtained on NAG media in Petri dishes. From 18 cultures on plates, 76 isolates were obtained, presented in Table 2.

A total of 76 bacterial isolates with different morphological appearances and pigmentation were then purified before testing the ice nucleation activity. Six isolates were frozen after testing the ice nucleation activity on all isolates. The ice nucleation activity of INA bacterial isolates is presented in Figure 2.

Table 2. Bacterial isolates obtained from each village

Name of village	Number of obtained isolates	The number of frozen isolates
Bakal	28	3
Diengkulon	23	2
Sembungan	25	1

Table 1. Potato sampling station in Dieng Plateau, Central Java, Indonesia

Station	Altitude (asl)	Morning (06.00) air temperature (°C)	Daytime (11.00) air temperature (°C)	Night (01.00) air Temperature (°C)	Name of village
1	1.800	17	21	12	Bakal Village
2	2.000	15	17	9	Diengkulon Village
3	2.500	11	16	2	Sembungan Village

Samples that experience freezing are thought to be the result of the activity of the INA bacteria. Colonies that can be categorized as positive for INA bacteria are colonies that have ice nucleation activity. The ice nucleation activity can be seen when the bacterial suspension in the microtube freezes after being put into a circulating alcohol bath at a temperature below -5°C for 10 minutes. This condition indicated that the microtube with the suspension contained INA bacteria which had a single protein as an initiator of ice core formation (Morris et al. 2008). On the other hand, if the bacterial suspension does not freeze, then the suspension is categorized as negative for INA bacteria.

Tests were carried out at -5°C, -7°C, and -10°C. Tests at different temperatures were carried out because not all samples at each station experienced freezing activity at the same temperature. The temperature difference was due to differences in the activity of each bacterial suspension that was tested. It depends on the protein activity of the INA bacteria that live on the leaves. It shows several bacteria in each sampling station with different activities, even in one station. In the ice nucleation activity test, it was found that 1 sample was frozen at -7°C, while five samples froze at -10°C (Table 3).

Kieft and Ruscetti (1990) stated that the formation of an ice core indicated a positive test for INA bacteria after an incubation period of 5 minutes. Tests were carried out at different temperatures. Based on their activity, INA proteins were divided into three main classes, namely classes A, B, and C. Class A was active in forming ice cores at temperatures > -2°C to -5°C, class B was active at temperatures ≥ -5°C to -7°C, and class C is active at ≥ -7°C to -10°C (Baertlein et al. 1992). Based on the tests on the ice nucleation activity of bacterial isolates, it was found that one bacterial isolate was classified into class B. In contrast, the other five isolates were class C. Class A bacteria were not found in this study.

INA bacterial species in potato plant leaves based on the 16S rRNA coding gene sequence

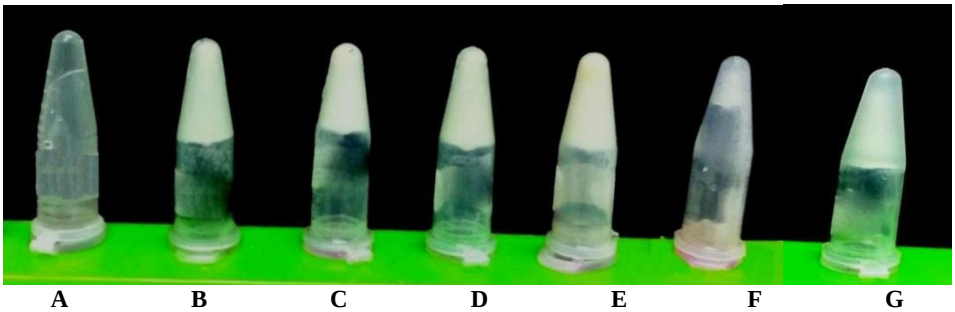
Ice core-forming bacteria or known as INA bacteria, have been widely studied and tested for ice nucleation activity by several researchers in various countries, such as Lindow (1983b), Gurian-sherman, and Lindow (1993), Arwiyanto (1996), and Waturangi et al. al. (2008). Identification has also been carried out to find out the species of INA bacteria that develop in an area. Based on Waturangi and Tjhen's (2009) research, the phenotype of INA bacteria has been found in the genera of three bacteria, namely *Pseudomonas, Xanthomonas,* and *Erwinia*. Still, other bacterial species may be found that are also included in the INA bacterial species. Several species of bacteria included in the INA bacteria are *E. ananas* (Arwiyanto 1996); *Chryseobacterium* sp., *Pseudomonas lurida, Enterobacter aerogenes, P. syringae* (Hendrawan 2014); *Pantoea* sp., *Pseudomonas* sp., *Rahnella* sp. (Arifin 2014); *P. viridiflava, P. fluorescens,* and *X. campestris* (Gurian-Sherman and Lindow 1993).

The identification carried out to determine the species of INA bacteria has different techniques and methods, such as standard methods (Hayward 1990; Arwiyanto 1996), biochemical test methods (Cappucino and Sherman 1978; Fu'adah 2014), and 16S rRNA gene sequencing methods (Castrillo et al. 2000; Arifin 2014). Identification using the 16S rRNA gene sequencing method is effective because the 16S rRNA gene is not easy to mutate and has the same function from one bacterium to another, with a length of ± 1,500 bp which is sufficient for identification purposes (Pangastuti 2006; Janda and Abbott 2007). Identification using the 16S rRNA gene sequencing has the highest data validity among other methods (Hauben et al. 1997; Janda and Abbott 2007).

Several species were obtained at each collection station based on identifying INA bacteria isolated from potato plants in the Dieng Plateau. Of the 76 samples purified based on their colony morphological characters, six isolates showed ice nucleation activity and were identified using the 16S rRNA gene sequencing method.

Table 3. Classification of INA bacteria based on differences in ice nucleation activation temperature from potato plant leaves

Isolate code	Freezing temperature	INA protein class
BK-2a2	-7°C	B
BK-1a6	-10°C	C
BK-1a5	-10°C	C
DK-1b1	-10°C	C
DK-2b3	-10°C	C
SB-3a2	-10°C	C



A B C D E F G

Figure 2. Ice formed from the activity of INA bacteria on potato leaves in microtubes. Note: A. Control, B. DK-1b1, C. DK-2b3, D. PW-3a2, E. BK-1a6, F. BK-1a5, G. BK-2a2

Amplification was carried out using the PCR method. The resulting PCR product was 1,324 bp. The amplification results were then carried out by electrophoresis and presented in Figure 3. All positive samples that were successfully electrophoresed were then identified by sending samples to 1st Base Singapore for sequencing. There were three species of bacteria suspected of causing *embun upas* on potato plants in the Dieng Plateau, presented in Table 4.

Each sampling station contained ice nucleation activity indicated by the freezing of the isolate in the previous test. Based on research and tests conducted by Waturangi and Tjhen (2009), gram-negative bacteria with the genus Pseudomonas is one of the bacteria that has ice nucleation activity. In other words, it can be said that this genus of bacteria is included in the category of INA bacteria. INA bacteria similar to *Pseudomonas azotoformans* were found in Sembungan and Diengkulon villages and are INA bacteria belonging to class C bacteria, which freeze between temperatures of -7°C to -10°C. In Bakal Village, two species of bacteria were found that were similar to *Arthrobacter sulfonivorans* and *Curtobacterium luteum*.

Pseudomonas azotoformans can express the gene encoding the protein INA (INP). The gene that bacteria express to initiate the occurrence of *embun upas* is the inaW gene. These genes can control the mechanism of ice crystal formation by activation of INPs located on the surface of bacterial cell membranes. INP will catalyze the formation of ice crystals called *embun upas* (Castrillo et al., 2000; Morris et al., 2008). No one has stated that *A. sulfonivorans* and *C. luteum* are INA bacteria. Still, these two bacteria have specific mechanisms in response to cold temperatures around their environment to tolerate cold temperatures. Arthrobacter sulfonivorans has cold shock protein (CSP) which helps the bacteria to tolerate cold temperatures. One way is by the mechanism of ice crystal formation (Mishra et al. 2010).

Estimation of the population density of INA bacteria in one gram of leaf using the multiple tube nucleation method

The calculation of the bacterial population was carried out using 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. This method focuses on diluting bacterial suspensions in liquid media and is calculated based on the number of tubes undergoing freezing (Cazorla et al. 1995; Watson and Lawrence 2003). According to Watson and Lawrence (2003), the tube nucleation test method can calculate the population of INA bacteria associated with plants and is based on and developed from the MPN (Most Probable Number) method. The MPN method is used to determine the amount of coliform in water. According to Montesinos and Vilardell (1991), using a tube assay to determine the number of ice-core-forming bacteria is based on the number of tubes that freeze in each dilution series.

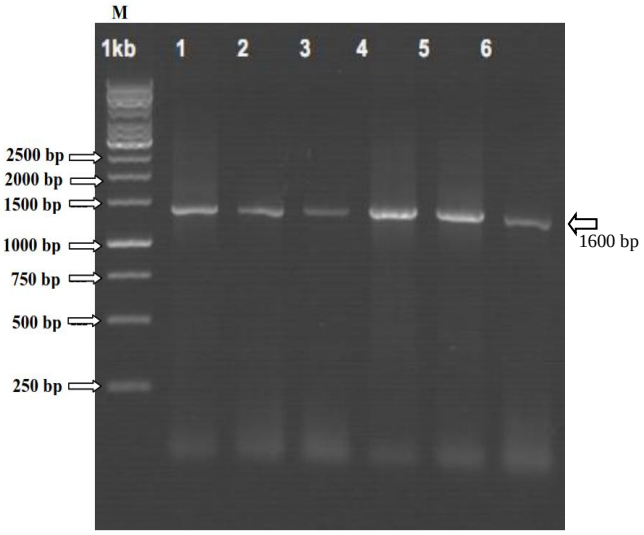


Figure 3. Electrophoresis of INA bacteria that ice nucleation activity. Description: M: Marker

Table 5. MPN values based on the Thomas formula MPN table series 3.3.3

Village	Dilution 10^{-1}	Dilution 10^{-2}	Dilution 10^{-3}	MPN INA/gram
Bakal	1	0	0	1,44 x 102
Diengkulon	3	1	1	3,00 x 103
Sembungan	3	3	1	1,824 x 105

Table 4. Species of INA bacteria found at each sampling station

Isolate code	Isolate description	Query cover	Ident.
BK1-a5	*Arthrobacter sulfonivorans* Strain All16S ribosomal RNA gene Partial Sequence	98%	98%
BK-1a6	*Curtobacterium luteun* Strain DSM 20542 16S ribosomal RNA genepartial sequence	99%	98%
BK-2a2	*Arthrobacter sulfonivorans* Strain All16S ribosomal RNA gene Partial Sequence	98%	98%
DK-1b1	*Pseudomonas azotoformans* Strain NBRC 12693 16S ribosomal RNAgene partial sequence	97%	98%
DK-2b3	*Pseudomonas azotoformans* StrainNBRC 12693 16S ribosomal RNA gene partial sequence	99%	99%
SB-3a2	*Pseudomonas azotoformans* Strain NBRC 12693 16S ribosomal RNAgene partial sequence	99%	99%

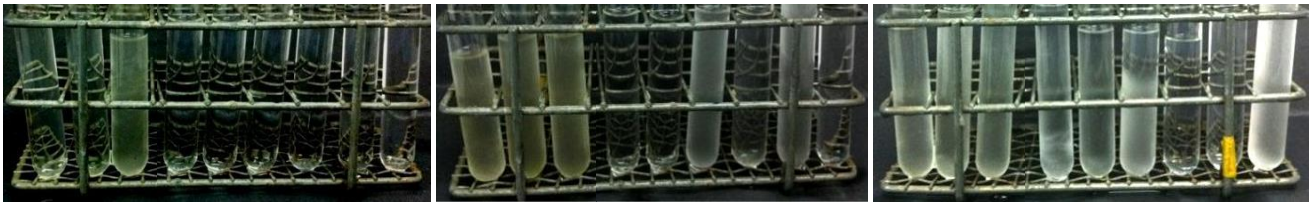


Figure 4. Freeze-positive tube in the MPN test. Note: A. Tube combination 1-0-0; B. 3-1-1 tube combination; C. 3-3-1 tube combination

Several researchers have carried out several studies on estimating the population of INA bacteria using the Thomas formula series 3.3.3. Hirano et al. (1985) stated that the optimum temperature for the estimation test was -5ºC. This study is supported by a test carried out by Baertlein et al. (1992) and Cazorla et al. (1995). At a temperature of -5ºC, it was sensitive enough to detect the activity of ice core formation by INA bacteria. In several other studies conducted by Lindow et al. (1982), Hirano and Upper (1986), and Olive and McCarter (1988), it is stated that a temperature of -5ºC for routine testing in determining the activity of INA bacteria is the right and optimum temperature. From the research conducted, each sampling station was found to have frozen positive tubes (Figure 4).

The bacterial population was estimated by matching the results obtained with the MPN table according to the Thomas series 3.3.3 formula with the number of frozen test tubes. The MPN method assumes that a frozen tube contains at least one ice core (Oblinger and Koburge 1975; Govindarajan and Lindow 1988). 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, the MPN value can generally be interpreted as an estimate of the number of individual bacteria. The obtained freezing series were then matched with the MPN table and calculated for different MPN results from each retrieval station (Table 5).

Based on the tests carried out, the results were that freezing occurred at all sampling stations. In sampling station 1, namely Bakal Village (1,800 m asl), the number of frozen positive tubes with a combination of 1-0-0 was then matched to the MPN table, and a calculation was made, showing the results of 1.44 x 102 MPN/gram. In sampling station 2, namely Diengkulon Village (2,000 m asl), the number of frozen positive tubes with a combination of 3-1-1 was then matched to the MPN table, which shows the results of 3.00 x 103 MPN/gram. In sampling station 3, namely Sembungan Village (2,200 m asl), the number of frozen positive tubes with a combination of 3-3-1 was then matched with the MPN table, which showed the results of 1.842 x105 MPN/gram.

Based on the research, the following conclusions were obtained: (i) INA bacteria could be isolated from potato plants in Batur District, Dieng, as many as six isolates. All isolates were found at all sampling stations, namely Bakal Village (station 1: 1,800 m asl) with three isolates, Diengkulon Village (station 2: 2,000 m asl) with two isolates, and Sembungan Village (station 3: 2,200 m asl) with one isolate. (ii) The INA bacterial species found in potato plants in Batur District, Dieng were *A. sulfonivorans* in sample BK-1a5 (98%), *C. luteum* in sample BK-1a6 (98%), *A. sulfonivorans* in sample BK-2a2 (98). %), *P. azotoformans* in sample DK-1b1 (98%), *P. azotoformans* in sample DK-2b3 (99%) and *P. azotoformans* in sample SB-3a2 (99%). (iii) The population of INA bacteria on potato leaves in Batur District, Dieng Plateau was 1.44 x 102 MPN INA/gram in Bakal Village, 3.00 x 103 MPN INA/gram in Diengkulon Village, and 1.824 x 105 MPN INA/gram in Sembungan Village.

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