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Identification and characterization of three endophytic bacteria from *Neesia altissima* (*Malvaceae*) antagonistic to diarrhea-causing bacteria

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ABSTRACT

Aims: Diarrheal disease is one of serious healthcare problems in developing countries. Endophytic bacteria have been known as a promising source of new antibiotics against susceptible and resistant forms of microorganisms. In this study, we identified three endophytic bacteria isolated from *Neesia altissima* and screened their antagonistic activity against diarrhea-causing bacteria in order to find new potential secondary metabolites.
Methodology and results: Samples of *N. altissima* were collected from mount Halimun-Salak national park. Endophytic bacteria were isolated from roots, barks, and fresh leaves of *N. altissima* by surface sterilized method. Screening of antagonistics activity was conducted against five diarrhea-causing bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Shigella flexneri*, and *Bacillus cereus* using crude extract dilution and diffusion disc methods. Three endophytic bacteria showed antagonistic activity against the pathogenic bacteria. Identification of the three potential endophytic bacteria using molecular analysis showed that two isolates determined as *Pseudomonas aeruginosa* and one isolate belongs to *P. azotoformans*.
Conclusion, significance and impact study: Crude extract of *P. aeruginosa* (strain 1.4.1A and 1.2.7D) and *P. azotoformans* (strain 1.8.7KB) showed growth inhibition activity to the diarrhea-causing bacteria. This is the first report of *P. azotoformans* exhibited antagonistic activities against diarrhea-causing bacteria. This data suggested that bacterial endophytes from *N. altissima* provided potential sources for the discovery of new secondary metabolites to combat the diarrhea-causing bacteria. This finding highlights potential prospects of endophytic bacteria utilization from endemic medicinal plants for the discovery of novel bioactive compounds.

Keywords: anti-diarrheal, endophytic bacteria, 16S, *Neesia altissima*, phylogeny

INTRODUCTION

Diarrheal disease is an important health problem worldwide, especially in developing countries. The disease is mainly caused by bacteria due to poorly of sanitary water, bad sanitation, and hyginization. The disease causes mortality of over 5-8 million infants and children (under 5 years old) every year in developing countries (Bodhidatta *et al.*, 2010).

According to the morbidity survey carried out by the Ministry of Health of Indonesia in 2003, about 200-374 diarrhea cased / 1000 residents occurred in 2003, and outbreak diarrhea were reported from 16 provinces in 2006 (WHO, 2004). Various bacteria have been recorded as causal agents of diarrhea. These include *E. coli*, *S. aureus*, *Salmonella* spp., *Shigella* spp., and *B. cereus*. Particular bacterium such as *S. aureus* has become problematic due to their resistance to available commercial antibiotics. Therefore, the World Health Organization (WHO) had initiated diarrhea disease control program to study traditional medical practices and other related aspects (Akuodor *et al.*, 2010). Although newer therapeutic agents such as daptomycin and linezolid have entered the clinical area (Levy and Marshall, 2004), the need for the discovery and development of newer antimicrobial agents are still important to combat resistant bacteria (Wenzel, 2004; Nathwani, 2005).

Microbial endophytes in tropical areas are relatively unstudied and unexplored particularly in relation to their potential use in medicine, agriculture, and another industries. Several reports found that the endophytic bacteria provide vast potential in producing various novel

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natural products, including secondary metabolites similar to their hosts (Castillo *et al.*, 2002; Castillo *et al.*, 2003; Castillo *et al.*, 2006; Mehanni and Safwat, 2010; Ding *et al.*, 2010; Ding *et al.*, 2011). Ecomycins, pseudomycins, munumbicins, xiamycins, and kakadumycins are examples of the novel antibiotics produced by endophytic bacteria (Ballio *et al.*, 1994; Castillo *et al.*, 2006). Two of these antibiotics, *viz*, the munumbicins and xiamycins were proved being effective against vancomycin-resistant *Enterococcus faecium* (VREF) (Kauffman, 2003) and MRSA (Dancer *et al.*, 2003). These showed that endophytic bacteria are promising source of new antibiotics against susceptible and resistant forms of various infectious microorganisms.

Neesia altissima Bl. is a large tree (grows up to ± 40 m) and distributed primarily in the rainforest of Malaysia and Indonesia (Sumatera, Borneo, and Java islands). In Indonesia, this endemic plant is used medically for treatment of gonorrhea, diuretic, and diarrhea (Rahayu *et al.*, 2002). Although having such an important medicinal value, studies on microbial endophytes from *N. altissima* in relation to discovery of alternative secondary metabolites are lacking. Therefore, it is important to explore endophytic bacteria from endemic medicinal plant such as *N. altissima*, and screened their potential in producing antimicrobial compounds. In this paper, we isolated endophytic bacteria from *N. altissima* and screened their potential against diarrhea-causing bacteria in order to find new potential sources of secondary metabolites. These bacteria were also characterized by molecular analysis based on nucleotide sequence generated from 16S rRNA region.

MATERIALS AND METHODS

Microorganisms

Escherichia coli ATCC 25922, *S. aureus* ATCC 25923, *S. typhimurium* ATCC 25241, *S. flexneri* ATCC 12022, and *B. cereus* ATCC 10876 were selected as infectious microorganisms in this study.

Sample collection and selective isolation of endophytic bacteria

Roots, stems, and leaves of *N. altissima* were collected from Halimun-Salak Mount, Bogor, West Java, Indonesia. The samples were kept in sterile sampling bags and processed immediately after arriving in the laboratory. Surface sterilization was performed for each sample to ensure the elimination of surface microorganisms. The samples were washed in running water to remove soil particles, and were surface sterilized by sequential immersion in 70% ethanol for 5 min and a solution of sodium hypochlorite (NaOCl) (0.9% available chlorine) for 20 min. The samples were further washed three times in sterile distilled water to remove surface sterilizing agents before being soaked in 10% $NaHCO_3$ solution to disrupt the plant tissues and to inhibit the growth of fungi (Cao *et al.*, 2005). Each sample was divided into small fragments (1-3 cm) under aseptic conditions and was plated on Nutrient Agar (NA). The NA plates were incubated at 37 °C for up to 18-24 h. Endophytic bacterial strains growing on media were purified and were preserved on NA slants for further studies.

Antibacterial activity assay

Antibacterial activity was evaluated on Mueller Hinton Agar (MHA) medium by the streak method against five selected diarrhea-causing bacteria The cultures were incubated in a rotary shaker at 37 °C for 18-24 h. After the cultivation, an aliquot of 15 mL of each pre-inoculum was transferred to 500 mL Erlenmeyer flask. The pellet containing bacteria cells remained in ethyl acetate and vapored with rotary evaporator for crude extract (Bauer *et al.*, 1996). Crude extract of metabolite compounds were assayed on NA medium in Petri dishes to determine the MICs of these compounds against the pathogenic bacteria using the diffusion and dillution methods (Elson *et al.*, 1994).

In the dillution method, the compound (0.3 mg) was dissolved in DMSO (300 µL), diluted serially in the same solvent, and added to NB medium at 48 °C. The medium (5 mL) was added to a 5 cm diameter petri dish. The final concentrations were set at 25.10^4 ppm, 5.10^4 ppm, 1.10^4 ppm, 5.10^3 ppm, 1.10^3 ppm, 500 ppm, 250 ppm, 125 ppm, 100 ppm, 62.5 ppm, 31.25 ppm, 15.625 ppm and 7.8125 ppm while in the diffusion method, a 6 mm diameter disc was placed on NA plates. 15 µL of compounds was dropped on the each of disc. The experiment was repeated twice. The antibacterial activity was confirmed by the visualization and measurement of inhibition zones.

Characterization and molecular identification of potential endophytic bacterial strains

The selected endophytic bacteria strains were identified based on molecular phylogenetic analysis of the nucleotide sequence generated from 16S rRNA region in combination with morphological, biochemical and physiological characteristics. In morphological characterization, macroscopic and microscopic features of the selected isolates were studied, additionally an array of biochemical and physiological tests including catalase test, oxidase test, starch utilization test, nitrate reduction test, motility test, and sugar utilization test were performed following the methods described by Gerhardt *et al.* (1994).

In molecular analysis, bacterial genomic DNA was obtained by using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan). A total 25 µL of PCR mix was prepared as follow: 1.25 µL of 10 µM 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primer pairs, 2 µL (10-100 ng) of DNA template, 25 µL Go Taq® master mix (Promega, USA) and 20.5 µL ultra pure water DNA/RNAse free. PCR reaction was conducted using Thermalcycler (Takara Shuzo Co. Ltd., Shiga, Japan)

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according to the following setting: pre-denatu ration at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec, and extension at 72 °C for 1.5 min. After completing 30 cycles, post-extension was conducted at 72 °C for 10 min and cooling down at 4 °C for 30 min. PCR products were run in 1% agarose gel by electrophoresis at 100 V for 30 min, and soaked in ethidium bromide (EtBr) for 15 min. Gel was then visualized using Gel Doc™ XR system (BIO-RAD, Germany). PCR products were sent to 1stBASE (Malaysia) for sequencing.

Nucleotide sequence obtained from the respective primer, 27F and 1492R, were assembled in Chromas Pro 1.41 (Technelysium Pty Ltd., Australia). The sequences were aligned with sequences retrieved from DNA databases (DDBJ, NCBI) using MUSCLE (*MUltiple Sequence Comparison by Log-Expectation*) (Edgar, 2004) in MEGA 6 (*Molecular Evolutionary Genetics Analysis*) (Tamura *et al*., 2013). *Escherichia coli* strain K-12 (AB681728) was assigned as outgroup. GenBank accession number, strain code, and taxon name used in this study are given in figure. Phylogenetic analysis was conducted using the neighbour joining (NJ) method implemented in MEGA 6. Maximum composite likelihood was used as the substitution model for the current analysis. Strength of the internal branches of the phylogenetic trees was tested with bootstrap (BS) analysis (Felsenstein, 1985) using 1000 replications. Other parameters used in the NJ analysis were selected according to the default standard in MEGA 6 software. Bootstrap values of 50% or higher were shown. Tree generated from NJ analysis was edited in TreeGraph version 2 (Stöver and Müller, 2010). GenBank accession number, sequence name and strain code used in the phylogenetic analysis were showed in Figure 1.

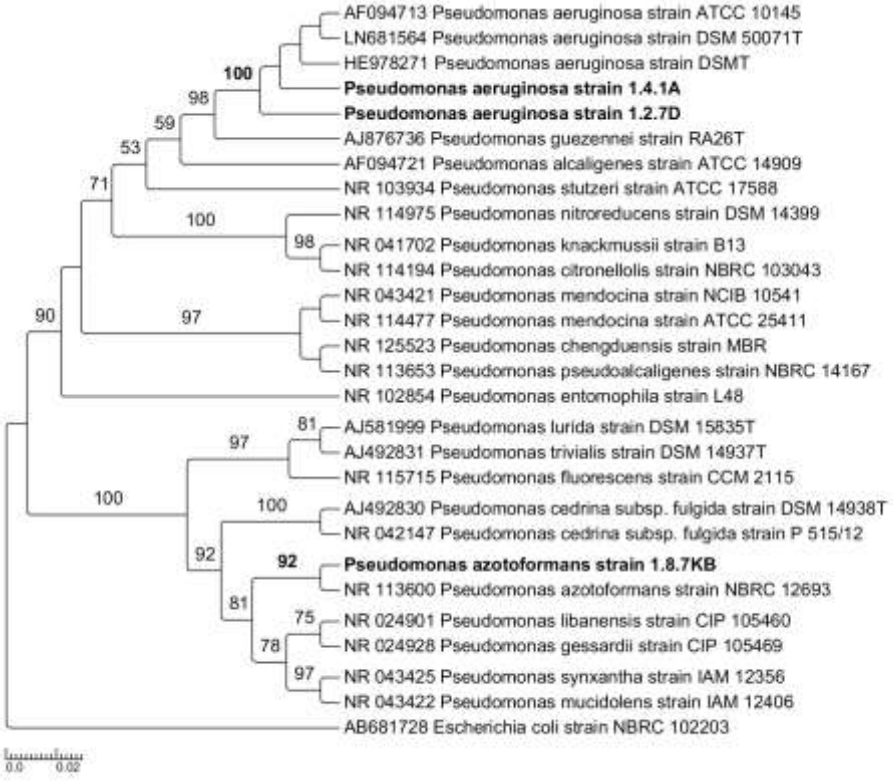

Figure 1: Phylogenetic tree of three endophytic bacteria isolates from *N*. *altissima* reconstructed by neighbor-joining (NJ) method. Bootstrap values greater than 50% from 1,000 replication bootstrap were showed at the nodes. *Escherichia coli* strain NBRC102203 was used as an outgroup.

RESULTS AND DISCUSSION

Isolation of endophytic bacteria from *N. altissima*

The microecology of microbial endophytes indicates that they occupy intercellular spaces of plants (Bacon and Hinton, 2002). In this study, a total 185 endophytic bacteria were isolated from leaves (104 or 56.21%), roots (30 or 16.21%), and stem barks (51 or 27.56%) of *N*. *altissima*. This showed that majority of endophytic bacteria were distributed inside the leaf tissue rather than other organs. Our study also found that the occurrence of endophytic bacteria inside the older leaf tissue (90%) is higher than younger leaf (10%). This data is in good agreement with other studies on endophytic microorganisms of which showed higher diversity of

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endophytes living in older leaves (Araújo *et al.*, 2000). This condition is probably occurred due to several factors, such as biochemistry of leaves at different ages (Fernandez *et al.*, 2011), chemical protection of younger leaves from alien organisms (Coley, 1988), and longer exposure time of older leaves provides more biomass for endophytes growth (Wilson and Carroll, 1994).

In contrast to this result, bacterial endophytes diversity was usually found highest in the roots and decrease progressively from the stem to the leaves (Quadt-Hallmann and Kloepper, 1996; Lamb *et al.*, 1996). With the exception of seed transmitted bacteria, bacterial endophytes must first colonize the root surface prior to entering the plant. Once inside the plant tissue, endophytic bacteria remain localized in a specific plant tissue, such as the root cortex, or colonize the plant systematically by transport or active migration through the conducting elements or the apoplast (Hurek *et al.*, 1994; James *et al.*, 1994; Mahaffee *et al.*, 1997). Although bacterial endophytes from roots of *N*. *altissima* possess lower number of isolates, however, their roles in plant development and health could not be abandoned. Several reports refer to bacteria acting in plant protection against pathogens and the influence of their metabolic products on plant growth and physiology (Gupta *et al.*, 1995; Kolomiets *et al.*, 1997).

Antibacterial activity assay

Discovery of new secondary metabolites to combat resistant pathogens to existing medicines becomes important and priority (Strobel and Daisy, 2003). Endophytic bacteria, reside in healthy tissues of living plants, have emerged as potential candidate as sources of novel secondary metabolites due to their unique properties such as producing secondary metabolites similar to their hosts (Castillo *et al.*, 2006; Nimnoi and Pongslip, 2009). Of the 185 endophytic bacteria tested in the ethyl acetate assay, three endophytic bacteria isolates was capable in inhibiting the growth of *S. aureus*, *B. cereus*, *S. flexneri, S. typhimurium,* and *E. coli* (Table 1).

Table 1: Antimicrobial activity of crude extract from endophytic bacteria against pathogenic selected bacteria.

Bacteria isolates	*E. coli*	*S. aureus*	*S. typhimurium*	*S. flexneri*	*B. cereus*
P. aeruginosa 1.4.1A	+	+	+	+	+
P. aeruginosa 1.2.7D	+	+	+	+	+
P. azotoformans 1.8.7KB	+	+	+	+	+

+, inhibition; -, absence of inhibition

Table 2: The mean of inhibitory zone (mm) of antibacterial activity from the crude extract of endophytic bacteria.

Isolates	*E. coli*	*S. aureus*	*S. typhimurium*	*S. flexneri*	*B. cereus*
P. aeruginosa 1.4.1A	3.00	2.75	9.50	1.50	6.50
P. aeruginosa 1.2.7D	2.50	2.00	3.62	2.12	6.25
P. azotoformans 1.8.7KB	1.75	2.00	1.00	2.37	4.00

The crude extract from the three endophytic bacteria isolates showed that *P. aeruginosa* strain 1.4.1A exhibited highest antibacterial activity against *E. coli*, *S. aureus*, *S. typhimurium*, *B. cereus*; while *P. azotoformans* strain 1.8.7KB produced highest antagonism activity against *S. flexneri* (Table 2). Based on this result, the minimum inhibition concentration (MIC) assay from the crude extract *P. aeruginosa* strain 1.4.1A using diffusion method was further conducted at various concentrations. In this assay, concentration of 1000 ppm showed 12.2-12.7 mm inhibition zone against *S. aureus* (Figure 2), at concentration 5000 ppm showed 6.7-7.1 mm inhibition zone against *B. cereus* and at 100 ppm exhibited 1.1 mm inhibition zone against *S. flexneri*. This data suggested that *P. aeruginosa* strain 1.4.1A and *P. azotoformans* strain 1.8.7KB from *N. altissima* provided potential for production of secondary metabolites against the diarrhea-causing bacteria. They indicated promising capability to produce useful bioactive compounds.

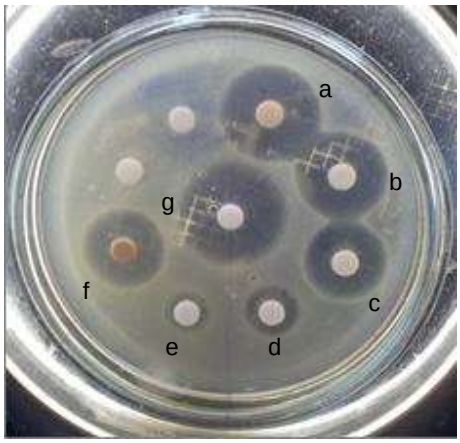

Figure 2: Inhibitory zone of *P. aeruginosa* strain 1.4.1A at various concentrations. a, 5.10^4 ppm; b, 1.10^4 ppm; c, 5.10^3 ppm; d, 1.10^3 ppm; e, 1.10^2 ppm; f, 25.10^4 ppm; g, K(+).

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Characterization and molecular identification of potential endophytic bacterial strains

The phylogenetic tree generated from 16S rRNA sequence showed that the sequence from all three potential isolates nested in the clade large clade of the sequences belonging to the genus *Pseudomonas* (Figure 2). Sequence of 1.4.1A and 1.2.7D nested in the clade containing the type strain of *P. aeruginosa* with 100% bootstrap support (BS), while sequence of isolate 1.8.7KB formed a monophyletic clade with *P. azotoformans* strain NBRC 12693 (92% BS). It is apparent that sequence of 1.4.1A and 1.2.7D belong to *P. aeruginosa* and sequence of isolate 1.8.7KB belongs to *P. azotoformans*. Phenotypic characterization showed that the optimum temperature for these strains is 37-42 °C. *Pseudomonas aeruginosa* (strain 1.4.1A and 1.2.7D) and *P. azotoformans* (strain 1.8.7KB) did not grow below 14 °C or above 45 °C. The colony characteristics between *P. aeruginosa* (strain 1.4.1A and 1.2.7D) and *P. azotoformans* (strain 1.8.7KB) were distinct. Colonies of *P. aeruginosa* (strain 1.4.1A and 1.2.7D) produced white or blue-green fluorescent pigment with small and round structures on the NA medium, while the colonies of *P. azotoformans* (strain 1.8.7KB) exhibited brown to yellowish pigment, larger colony size, and knob-like structures (Figure 3). The biochemical and physiological characterization showed that all these bacteria exhibited positive results for catalase and oxidase tests (Table 3). Likewise, *P. aeruginosa* (strain 1.4.1A and 1.2.7D) showed positive results in motility test.

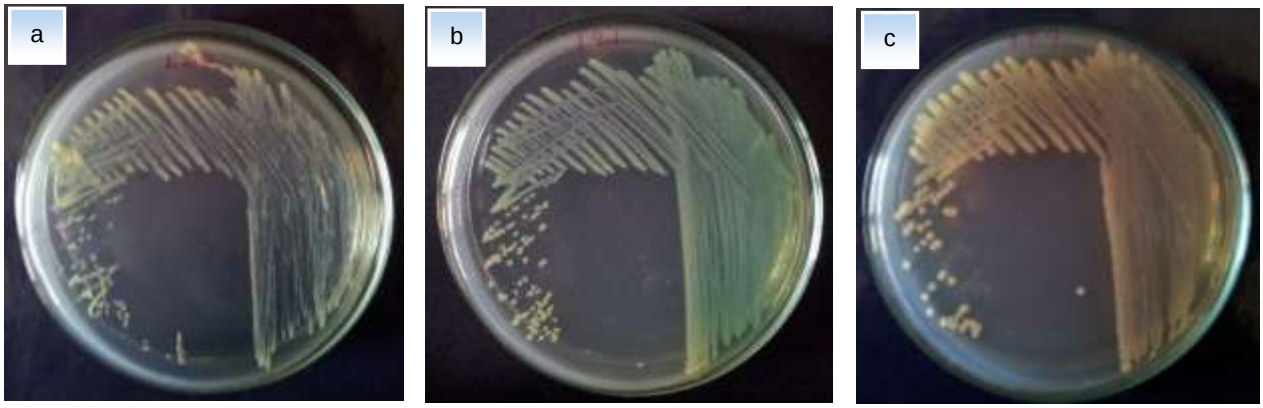

Figure 3: The colony characteristics of endophytic bacteria isolated from *N. altissima*. (a) *P. aeruginosa* strain 1.2.7D; (b) *P. aeruginosa* strain 1.4.1A; (c) *P. azotoformans* strain 1.8.7KB.

Table 3: Biochemical and physiological characteristics of the selected endophytic bacteria.

	Bacteria isolates		
Biochemical Test	*P. aeruginosa* strain 1.4.1A	*P. aeruginosa* strain 1.2.7D	*P. azotoformans* strain 1.8.7KB
Indole	-	-	-
MR	-	+	-
VP	+	+	+
Catalase	+	+	+
O	+	+	-
F	-	-	-
Motility	+	++	-

++, strong positive; +, positive; -, negative

In relation to the endophytic bacteria community, members of *Pseudomonas* have frequently been found as dominant endophytic bacteria. Among common Gram positive and Gram negative bacterial endophytes such as *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Bacillus*, *Brevibacterium*, *Burkholderia*, *Chromobacterium*, *Curtobacterium*, *Enterobacter*, *Kocuria*, *Lysinibacillus*, *Methylobacterium*, *Microbacterium*, *Paenibacillus*, *Pantoea*, *Phyllobacterium*, *Pseudomonas*, *Pseudoalteromonas*, *Rahnella*, *Rhodanobacter*, *Stenotrophomonas*, *Actinomycetes*, *Streptomyces*, and *Xanthomonas*, members of *Pseudomonas* usually occupy about 40% of bacterial endophytes living in the plant tissues (Rashid *et al.*, 2012; Ma *et al.*, 2013; Sun *et al.*, 2013). *Pseudomonas* species, in general, have simple nutritional requirements and are readily isolated from a variety of environments. In the laboratory, they grow well in common bacteria medium (NA), at neutral pH, and at temperatures in the mesophilic range.

In the discovery of antimicrobial compounds, several endophytic *Pseudomonas* species were reported of producing antibacterial and antifungal agents for various human pathogenic bacteria and fungi (El-Deeb *et al.*, 2013). For example, root endophytic *P. putida* (Andreote

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et al., 2009) and *P. syringae* of which producing pseudomycins (Ballio *et al.*, 1994). Strains of *P. aeruginosa* were also frequently reported of producing antimicrobial compounds (El-Shouny *et al.*, 2011; Menpara and Chanda, 2013). Members of *P. fluorescens* group have also been known for their capability to produce antimicrobial compounds such as pseudomonic acid A (Mupirocin) (Fritz *et al.*, 2009; Matthijs *et al.*, 2014). Mupirocin is an antibiotic compound belongs to polyketide with broad antibacterial activity including strongly antagonistic activity against *S. aureus* (Matthijs *et al.*, 2014). Although *P. azotoformans* belongs to *P. fluorescens* group, however, the finding of *P. azotoformans* as antagonistic bacterium against several pathogenic bacteria in this study is probably new, because this species is indeed known as nitrogen fixation bacteria, and involved in bacteria-plant growth promotion system (Xie *et al.*, 2006). Therefore, it is important to determine the structure of antibacterial compounds produced by *P. azotoformans* in the further study.

CONCLUSION

Three endophytic bacteria isolated from *N. altissima* showed their antagonistics activity against diarrhea-causing bacteria, include *E. coli*, *S. aureus, S. typhimurium, S. flexneri,* and *B. cereus*. Based on phylogenetic analysis of nucleotide sequence generated from 16S rRNA region, two isolates determined as *P. aeruginosa* and one isolate belongs to *P. azotoformans*.

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