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Isolation and Identification of Ice-Nucleating-Active Bacteria from Indonesian Edible Leafy Plant *Poh-Pohan* (*Pilea glaberina*)

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Two ice-nucleating-active (INA) bacteria (isolates C and 6) were isolated from *poh-pohan* (*Pilea glaberina*), an Indonesian edible leafy plant (*lalaban*). The maximum nucleation temperature of aqueous suspensions of the two isolates is -5 °C. They were classified as a type II ice nucleator. Microscopic and morphological determination showed that these isolates had yellow pigmentation, rod shape, and were Gram negative. Biochemical analysis indicated that the isolates were exhibited catalase activity, but negative in oxidase and indole assays. DNA sequencing of 16SrRNA gene of isolate A3 showed a 94% similarity to *Pseudomonas* sp. while isolate A4 showed a 97% similarity to *Xanthomonas campestris*. To our knowledge, this is the first report of INA bacteria isolated from a tropical edible leafy plant.

Keywords: Ice-nucleating bacteria, Indonesian edible leafy plant, *Pilea glaberina*

Leaf microbial communities of leaves are diverse and include many different genera of bacteria, filamentous fungi, yeasts, algae, and less frequently, protozoa and nematodes (Andrews and Harris 2000) Bacteria are by far the most abundant inhabitants of the phyllosphere and ephiphytic bacterial populations differ sharply in size among and within plants of the same species (Lindow *et al.* 1978). One of the bacterial components of leaf microbial communities are ice-nucleation-active (INA) bacteria (Lindow and Brandl 2003).

These bacteria have been shown to incite frost damage to some plants (Hirano and Upper 2000). The bacteria commonly found on plants comprise strains of several species (such as *Pseudomonas syringae* and *Erwinia herbicola*) which produce a protein able to induce ice nuclei at temperatures as high as -2°C. Leaf surface populations of these bacterial species limit supercooling in the plant parts on which they reside by initiating damaging ice formation at temperatures of -2 to -4°C. Since plants do not have intrinsic ice nuclei active at these temperatures, these bacteria play an important role in initiating frost damage (Lindow *et al.* 1978). While data of the presence of INA bacteria on the leaf surface are commonly found from subtropical areas, little is known about the presence of these bacteria in tropical areas. Riupassa *et al.* (2005) investigated the abundance of phyllosphere bacteria in a number of Indonesian edible leafy plants (*lalaban*) which could reach up to 3×10^8 CFU/g. In this study we are interested to focus on the isolation and identification of ice-nucleating active bacteria from an Indonesian edible leafy plant *poh-pohan* (*Pilea glaberina*).

MATERIALS AND METHODS

Sampling Technique. Isolations of INA bacteria were done from many edible leafy plants such as *poh-pohan* (*P. glaberina*), *selada* (*Lactuca sativa*), *kemangi* (*Ocinum sanctum*), and *taoge kacang hijau* (*Vigna radiata*). Samples were placed in sterile plastic bags and plated immediately upon return to the laboratory.

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Samples of 2 grams of leaves were cut into pieces and washed with sterile distilled water 3x. Samples were placed in sterile tubes containing 10 mM phosphate buffer (pH 8.0), vortexed for 2 minutes and then diluted to 100 fold. The suspensions were spread on King's B medium agar and incubated at room temperature (28-30°C) for 2 days.

Detection of INA Colonies by Replica Freezing Assay. Colony growth from the isolations were picked and prepared for ice-nucleation assay by the method of Lindow *et al.* (1978). *Escherichia coli* DH5α carrying pJL1703 was used as a positive control. Ice nucleation activity was assessed with the drop-freezing assay. Frequencies of the ice nucleation activity of the isolates were measured using the formula described by Vali (1971).

$$N(t) = (- \ln f)/V,$$

where N (t) is the nucleation frequency at temperature T, f is the proportion of droplets unfrozen and V is the volume of individual droplets.

Biochemical Tests. Biochemical properties of these isolates were determined for indole, catalase, and oxidase assays.

PCR Amplification, Cloning, and Sequencing of 16S rDNA. Genomic DNA was extracted and purified using the Wizard Genomic DNA Purification Kit (Promega, USA). The extracted DNA was used as a template for PCR amplification of the 16S rRNA gene using 63f (5'-CAGGCCTAACACA TGCAAGTC-3') primer and 1387r primer (5'-GGGCGGWGTG TACAAGGC-3') (Marchesi *et al.* 1998). The reactions were performed in a volume of 25 µl containing 1 µl of DNA template, 1 µl of each primer (25 pmol/µl) (Research Biolabs), 0.5 µl of dNTP mix (Reseearch Biolabs) and 0.5 µl (2.5 U) of *Taq* polymerase, 2.5 µl of 10 x buffer (New England Biolabs, Mass. USA). The total volume was adjusted to 25 µl with sterile double-distilled water. The following cycles were applied for PCR: 94°C for 5 minutes then 25 cycles: 94°C for 30 seconds denaturation, 55°C for 30 seconds annealing and 72°C for 1 minutes elongation. After the amplification, an 8 µl of amplified DNA was mixed with 3 µl of loading buffer and then loaded onto 1% (w/v) gel electrophoresis (Mini-Sub

Cell GT; Biorad, Richmond, CA) which was run for 60 minutes at 55 V prior to visualization using a UV transiluminator. The resulting amplicons were purified using a *QIAquick PCR Purification Kit* (Qiagen, Germany) and then cloned using the pGMT *easy vector System I* (Promega, USA) and transformed into *E. coli* DH5á. *E. coli* transformants were selected on LB agar supplemented with X-gal (40 µg/ml). Recombinant plasmids were isolated using *Plasmid Wizard Plus SV Minipreps DNA Purification System* (Promega, USA). The cloned amplicons were sequenced (ABI Prism 3130x genetic analyser) on both strands using M13 reverse and forward primers. The sequences were compared to GenBank sequences by using a standard nucleotide basic local alignment search tool (BLAST) search.

RESULTS

We recovered two isolates of bacteria (isolate A3 and A4) which showed ice-nucleation activity from *poh-pohan* (*P. glaberina*) (Fig 1). The ice-nucleation-activity of isolate A4 is higher than isolate A3 with an ice-nucleation frequencies of 59.9/ml and 37.9/ml respectively (Table 1). Microscopic and morphological determination showed that these isolates had yellow pigmentation, rod shape and were Gram negative. Biochemical analysis indicated that the isolates were exhibited catalase activity, but negative in oxidase and indole assays. DNA sequencing of 16SrRNA gene of isolate A3 showed a 94% similarity to *Pseudomonas sp* while isolate A4 showed a 97% similarity to *Xanthomonas campestris*. The DNA sequences have been submitted to Genbank with the accession number EU563218 for isolate A3 and EU531514 for isolate A4.

DISCUSSION

There are two isolates (A3 and A4) from *poh-pohan*, Indonesian edible leafy plant, which were identified as ice-nucleation bacteria. These two isolates are categorized as type two ice-nucleation bacteria, having the maximum nucleation temperature of aqueous suspensions at -5°C. Turner *et al.* (1990) classified ice nucleation proteins into three classes: class A which showed the ice nucleation activity between temperature -2°C to -5°C, class B within the temperature range of -5°C to -8°C, while class C is below -8°C. Based on that system, these two isolates were categorized as class B.

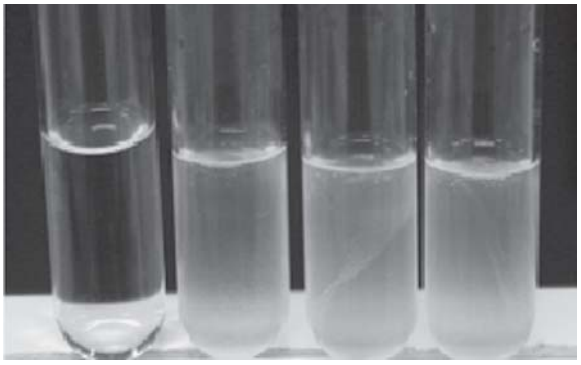

Fig 1 Assay of ice nucleation activity. a, negative control; b, positive control *E.coli* DH5α carrying pJL1703; c, isolate A3; d, isolate A4.

Table 1 Ice-nucleation activity of bacteria form *poh-pohan* (*Pilea glaberina*)

Isolates	*f*	N(t) (-8 °C)
A3	17/20	37.9/ml
A4	19/20	59.9/ml

N(t) = Ice-nucleation activity per ml.

The presence of INA-bacteria on leaf surfaces could alter leaf habitats at subzero temperatures. Frost-sensitive plants are injured when ice forms within plant tissues. Without ice formation, such injury does not occur, and perhaps many ice-nucleation genes of INA-bacteria recovered from tropical leaf might not being expressed. This finding might raise question such as: What is the role of these bacteria on tropical leaves?; are these bacteria transmitted from subtropical habitats?; how is the distribution on plants in tropical areas?

From the measurement of ice-nucleation frequency (Table 1), isolate A3 have ice-nucleation activity of 37.9/ml, while isolate A4 is 59.91/ml. Isolate A4 showed higher ice-nucleation activity than A3. Identification of the isolates through PCR amplification, cloning and DNA sequencing indicated that isolate A3 showed a 94% similarity to *Pseudomonas* sp. while isolate A4 showed a 97% similarity to *Xanthomonas campestris*.

Loper and Lindow (1994) reported that the ice nucleation protein from *Pseudomonas* sp. was classified as IceC. While the ice nucleation protein from *X. campestris* was InaX (Turner *et al*. 1990; Edwards *et al*. 1994). However, all of the publication data were derived from subtropical areas. To our knowledge, this is the first finding of INA bacteria from a tropical edible leafy plant.

Finding of *Pseudomonas* sp. and *X. campestris* on *P. glaberina* was interesting and we need more studies on characterization of the activity of the protein and their role in their natural habitats.

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