Biosaintifika 9 (3) (2017) 600-607

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Endophytic Actinobacteria from *Rhododendron* spp. as an Antibacterial Agent

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DOI: 10.15294/biosaintifika.v9i3.10323

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History Article

Received 16 July 2017
Approved 30 November 2017
Published 31 December 2017

Keywords

Actinobacteria; Antibacterial activity; Endophyte; Rhododendron spp.

Abstract

Rhododendron has been known to treat various diseases including diarrhea, but diversity and potency of its endophytic actinobacteria have not been studied. The objectives of this research were to explore the existence of endophytic actinobacteria from *Rododendron* spp. and assessed their antibacterial activity, as an effort to control the growth of bacterial pathogen resistant to some antibiotics. The endophytes were isolated from *Rhododendron* spp. using HV medium, and purified in the ISP2 medium. The antibacterial activity was assayed against Enteropathogenic *Escherichia coli* (EPEC) K1.1 resistant to ampicillin and *Bacillus pumilus*. The Minimum Inhibitory Concentration (MIC) value, macroscopic and microscopic were examined. Twenty-three of endophytic actinobacteria were successfully isolated from 7 *Rhododendron* species. Two of them, i.e., RJkb1 and RJkb3 isolates, had high antibacterial activity, with 17.2 mm and 14.5 mm inhibition zone against EPEC K1-1, respectively; and 12.4 mm and 16.1 mm inhibition zone against *B. pumilus*, respectively. The highest antibacterial activity for both RJkb1 and RJkb3 isolates was achieved at day 15, at 28 °C. At 250 µg/mL to 1750 µg/mL either RJkb1 or RJkb3 supernatant showed no activity against EPEC K1-1. The MIC value against *B. pumilus* was at 1250 µg/mL for both tested isolates. Under an electron microscope observation, cell morphology of the treated *B. pumilus* showed elongated cells and viewer in cell number, compared with the untreated one. From this work, the existence of endophytic actinobacteria from *Rhododendron* spp. and their antibacterial activity contributes to the understanding of their diversity and potency as an antibacterial agent.

How to Cite

Fitriandini, R., Budiarti, S., & Lestari, Y. (2017). Endophytic Actinobacteria from *Rhododendron* spp. as an Antibacterial Agent. *Biosaintifika: Journal of Biology & Biology Education*, 9(3), 600-607.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Actinobacteria are gram-positive bacteria that commonly live in soil and also associated with plants as endophytes. Actinobacteria are known to produce bioactive compounds with various biological functions. From 16500 antimicrobial compounds isolated from microbes, 52.7 % (8700 antimicrobials) were from Actinobacteria, while the remaining 29.7% and 17.6%, respectively, were isolated from fungi and bacteria (Bérdy, 2005). Actinobacteria from tropical regions like Indonesia have also proved to have antimicrobial potency (Hidayati *et al.,* 2013, Sipriyadi *et al.,* 2016). Most of the reported data were based on actinobacteria with soil origin, while antimicrobial activity from endophytic actinobacteria is rarely reported.

Rhododendron spp. is generally cultivated as an ornamental plant, but it can also be used as a medicinal plant. Bioactive compounds produced by *Rhododendron* plant was reported to function as an antioxidant due to its flavonoid activity *(Jung et al.,* 2007). Moreover, Rezk *et al*. (2015) had shown that the leaves methanol crude extract for 17 species of *Rhododendron* spp. inhibited the growth of both gram-positive and gram-negative bacteria. Various types of bioactive compounds with diverse functions contained in plants, thought can also be produced by the endophytic microbes in the plant (Strobel & Daisy, 2003).

Endophytic bacteria are bacteria that live in plant tissues without causing harm, and they can be isolated from sterilized plant tissue surface or extracted from plant tissue (Hallmann *et al.,* 1997). The endophytic bacteria that colonize plant tissues obtain the nutrients and protection from the host plant (Hasegawa *et al.,* 2006). Furthermore, the endophytes may produce similar bioactivity as shown by their host plant. Endophytic actinobacteria were found in *Rhododendron* and had potency as an antimicrobial agent (Shimizu *et al.,* 2000). The existence of endophytic actinobacteria from *Rhododendron* spp. from Indonesian origin and their antibacterial activity have not yet been explored. This reported work focussed on investigating the presence of endophytic actinobacteria from *Rhododendron* spp. grown in rainforest conservation area, Cibodas, Indonesia. Their ability to inhibit the growth of diarrheal bacteria, such as *Enteropathogenic Escherichia coli* (EPEC) was assessed. As commonly known that diarrhea is one cause of death in Indonesia, which 31.4% of infants and 25.2% in toddlers (Kemenkes RI, 2014). The EPEC is the *E. coli* pathogen causing diarrhea in infants and toddlers (Nataro & Kaper, 1998). EPEC K1-1 isolated from the feces of diarrhea children were known to have resistance to ampicillin by producing the β-lactamase enzyme (Wahyuni, 2006). Other bacteria causing diarrhea is *Bacillus pumilus* (Kusmiatun, 2014). The high use intensity of antibiotics to treat the infection can lead to the emergence of bacterial resistance and may give negative impact on economic and social aspects. A way to control antibiotics resistant bacterial pathogen is by finding new antibacterial compounds that can control the resistance pathogens. This work aimed to explore the existence of endophytic actinobacteria from *Rhododendron* spp. and their potency as a producer of antibacterial compounds. The information from this study would be beneficial for the development of the microbial-based product to control the growth of pathogenic bacterial resistance to antibiotics in society.

METHODS

Rhododendron spp. were collected from Cibodas Botanical Garden, Indonesia, by placing in a plastic bag and stored at 4 °C until ready to be used. The endophytic actinobacteria were isolated from the stem and leave samples of collected *Rhododendron* spp. The isolation method was based on Coombs & Franco (2003). The amount of 100 µL sample extract was spread on to Humic Acid Vitamin (HV) medium and incubated at room temperature (28 °C) for four weeks. To ensure that the obtained isolates were endophytes, a last water immersion from the surface sterilization was also spread on HV medium. The colonies which appeared in HV medium were purified using International *Streptomyces* Project 2 (ISP 2) medium for 14 days at 28 °C.

The macroscopic observation was conducted based on the color of aerial and substrate mycelia as well as the pigmented medium. The spore chain type was observed under a light microscope at 400x magnification.

Screening of antimicrobial was performed by using agar plug method in a double layer Nutrient Agar (NA) medium. Actinobacterial colonies were cut ± 6 mm in diameter, and placed on NA medium containing 10^6 cfu/mL of EPEC K1.1 or *B. pumillus*, and incubated for 24 hours at 36 ± 1°C. Antimicrobial activity was observed by a clear zone around the tested isolates, and the inhibitory activity was obtained by measuring the diameter of a clear zone around the strains. The strains that have high inhibition activity were selected for further antibacterial activity assay.

The selected endophytic actinobacteria

were grown on ISP2 agar medium, ± 6 mm diameter of the colony was inoculated on ISP2 liquid medium (1 inoculum in 30 mL ISP2 liquid medium). The culture was placed on an incubator shaker, at 120 rpm, incubated at 28 °C for 5, 10, 15 and 20 days. For each of incubation period, the culture was centrifuged at 6000 rpm for 20 min at 4 °C. The collected supernatant was tested for its inhibitory activity against EPEC K1-1 and *B. pumillus* using a Kirby-Bauer method (Bauer *et al.,* 1966). Paper disc with 6 mm in diameter was added with 15 uL of supernatant and then placed on the Nutrient Agar (NA) medium containing bacterial target (10^6 cfu/mL), and incubated at a temperature of 36 ± 1 °C for 24 hours. The ISP2 liquid medium used as a negative control.

Supernatant at various concentrations, i.e., 1750 ppm, 1500 ppm, 1250 ppm, 1000 ppm, 750 ppm, 500 ppm, 250 ppm were tested against EPEC K1-1 and *B. pumilus*. Nine milli litter of bacterial inoculum (10^6 cfu/mL in Nutrient Broth, NB) was placed into the tubes.Then each tube was added with the above concentrations of supernatant of selected endophytic actinobacteria. Cultures were incubated for 24 h at 36 ± 1 °C C. The number of living bacterial cells was calculated by spreading the 0.1 mL treated culture on NA medium, and incubated for 24 h at 37 °C. The colonies that appeared on the medium were calculated. The lowest concentrations were determined by the lowest number of colonies grown in the culture medium, which then considered as the Minimum Inhibitory Concentration (MIC).

Morphological observation of the treated bacterial cell target was conducted by using an electron microscope, JSM-5000 models. Treated bacterial cells in the microtube were centrifuged to separate the cell and supernatant. The supernatant was discarded, and then the pellets were added with 2% glutaraldehyde and soaked for a few hours. The glutaraldehyde was then separated by centrifugation, removed and subsequently, the cacodylate buffer solution was added to the sample. After soaking the sample for 10 min, the solution was centrifuged and removed by soaking with 1% osmium tetroxide for 1 h. Samples were re-centrifuged then the solution was discarded, followed by soaking with 50% alcohol for 10 min, then added 70%, 80%, 95% and 99% alcohol, with each immersion time for 10 min. Alcohol was removed by centrifugation, then adding the t-butanol into the sample. Coverslip (cover glass cut to size 0.25 cm^2) washed with absolute alcohol and the bacterial suspension was applied on it after the cover glass dry. Further, samples were frozen drying and coated with Au ion. All phases of centrifugation were performed at a speed of 4,000 x g for 5 min at 4 °C.

RESULTS AND DISCUSSION

In our work, there were 23 of endophytic actinobacteria which were successfully isolated from 7 *Rhododendron* species, which many of them occupied in stems compared with roots. Sardi *et al*. (1992) reported that actinobacteria commonly found in soil, some of them can enter and reside in the plant parts, and can be more dominant on the closer plant parts to the soil, especially live in root tissue.

The endophytic actinobacteria from *Rhododendron* spp. showed to diverse in morphological characteristic based on the color of aerial mycelium, substrate mycelium, and spore chain type (Table 1). The various morphological colonies, when grown on ISP2 medium for 14 days, can be seen in Figure 1.

There were two endophytic actinobacteria, amongst the 23 isolates that were selected based on their potential inhibitory activity against EPEC K1-1 and *B. pumilus*. The selected isolates were RJkb1 and RJkb3, with 17.2 mm and 14.5 mm inhibition zone against EPEC K1-1, respectively; and 12.4 mm and 16.1 mm inhibition zone against *B. pumilus*, respectively. The antibacterial activity is indicated by a clear zone around the isolates as can be seen in Figure 2.

The level of inhibition produced by both RJkb1 and RJkb3 was categorized as medium level. Inhibition level is obtained by calculating inhibition zone minus diameter of the actinobacterial colony. Inhibition level of 5 < 10 mm is considered weak category, 10 < 20 mm as a medium category, and > 20 mm as strong category (El-tarabily *et al.,* 2000).

The optimum time of both RJkb1 and RJkb3 to produce antibacterial compounds qualitatively indicated by the most significant inhibition zone, which in this experiment was found at 15 days incubation, either against EPEC K1-1 or *B. pumilus* (Figure 3).

Actinobacteria grow and form spores optimally at 10-14 days old culture. At that age of culture, the production of secondary metabolites was also increased. The production of secondary metabolites and sporulation occurred almost simultaneously in the early stationary phase (Bibb, 2005).

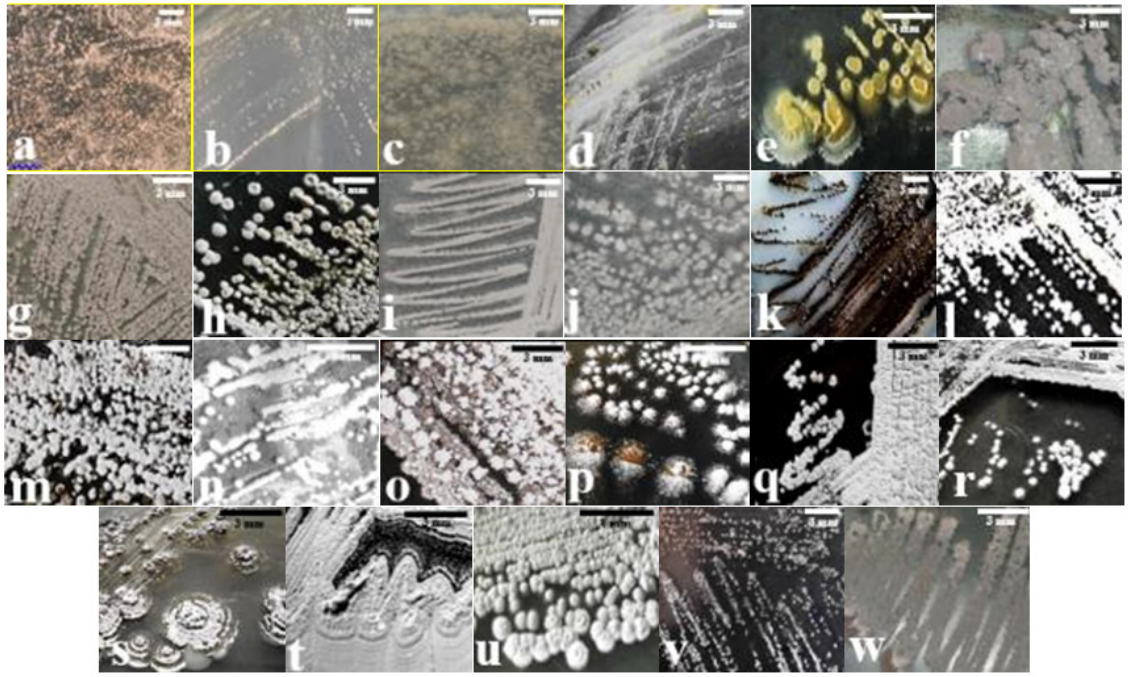

Figure 1. Diversity of morphological colony of endophytic actinobacteria with origin from *Rhododendron sp*. hybrid (*R. javanicum* and *R. sessilifolium*) (a) RJkb1, (b) RJkb2, (c) RJkb3; from *R. multicolour* Bengkulu (d) RMBd, (e) RMBb; from *R. javanicum* Jawa (f) RJvJwb1, (g) RJvJwb2, (h) RJvJwb3; from *R. javanicum* Jambi (i) RJvJmb1, (j) RJvJmb2, (k) RJvJmb3; from *R. sessilifolium* Sumatera Utara (l) RSSd1, (m) RSSd2, (n) RSSd3, (o) RSSd4, (p) RSSb; from *R. zoeleri* Papua (q) RZPd1, (r) RZPd2, (s) RZPd3, (t) RZPb1, (u) RZPb2, (v) RZPb3; from *Rhododendron* sp. Bengkulu (w) RSpB, grown for 14 days at ISP2 agar medium.

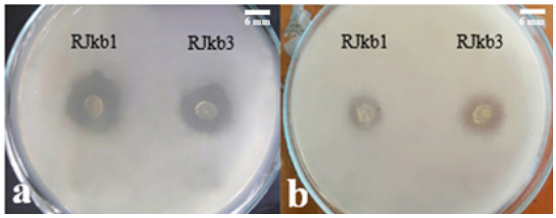

Figure 2. Inhibition activity of endophytic actinobacteria, RJkb1 and RJkb3: (a) against EPEC K1-1; (b) against *B. pumilus*

In the stationary phase, there is no increase or decrease in the number of microbial cells, so they form a balance condition. One of the causes of the stationary phase appears to be the limitation of the availability of a nutrient in the medium. The nutrient deficiencies can lead to accumulation of secondary metabolites inducer enzymes, releases the genes to synthesize secondary metabolites (Madigan *et al.,* 2009), and supports sporulation (Moat *et al.,* 2002).

The supernatant containing antibacterial gave smaller inhibition zone compared with direct antagonist colony test (Figure 4). Decreased in antibacterial activity may be caused by less secondary metabolite compounds in the supernatant, as the supernatant may also consist of various compounds including the medium. Hwang *et al*. (2001) had shown that increased supernatant concentration, may result in broader inhibition zone. However, for both RJkb1 and RJkb3, their supernatant showed no antibacterial activity against EPEC K1-1 (Figure 6a).

Determination of MIC was conducted to know the MIC value of antimicrobial compounds in the tested supernatant to inhibit the growth of the bacterial pathogen. The result showed that no antibacterial activity against EPEC K1-1 at 250 µg/mL to 1750 µg/mL, for both RJkb1 and RJkb3 as indicated by the abundance of colonies appeared in the tested medium (Figure 5).

They were many colonies which still able to grow, even at the highest concentration (1750 µg/mL) of the supernatant of both tested isolates, indicating that it could not inhibit the growth of EPEC K1-1. Gram-negative bacteria which are more resistant to antibacterial compounds may due to their complexity of cell structure, i.e., consisted of an outer layer form of lipoproteins, middle layer form of lipopolysaccharide, and an inner layer form of peptidoglycan (Silhavy *et al.,* 2010).

Table 1. Morphological characteristics of endophytic actinobacteria isolated from *Rhododendron* spp. grown for 14 days at ISP2 medium

Plants	Code of isolate	Colour of aerial mycelium	Colour of substrate mycelium	Spore chain type
Rhododendron sp. Hybrid West Java	RJkb1	Peach	Light brown	spira
	RJkb2	Light brown	Light brown	-
	RJkb3	White yellowish	White yellowish	-
Number of isolates	3			
R. multicolor Bengkulu	RMBd	White	White yellowish	-
	RMBb	Yellow	Cream	Biverticillus
The number of isolates	2			
R. javanicum West Java	RJvJwb1	Grey	Light brown	Spira
	RJvJwb2	Peach	Dark brown	Spira
	RJvJwb3	White	Dark brown	Spira
Number of isolates	3			
R. javanicum Jambi	RJvJmb1	Grey	Dark brown	Spira
	RJvJmb2	Grey	Dark brown	Spira
	RJvJmb3	Black	Dark brown	Monoverticillus
Number of isolate	3			
R. sessilifolium North Sumatera	RSSd1	White	White yellowish	Monoverticillus
	RSSd2	White	Dark brown	Monoverticillus
	RSSd3	White	Dark brown	Rectus
	RSSd4	White	Cream	Rectus
	RSSb	White	Cream	Rectus
Number of isolates	5			
R. zoeleri Papua	RZPd1	Grey	Yellow greenish	Spira
	RZPd2	Grey	Light brown	Spira
	RZPd3	Grey	Dark brown	Spira
	RZPb1	Grey	Dark brown	Spira
	RZPb2	Grey	Light brown	Spira
	RZPb3	Grey	Cream	Retinaculum apertum
Number of isolates	6			
Rhododendron sp. Bengkulu	RSPBd	Grey	Dark brown	Spira
Number of isolates	1			
	Total number of isolates			23

The antibacterial activity of the selected endophytic actinobacteria could inhibit the growth of *B. pumilus* with MIC value at 1250 µg/mL. As it can be seen in Figure 6, the addition of 1250 µg/mL concentration of RJkb1 and RJkb3 supernatant lowered the growth of *B. pumilus*. The plated samples only consisted of 30 cfu/mL and 20 cfu/mL, respectively.

The *B. pumilus* is a Gram-positive bacterium that more sensitive to antibacterial compounds. This phenomenon may be caused by the less complexity of cell wall compared with the Gram-negative bacteria like EPEC. The cell wall of Gram-negative bacteria consisted of a single-layered of cell wall structure, and this may make it easier for antibacterial compounds to enter the cell and to find targets for inhibiting bacterial growth (Pelczar & Chan, 2008).

It was reported that endophytic *Streptomyces galbus* from *Rhododendron* sp. had antibacterial activity against gram-positive bacteria such as *Bacillus subtilis* M-45 (Shimizu *et al.,* 2004)

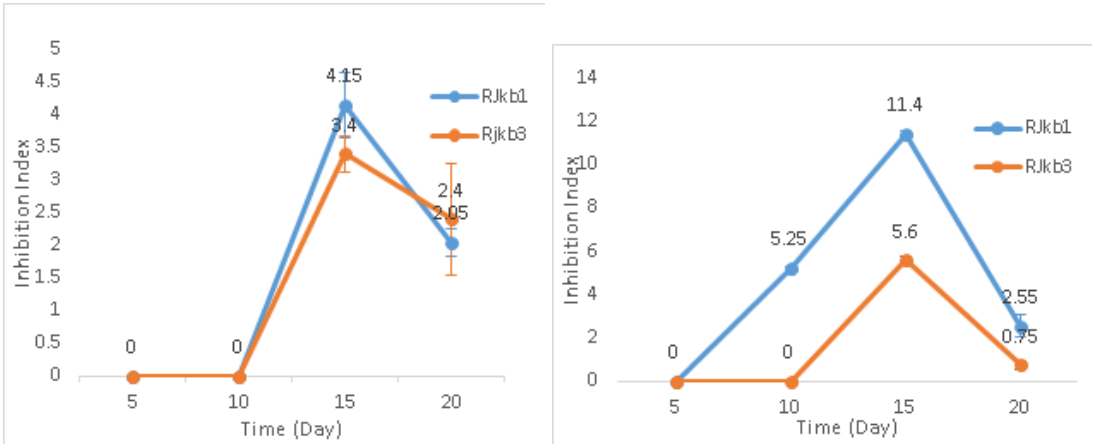

Figure 3. Antibacterial activity pattern of RJkb1 and RJkb3 (a) against EPEC K1-1; (b) against *B. pumilus*.

endophytic Streptomyces sp. R-5 isolated from a field-grown rhododendron was able to confer disease resistance on tissue-cultured seedlings of rhododendron when applied to medium surfaces in flasks. Here, the isolate was identified as Streptomyces galbus based on various physiol. characteristics and anal. of the 16S rDNA sequence. Its major antimicrobial metabolites were identified as actinomycin X2 and fungichromin by analyses using liq. chromatog./mass spectrometry and NMR. [on SciFinder (R. In our work, observation under the microscope electron, for the treated cell of *B. pumilus* with RJkb1 supernatant containing antibacterial compounds showed to have different in cell morphology and density compared with the untreated cells (Figure 7).

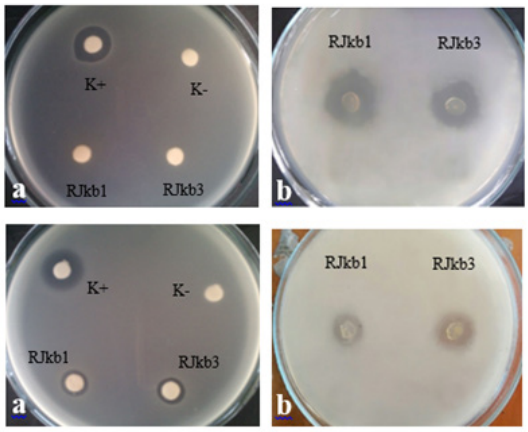

Figure 4. Antibacterial activity of RJkb1 and RJkb3: (a) antibacterial activity of supernatant (b) antibacterial activity of direct antagonist colony test. Against EPEC K1-1, Polymixin B used as positive control ((K+ above), and *B. pumilus,* ampicillin as a positive control (K+ below).

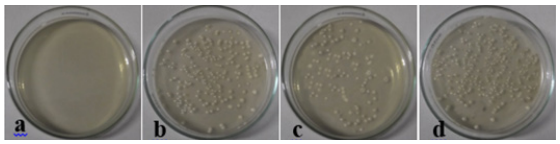

Figure 5. Colonies of EPEC K1-1 growing in NA medium after treated with supernatant of endophytic actinobacteria: (a) positive control with the addition of polymixin B, showing no growth; (b) negative control, abundance of colonies; growth colonies previously treated with (c) 1750 µg/mL RJkb1 supernatant; (d) 1750 µg/mL RJkb3 supernatant.

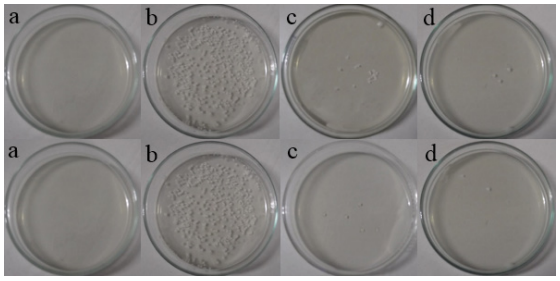

Figure 6. The growth of the bacterial colony in NA medium, as the result of MIC test for RJb1 (above), and RJb3 (below) against *Bacillus pumilus*: (a) Positive control with the addition of ampicillin; (b) negative control; (c) Addition of 1000 µg/mL supernatant; (d) Addition of 1250 µg/mL supernatant.

Observation of cell morphology of *B. pumilus* under the electron microscope showed viewer cell number and elongated treated cells, compared with more cell number and the shorter cell for the untreated one. The adding of antibacterial compound from the selected endophytic

actinobacteria from *Rhododendron* did not damage the cell wall of bacterial target, indicating another inhibitory mechanism. Antibacterial compounds have a various mechanism of action to inhibit bacterial growth. The bioactive compounds may inhibit the synthesis of the cell wall, cell membrane, protein, DNA, RNA and folic acid synthesis (Tenover, 2006). An antibacterial compound which inhibits DNA synthesis, acts by binding 50s ribosome subunit, inhibits peptidyl transferase enzyme as a form of peptide catalyst (Katzung, 2014). Antibacterial compounds produced by nonendophytic actinobacteria were reported to inhibit the synthesis of the cell wall of EPEC K1.1 (Lestari, private communication), but in our work, this has not been observed, since the supernatant of tested RJkb1 and RJkb3 could not inhibit EPEC K1-1. These research findings give a new information regarding the existence of endophytic actinobacteria in *Rhododendron* spp. from Indonesia and their potency as antibacterial producer. The data may also be used for further development of a microbial-based product to control bacterial pathogens resistant for some antibiotics in the environment.

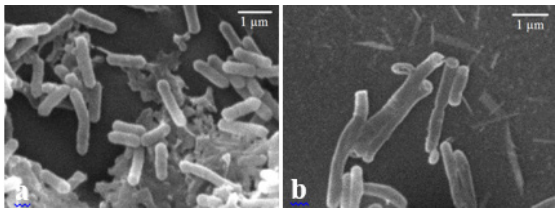

Figure 7. Cell morphology of *Bacillus pumilus* observed under an electron microscope (magnification 10,000x) (a) control negative, without the addition of supernatant RJkb1; (b) treated cell, with the addition of 1000 µg/mL RJkb1 supernatant.

CONCLUSION

There were 23 endophytic actinobacteria successfully isolated from *Rhododendron* spp. Based on the direct colony assay, two of the selected isolates, i.e., RJkb1 and RJkb3 capable of inhibiting the growth of EPEC K1-1 and *B. pumilus*. The supernatant of the two colonies could only inhibit *B. pumilus.* Under the electron microscope, the treated cells of *B. pumilus* showed decreased in number and elongated cell. Both RJkb1 and RJkb3, endophytic actinobacteria from *Rhododendron* spp. have the potency as an antibacterial agent which may be further developed to overcome resistance pathogen to antibiotics.

ACKNOWLEDGEMENT

We thank the Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, for the facilities. This work was supported by University Research Priority (PUPT) Contract No. 011/SP2H/LT/DRPM/IV/2017, Directorate for Research and Community Services, Directorate General for Research and Development, Ministry of Research, Technology, and Higher Education, Republic Indonesia.

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