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Bacterial endophytes from mangrove leaves with antibacterial and enzymatic activities

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ABSTRACT

Aims: The aim of this study was to screen bacterial endophytes with antibacterial and enzyme activity from mangrove leaves of Indonesia.
Methodology and results: Bacterial endophytes were isolated and evaluated for antibacterial activity against five strains of pathogenic bacteria. Enzymatic Index (EI) was measured to evaluate the production of protease, amylase and cellulase. Hemolysin test was performed on Blood Agar and the sensitivity to antibiotic was performed. Bacterial endophyte Strain 1-1 isolated from *Bruguiera gymnorrhiza* showed strong inhibition against *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus* and *Pseudomonas aeruginosa* with inhibition zone of 12.6 ± 1.4, 8.8 ± 4.1, 12.5 ± 2.3 and 8.4 ± 0.9 mm respectively. Isolate 1-16 which isolated from *B. gymnorrhiza* exhibited antibacterial activity against *E. coli* and *P. aeruginosa*, while Isolate 6-10 isolated from *Avicennia lanata* exhibited strong inhibition on *Salmonella* sp. (13.1 ± 3.3 mm). All of those three isolates produced protease, non-haemolysin-producing strain and sensitive to gentamicin or kanamycin but resistant to ampicillin, tetracycline and chloramphenicol. Those three isolates were identified based on homology of 16S rDNA sequence. Strain 1-1 and 1-16 were identified as *P. aeruginosa*, while strain 6-10 identified as *S. marcescens*.
Conclusion, significance and impact of study: This finding was showed the potential endophytic bacteria from Indonesian mangrove plants with antibacterial and enzyme production.

Keywords: Antibacterial, bacterial Endophyte, enzymatic index, *Pseudomonas aeruginosa*, *Serratia marcescens*

INTRODUCTION

The demand of antimicrobial substances is increasing due to the emerging of antibiotic resistant pathogens and the sharp reduction of new discovery of active metabolites in the last decades. The prominent source of active compounds was from cultivated soil bacteria, Actinobacteria, the genus of *Streptomyces*. However, the potential of more novel actinobacterial taxa is under established, therefore the new source of niche of metabolite-producing bacteria is needed. Compared with soil bacteria, bacterial symbions especially which is living inside plants or endophyte is less investigated for metabolic potential. Endophyte are of interesting source of active compounds since may inhabit high number of niches and resource, such as terrestrial and marine ecosystem. It colonized plant tissues as part of their lifetime and act as non-phytopathogenic organism (Brader *et al*., 2014).

Mangrove forest is potential source of endophytes due to it living in the most productive natural ecosystems. Mangrove ecosystem alone is source of food supply, building material and medicines. Since mangrove is living in one of most dynamic conditions of environment, it is believed that the organisms have acquired defense system to survive and adapt in the mangrove habitat. The stress of environment such as fluctuation of tidal gradient as well as salinity also believed lead to the production of distinct metabolites with wide array of activities. *Streptomyces* species from mangrove soil were previously reported produce antioxidant, while other reported *Streptomyces* of mangrove soil produce

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antibacterial activity (Tan *et al*., 2018).

The adaptation and survival of plants in stress condition such as infection of pathogen, salinity, contaminants and drought are resulted from co-evolution of plants and endophytic bacteria. It was reported that endophytic bacteria alleviate the toxicity of metal ions by efflux of metal ions on the surface of the cell or intracellular polymers, adsorption, precipitation and biomethylation, therefore it suggested that inoculation of endophyte may improve survival of plants. Endophytic bacteria also reported produce plant growth regulator by stimulating of plant development and soil fertility (Phetcharat and Duangpaeng, 2012) or improve the survival by inhibiting pathogens (Ma *et al*., 2016).

Endophytic bacteria are living inside different plant tissues at least part of their live and do not cause severe or pathogenicity to their host (Strobel and Daisy, 2003) and found in wide plant species as latent state or colonizing plant tissue actively (Afzal *et al.*, 2014). Endophyte may produce defense substance against herbivores, inhibit or suppress disease, induce the resistance of systemic of the plants and improving plant growth (Golinska *et al*., 2015).

Antagonistic activities of endophyte were reported as potential novel source of bioactive compounds which may utilized in drug or agricultural industry (Golinska *et al*., 2015). It was reported that endophyte produce higher bioactivity than epiphytic or soil microbes due to endophyte are living within plant tissue. Many antagonistic activity biological products were isolated from endophyte, consist of various structural group (flavonoids, steroids, terpenoids and peptides (Strobel and Daisy, 2003; Yu *et al*., 2010) therefore, it is believed that endophytic bacteria may be as an alternative source of antibiotic which produce highly effective, low environmental impacts, low toxicity antibiotics to prevent infection of antibiotic resistant pathogens (Das *et al*., 2017).

Bacterial endophyte are reported to have positive effect on host such as enhancement of nitrogen fixation, production of phytohormones, inhibition of ethylene biosynthesis as respons to abiotic stressor, and solubilize phosphate. Endophytic actinobacteria from various genera were isolated and 17 stains were reported having antimicrobial activity (Dudeja and Giri, 2014; Salam *et al*., 2017), while antimicrobial produced are classified as natural active compounds (Guo *et al*., 2008) such as alkaloids, quinines, terpenoids, peptides, flavonoids, and phenols (Yu *et al*., 2010). Zhao *et al*. (2011) reported that of 26 medicinal plants from Panzi plateu, there are large number of endophytic actinomycetes with novel bioactive compounds (Singh *et al*., 2017). Diverse community of microorganisms (bacteria and fungi) with antimicrobial and or enzymatic activities are harbored in mangrove plants and ecosystem (Bibi *et al*., 2017; Saravanakumar *et al*., 2016). Bibi *et al.* (2017) reported of 317 rhizo- and endophytic bacteria from mangrove roots, soil, and leave tissue and found 25 of bacterial isolate active against oomycetes fungal pathogen. Those bacteria consisted of five different classes of bacteria and endophytic bacteria isolate EA218 identified as *Bacillus amyloliquefaciens* subsp. *plantarum* was showed strong activity against oomycetes while weak or inative against other fungi.

Some of bacteria and fungi survived from extreme condition such as mangrove ecosystem which has fluctuative change of salinity, have unique life habitats and structure which produce novel enzyme system. Enzymes of microorganism are more stable than of plants and animals (Saravanakumar *et al*., 2016). Microorganisms are important source of enzymes because of the genetic and biochemical diversity (Saravanakumar *et al*., 2016). Enzyme of microorganisms play important role as food additives or industrial applications. However, report on bacterial endophyte from mangrove plants of Indonesia which produce antibacterial and enzyme is limited. Therefore, the aim of this study was to isolate and identify the bacterial endophyte from mangrove plants leaves of Mangrove Forest of Surabaya, Indonesia, that producing antibacterial and enzyme for food or industrial application.

MATERIALS AND METHODS

Mangrove leaf collection

Leaves of seven different mangrove plants species from Wonorejo Mangrove Forest, Surabaya, Indonesia, were randomly picked and stored in sterile polyethylene plastic and were brought to the laboratory for bacterial isolation. The mangrove plants sampled were identified as *Avicennia alba*, *A. lanata*, *A. marina*, *B. cylindrica*, *B. gymnorrhiza*, *Rhizophora apiculata*, and *Sonneratia casiolaris*.

Bacterial isolation

Bacterial endophyte was isolated following method previously described by Arora *et al.* (2014). Samples of leaves of mangrove plants were randomly picked from the tree and were washed in running tap water and dip in 70% ethanol for 2 min. The leaves were then dipped in 2% sodium hypochlorite for 1 min and rinsed with sterile distilled water for 2 min and dried in laminar airflow. After pretreatment, the leaves were crushed in sterile distilled water using mortar and pestle. A total of 1 mL of sample was diluted up to 10^{-4} and aliquot of 100 µL of diluted sample was spread onto Tryptic Soya Agar (TSA, Oxoid) and incubated at 28 °C for 3-5 days. The colony growth in medium were then selected and purified with streak plate method on TSA. The Gram staining performed

Antibacterial assay

The antibacterial assay was evaluated with disc diffusion assay against indicator bacteria *B. subtilis*, *Salmonella* sp., *E. coli*, *P. aeruginosa*, and *S. aureus* (Haque *et al*., 2016). Briefly, a total of 10 µL of overnight culture containing approximately 10^8 CFU/mL of bacterial endophyte was added in sterile paper disc. The paper disc was then placed on the plates that previously

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swabbed with indicator bacteria (10^8 CFU/mL) and incubated at 30 °C for 24-48 h. The estimation of antibacterial activity was evaluated by measuring the diameter inhibition zone around the disc.

Enzymatic activity assay

Enzymatic activity was evaluated with method described by Castro *et al*. (2014). The measurement of cellulase, protease and amylase was evaluated by initially growing the bacterial endophyte on tryptic soya broth (TSB, Oxoid) for 24 h at 30 °C. The culture was then centrifuged at 3,000 rpm for 15 min to get pellet. The pellet was then washed and diluted in physiological solution (0.85% NaCl) until the turbidity of 10^8 CFU/mL with standard McFarland 1 and diluted a third. Approximately, 10 µL of the culture was inoculated on sterile paper disk on specific medium. After incubation at 30 °C for 24 h, clear hollow around the disc were measured and the enzymatic index (EI) was measured by the ratio between the halo and bacterial colony (Hankin and Anagnostakis, 1975).

The amylolytic activity was performed by inoculation of bacterial culture at TSA medium containing 1% starch and incubated at 30 °C for 24 h. Incubated plate was then added with 1% iodine solution to visualize the clear halo around the colony (Hankin and Anagnostakis, 1975). The proteolytic activity was measured according to Castro *et al*. (2014). A culture containing skimmed milk was used (using Skim milk agar (Himedia) medium). The formation of halos wasconsidered indicative of enzymatic activity. The cellulolytic activity of the bacterial endophyte was measured following e Silva *et al*. (2016). The 1% of Carboxyl Methyl Cellulose (CMC) was added on Bacto Agar (Oxoid) and sterilized before plated on sterile petridish. The bacterial culture was placed on sterile disc and put at the surface of the medium and incubated at 30 °C for 24 h. A total of 10 mL of Congo Red Dye (1%) was added to visualized the presence of halo as indicator of cellulolytic activity.

Hemolysin test

The hemolysin types of the bacterial endophyte were characterized by the radical streak method on the blood-agar plate (5% sheep blood) in triplicate. A loopful of the culture was streaked on blood-agar and incubated at 37 °C for 24 h. The hemolysin-producing strain was separated based on clear or halo zone around streak. Alpha-hemolysin-producing strain was indicated by complete lysis with hazy edges, while darkening area around colony considered as β-hemolysin and δ-hemolysin described as complete clear zone around colony (Haque and Baldwin, 1964).

Antibiotic resistance test

The antibiotic susceptibility test was performed by the Kirby-Bauer disc diffusion method on Mueller Hinton Agar (Hi-Media, India) following CLSI standard (2019). Strain of bacterial endophyte was growth on Tryptic Soy Broth (TSB) at 37 °C for 24 h and was then centrifuge 3,000 rpm for 5 min. The pellet was diluted on normal saline solution (0.85% NaCl) until equal to standard McFarland 0.5 and diluted to 10^8. The culture was then swabbed on Mueller-Hinton Agar (Himedia, India) and the antibiotics were placed on the surface of the plate. The following antibiotics disks (Oxoid) were tested: ampicillin (10 µg), gentamicin (10 µg), kanamycin (30 µg), tetracycline (30 µg) and chloramphenicol (30 µg). The cut-off of considered as resistant if the inhibition zone diameters were less than 19 mm for ampicillin, 14 mm for tetracycline, and 13 mm gentamicin, kanamycin and chloramphenicol (CLSI, 2019).

Bacterial identification using 16S rRNA gene sequencing

The molecular identification was performed using Toualbia *et al.* (2018). DNA was extracted with placing fresh colony into concentration of 10^6 cell/mL and heated for 10 min at 95 °C to facilitated lysis of cells. Heated tube was then centrifuged to remove the debris and the DNA was used for PCR amplification. Amplification of the sequence of 16S rRNA gene was employing 27F(5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). PCR was carried out in Kappa ready mix (1.5 mM $MgCl_2$, of 1U *Taq* polymerase, buffer, 0.5 µM forward primer, 0.5 µM reverse primer, and 5 µL of template. The PCR cycling performed with heating of 95 °C for 7 min, cycle denaturation 95 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 45 min and final extension at 72 °C for 10 min. The PCR product was then sequenced with ABI Sequencer, Singapore. The sequence was then analyzed with Blasn of GeneBank and the phylogenetic tree was constructed with online software of www.phylogeny.fr (Dereeper *et al.*, 2008).

RESULTS

Bacterial endophyte isolation

A total of 82 isolates of bacterial endophyte were successfully isolated from seven species of mangrove leaves (Table 1). Based on Gram-staining, total of 64 isolates 78.05% were Gram-positive bacteria, while 18 isolates (21.95%) were Gram-negative bacteria. Based on Table 1, Genus *Avicennia* was the highest source of endophytic bacteria with 47 isolates (57.31%); while *Sonneratia* was the lowest number with 7 isolates (8.53%).

Antibacterial assay

Five bacterial endophytes (6.09%) were active against at least one of indicator bacterial tested (Table 2). From those five bacterial endophytes, three isolates were isolated from *B. gymnorrhiza*, one from *A. lanata* and one isolate from *B. cylindrical* (Table 2).

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Table 1: Total bacterial endophytes isolated from seven different mangrove's leaves species from Surabaya, Indonesia.

Species of mangrove	Total bacterial endophyte isolated
Avicennia alba	14
Avicennia lanata	27
Avicennia marina	6
Bruguiera cylindrica	1
Bruguiera gymnorrhiza	10
Rhizopora apiculata	17
Sonneratia casiolaris	7
Total	82

Enzymatic activity of bacterial endophyte

A total of 65 isolates (79.27%) out of 82 isolates exhibited at least one enzymatic activity of amylase, protease or cellulase. Among 65 enzyme positive strains, 17 isolates were produce more than one enzyme and only one isolate (Isolate 3-5 isolated from *R. apiculata*) produce amylase, protease and cellulase (Table 3).

Hemolytic test

Three bacterial endophytes were chosen based on the ability on production of high antibacterial and enzyme activity, i.e. isolate 1-1, 1-16 and 6-10. Those three strains were tested on their ability to produce hemolysin on Blood Agar. Based on the test, all of those isolates were negative hemolysin production indicated that the bacterial endophyte isolated from mangrove plants leaves of Surabaya, Indonesia were non-pathogenic to human.

Antibacterial susceptibility

Antibacterial susceptibility test was illustrated in the Table 4. Based on the diameter zone of inhibition of antibiotic, all isolates were resistant to ampicillin. Beside ampicillin, isolate 1-1 and 1-16 isolated from *B. gymnorrhiza* were resistant to kanamycin, tetracycline and chloramphenicol. On the other hand, isolate 6-10 isolated from *A. lanata* leaf was sensitive to all antibiotic except ampicillin.

Bacterial identification using 16S rRNA gene sequencing

The bacterial identification of bacterial endophyte with potential antimicrobial production was performed with sequencing of 16S rRNA gene. The homology of the sequences was analyzed with the database (BLAST-N, www.ncbi.nlm.nih.gov). Phylogenetic tree analysis of this study indicated 99% homology of the sequence of 16S rDNA of 1-1 and 1-16 with *P. aeruginosa*, while isolate 1-6 with *S. marcescens* (Figure 1).

DISCUSSION

The mangrove ecosystem is an important source of bacterial endophyte. This study was successfully isolated of 82 isolates of bacterial endophyte. Those isolates were purified, and morphological characteristics were recorded. Previous study by Castro *et al*. (2014) successfully isolated 14 genera of bacterial endophyte from Brazilian mangroves forest (*R. mangle* and *A. nitida*) with potential of antibacterial and enzymatic production.

Our finding on this study on the antibacterial activity of bacterial endophyte was different with Gayathri *et al.* (2010) who stated the most of endophytic bacteria (77%) isolated from leaves of mangrove and salt-marsh plant showed inhibition against test strain bacteria with 55% of them were had broad spectrum activity against

Table 2: Total bacterial endophyte exhibited antibacterial activity against indicator bacteria.

Bacterial endophyte isolate	Mangrove source	Diameter inhibition (mm) ± SD				
		Escherichia coli	*Salmonella* sp.	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Bacillus subtilis*
1-1	*Brugueira gymnorrhiza*	12.6±1.4	8.8±4.1	12.5±3.2	8.4±0.9	3.1±0.8
1-3	*Brugueira gymnorrhiza*	10±0.7	4.8±1.5	11.3±1.1	6.3±1.5	NA
1-16	*Brugueira gymnorrhiza*	9.3±2.6	4.4±2.2	7.1±1.7	4±1.8	NA
6-9	*Avicennia lanata*	NA	6.5±4.8	NA	NA	NA
6-10	*Bruguiera cynlindrica*	NA	13.1±3.3	NA	NA	NA

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Gram positive and Gram negative bacteria. Arunachalam and Gayathri (2010) described eight bacterial endophytes with antibacterial activity against fish and human pathogens. Six of those isolates had wide spectrum of activity. Antibacterial is needed due to emergence of antibiotic pathogenic microorganisms resistance. The antibiotic resistance of pathogens is caused by antibiotic resistance gene which is spread in environmental and human pathogens and may be horizontally transferred to other bacteria (Arunachalam and Gayathri, 2010).

The capability of production of novel enzyme and its activity to catalyzing reactions of biochemical from mangrove bacteria and fungi should be explored (Castro *et al*., 2014) due to the production of hydrolytic enzyme by halophilic bacteria is important for denaturation of proteins (Ventosa and Nieto, 1995). However, screening of enzyme production with quantitative method was time intensive that may reduce the number of microorganisms to evaluate. Enzymatic index was a practical and fast tool for screening the production of enzyme by different bacterial strains. The value of enzymatic index above 1.0 was indicated of secretion of enzyme to the medium (Carrim *et al.*, 2006). Our study also in accordance with Castro *et al.* (2014) which showed that the bacterial endophytes isolated from mangrove were capable of producing extracellular enzymes such as amylase, protease and lipase that important for industrial applications, such as detergent additives (Saeki *et al.*, 2007).

The production of protease from bacterial endophyte isolated in this study was predominant compared to amylase and cellulase. This pattern was different with previously reported by Dias *et al.* (2009) showed that microorganisms from sediments of mangrove from Brazil were dominated by Bacillales and Vibrionales that were capable to producing extracellular protease, amylase, lipase and esterase. Dias *et al*. (2009) founded that the strong producer of amylase and protease was *Bacillus* spp. strain from mangrove sediments, while Castro *et al.* (2014) reported that the endophytic *Bacillus* sp. 1A339 strain from mangrove were strong producer of lipase with enzymatic index of 4.8. Endophytic bacteria from Thailand mangrove's plants was studied by Khianngam *et al.* (2013) and found 20 isolates had ability to produce lipase, protease, cellulase or amylase. They reported that a bacterial endophyte isolated from *Rhizophora mucronata*, Rhf-2 strain, produced all enzyme tested and was identified as *Bacillus safensis*. This finding also suggested that the enzymatic activity of bacterial endophyte may depend on source or niche of the bacterial.

Our finding showed that cellulase-producing strain was only three isolates, isolate 5-3 isolated from *R. apiculata* and isolate 6-12 and 6-13 isolated from *A. lanata*. This finding was less reported compared to Tabao and Moasalud (2010) who evaluated the bioprospecting potential of the bacterial community found in mangroves in the Philippines, and found four promising cellulase-producing bacteria that identified as *B. cereus*, *B. licheniformis*, *B. pumilus* and *Bacillus* sp.

Antibacterial-producing agent such as bacteria or fungi should not harm the host 34 therefore, hemolysis test is performed. Based on the test, all of bacterial endophytes which showed antibacterial and enzyme production were non-hemolysin-producing strain. Bacterial endophytes as antagonist an agent can be applied if only non-pathogenic to nematodes, bacteria and human or mammalians (Yousif *et al.*, 2017). Some hemolysin-producing strains were reported consisted of Gram-positive and Gram-negative bacteria.

Based on Arunachalam and Gayathri (2010) described most of bacterial endophytes isolated were sensitive to gentamycin, amikacin, chloramphenicol, erythromycin, ciprofloxacin and amicillin while some are resistant to the antibiotics tested. However, this study was in line with Gagne-Bourgue *et al.* (2013) who reported that isolated bacterial endophyte with phosphor-solubilization activity, identified as *Bacillus subtilis*, *Pseudomonas flourescens* and *P. ananatis* and produce chitinase, pectinase and cellulase were resistant toward antibiotic. In the study Yousif *et al.* (2017) *P. flourescens* B25 was the most resistant strain.

Mangrove endophyte play important role in adaptation of plants towards adaphic factor and effect of detrimental of soil. The finding of this study was different with previously reported by Deivanai *et al*. (2014) who identified bacterial endophyte from mangrove twigs and petiole based on 16S rDNA sequence and revealed that bacterial endophyte found in *Rhizophora apiculata* Blume of India dominated with genus of Bacillus. Our finding also different with previously reported by Gayathri *et al.* (2010) who founded that bacterial endophyte from black mangrove of mangrove forest of Pitchavaram, Tamil Nadu, India were dominated by *Bacillus* which showed antibacterial activity against *Escherichia coli*, *Vibrio parahaemolyticus*, *V. anguillarum*, *Bacillus subtilis* and *Staphylococcus aureus*. On the other study, Actinomycetales, Vibrionales and Bacillales were reported as dominant mangrove associated bacteria in mangrove (Dias *et al*., 2009). This finding also suggested that different location and environment has different potential bacteria due to environmental factors such as mangrove plants' species, abundancy of soil microorganisms and sample (twig, root, leaves).

Serratia marcescens was previously reported as nosocomial pathogen or non-pathogenic bacterial endophyte. Endophytic *Serratia marcescens* strain MSRBB2 was reported to produce prodiginines and serratamolides and was evaluated on it biosynthetic pathway with biochemical analysis of feeding experiments (Eckelmann *et al*., 2018). Prodiginines produced naturally by microorganisms are group of tripyrrole alkaloid which has wide bioactivity, including antibacterial, antiprotozoal, and antifungi (You *et al*., 2019). This also indicated that the antibacterial activity of bacterial endophytes from mangrove leaves in this study may due to the production of secondary metabolites such as prodiginines.

Based on genome analysis of *S. marcescens* pathogenic and nonpathogenic strains was share 96-98% similarity (Vicente *et al*., 2016). The genus of *Serratia* is

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Table 3: Enzymatic activity of bacterial endophyte isolated from mangrove leaves of Surabaya, Indonesia.

Mangrove species	Cell form	Gram-staining	Code of Isolate	Enzymatic index		
				Amylase	Protease	Cellulase
Bruguiera gymnorhiza	Rod	Positive	1-1	-	2.73	-
	Rod	Negative	1-3	-	2.96	-
	Rod	Positive	1-4	-	1.41	-
	Coccus	Positive	1-7	-	2.19	-
	Coccus	Positive	1-11	-	-	-
	Coccus	Positive	1-13	-	1.78	-
	Coccus	Positive	1-14		1.59	-
	Coccus	Positive	1-16	-	2.36	-
	Coccus	Positive	1-18	-	-	-
	Coccus	Positive	1-21	1.27	1.55	-
Avicennia marina	Coccus	Positive	2-1	1.27	1.55	-
	Coccus	Positive	2-2	1.45	1.22	-
	Coccus	Positive	2-4	1.96	1.6	-
	Rod	Positive	2-9	1.91	1.33	-
	Rod	Negative	2-10	1.36	1.33	-
Avicennia alba	Rod	Negative	3-1	1.99	-	-
	Rod	Negative	3-2	1.25	-	-
	Rod	Negative	3-3	-	-	-
	Rod	Negative	3-4	-	1.24	-
	Rod	Negative	3-5	1.46	-	-
	Coccus	Positive	3-6	1.47	-	-
	Coccus	Positive	3-7	1.36	-	-
	Coccus	Positive	3-8	-	-	-
	Coccus	Positive	3-9	-	-	-
	Coccus	Positive	3-1	-	-	-
	Coccus	Positive	3-11	1.4	-	
	Coccus	Positive	3-12	1.21	-	-
	Coccus	Positive	3-13	1.31	-	-
	Coccus	Positive	3-14	-	-	-
	Rod	Negative	3-15	-	-	-
Sonnetaria caseolaris	Coccus	Positive	4-1	1.63	-	-
	Coccus	Positive	4-2	1.57	-	-
	Coccus	Positive	4-3	1.04	-	-
	Coccus	Positive	4-4	1.81	-	-
	Coccus	Positive	4-5	1.65	-	-
	Coccus	Positive	4-6	1.21	-	
	Coccus	Positive	4-7	1.64	-	-
Rhizopora apiculata	Rod	Positive	5-1	-	-	
	Rod	Positive	5-2	-	-	-
	Coccus	Positive	5-3	1.67	2.39	1.44
	Rod	Positive	5-4	-	1.4	-
	Rod	Positive	5-5	2.24	-	-
	Rod	Positive	5-6	2.18	-	-
	Rod	Negative	5-4-1	1.29	-	-
	Rod	Negative	5-7	2.36	-	-
	Rod	Negative	5-8	1.93	1.4	-
	Coccus	Positive	5-9	1.4	-	-
	Rod	Positive	5-10	1.12	-	-
	Rod	Positive	5-11	-	-	-
	Rod	Positive	5-12	1.38	2.58	-
	Rod	Positive	5-13	1.83	-	-
	Coccus	Positive	5-14	2.71	-	-

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Table 3: Continued

	Rod	Positive	5-15	1.13	-	-
	Rod	Positive	5-16	1.5	1.37	-
Avicennia lunata	Coccus	Positive	6-1	1.36	-	-
	Rod	Negative	6-2	1.98	1.89	-
	Coccus	Positive	6-3	1.5	1.33	-
	Rod	Negative	6-4	1.04	-	-
	Rod	Negative	6-5	2.1	1.58	-
	Rod	Positive	6-6	2.34	1.06	-
	Coccus	Positive	6-7	-	-	-
	Rod	Positive	6-8	-	-	-
	Coccus	Positive	6-9	-	2.36	-
	Coccus	Positive	6-10	-	2.2	-
	Rod	Positive	6-11	1.47	1.36	-
	Rod	Positive	6-12	-	1.94	1.21
	Rod	Negative	6-13	-	-	1.45
	Rod	Positive	6-14	-	-	-
	Rod	Positive	6-15	1.86	-	-
	Rod	Positive	6-16	-	1.35	-
	Rod	Positive	6-17	-	1.63	-
	Rod	Positive	6-18	-	1.38	-
	Rod	Positive	6-19	-	1.56	-
	Rod	Positive	6-20	-	1.52	-
	Rod	Positive	6-21	-	-	-
	Rod	Positive	6-22	-	-	-
	Rod	Positive	6-23	-	-	-
	Rod	Positive	6-24	1.98	-	-
	Coccus	Negative	6-25	-	1.76	-
	Coccus	Negative	6-26	-	1.33	-
	Coccus	Positive	6-27	-	1.88	-
Bruguiera cylindrica	Coccus	Positive	7-1	1.82	1.5	-
Total isolate				44	36	3

Table 4: Antibacterial susceptibility assay of bacterial endophyte with antibacterial and enzyme production.

Isolates	Inhibition zone (mm) ± standard deviation				
	Gentamicin	Kanamycin	Ampicillin	Tetracycline	Chloramphenicol
1-1	14.3±0.9	7.1±0.9	0.0±0.0	4.9±1.7	6.0±0.5
1-16	14.5±0.5	8.7±0.6	0.0±0.0	5.9±1.0	7.8±1.5
6-10	19.3±0.5	19.3±2.5	0.0±0.0	13.2±0.8	19.0±0.9

easily found in wide range of ecology due to it adaptability as living or host opportunistic bacteria in soil, water, plants, animals and insects. This genus has 19 species and 4 subspecies described and genome sequenced. *Serratia marcescens* was reported as common multidrug-resistant pathogen, while strains of *S. marcescens* AGPim1A reported as plant pathogen. However, *S. marcescens* PWN146 also reported as non-pathogenic bacterium. *Serratia marcescens* PWN146 described as multidrug resistant, formation of biofilm, cellulase activity, has no ACC deaminase activity and phosphate solubilization, and do not promote root elongation but positive production of exopolysaccharides (EPS) and indole acetic acid (IAA) (Richter and Rosselló-Móra, 2009). In addition, nonpathogenic *Serratia marcescens* AL2-16 isolated from medicinal plant of *Achyranthes aspera* L was reported to producing indolic acetic acid (IAA) with amount of 123.2 mg/mL with supplementation of L-tryptophan.

Our finding showed that *S. marcescens* from mangrove leaves has ability to produce antibacterial and protease, while previous study on endophytic *S. marcescens* mainly focusing on the ability of production of IAA.

Our study also finding that *P. aeruginosa* strain 1-1 and 1-16 isolated from *B. gymnorhiza* showed antibacterial and proteus production ability. This finding also in accordance with previously reported by Kumar *et al.* (2005). *Pseudomonas aeruginosa* PUPa3 isolated from rhizosphere soil of rice was previously reported has

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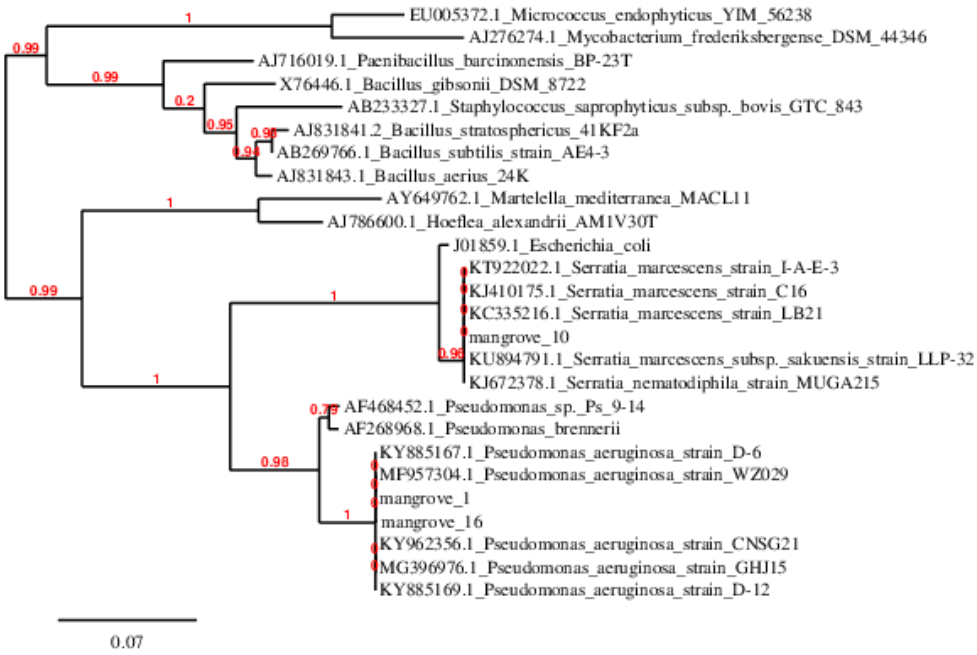

Figure 1: Phylogenetic tree of bacterial endophyte isolated from mangrove leaf of Surabaya, Indonesia.

ability to produce broad-spectrum antifungal activity, phenazine-1-carboxamide (PCN) based on NMR and MS data. In other study, Devi *et al.* (2017) evaluated 73 isolates of endophytic bacteria of leaves and stems of *Achyranthes aspera* L. and found one isolate (AL2-14B) has excellent plant growth stimulating attrivutes. This strain was identified as *P. aeruginosa* based on biochemical and molecular identification employing sequencing of 16S rRNA gene.

Acinetobacter, *Enterobacter*, *Pantoea*, *Pseudomonas*, *Serratia* and *Stenotrophomonas* are group of largely found endophytic Gammaproteobacteria. Some of *Enterobacter* and *Pseudomonas* were described as opportunistic or beneficial endophyte. Induced systemic resistance (ISR) and secondary metabolites antibiotic production are importance role of endophyte in plant defence system. Bacteria endophyte strain of *Pseudomonas* and *Bacillus* are considered as the most common genera of bacteria which inducing ISR due to its production of antibiotics, flagella, salicylic acid, N-acyl-homoserine lactones, siderophores, jasmonic acid, acetoin and lipopolysaccharides (Hardoim *et al*., 2015).

Member of *Pseudomonas* are described as non allergenic, non-pathogenic, and non toxic toward people, wild life or domestic animal. *Pseudomonas* also has great potential on producing antibiotic bioactive potential such as indole derivatives, phenazines, pyrrolnitrin-type antibiotics, glycolipids, another substances (Pratiwi *et al*., 2017). Wu *et al.* (2018) evaluated *P. aeruginosa* L10 as biosurfactant producing and excellent hydrocarbon-degrading bacteria isolated from roots, stems and leaves of a Reed *Phragmites australis*).

Based on test of hemolytic, all of strains isolated from mangrove plants leaves of Surabaya, Indonesia was non-pathogenic, however *P. aeruginosa* are also described as human pathogen. Based on the possibility of pathogenic characteristic, it was suggested that *P. aeruginosa* was suggested not suitable candidate for application of biocontrol or plant growth promotion application if not supported by strong data on its ability to infecting animals or humans (Thomas and Sekhar, 2016). Kumar *et al*. (2005) studied endophytic isolate of *P. aeruginosa* from pepper and clinical strains and they share high genomic similarity, therefore it application on agriculture is still concerned particularly it interaction with native endophytes and potential transmission to different plant.

Marine normal flora such as *Pseudomonas*, *Micrococcus*, *Bacillus* and *Pediococcus* are predominant microorganisms found in seafood or fermented seafood products (Dewapriya and Kim, 2013).The bacterial endophyte products such as enzyme or antibacterial substances may be applied as the addition of functional food and or nutraceuticals. Based on Dewapriya and Kim (2013), the wild type isolates from marine and environment should meet food regulatory requirements before applied as functional food or nutraceuticals. However, it became major challenges because of lack of knowledge in this area even though it was no legal definition of food-grade bacteria. The safe application description were based on undesirable properties, taxonomy, opportunistic infections, virulence factors and antibiotic resistance, and toxic metabolites as major criteria for safe use of microorganisms (Dewapriya and Kim, 2013). Therefore, based on the evaluation of the

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strains, it was suggested that the application of the bacterial endophyte of our study was not suitable for food application but for other industry such as additive of detergent.

CONCLUSION

A total of 82 isolates of bacterial endophytes were isolated from seven mangrove plants leaves from mangrove forest of Surabaya, Indonesia. Among those isolates, a total of three isolates were showed antibacterial activity and protease production. Based on biochemical and molecular approach, isolate 1-1 and 1-16 isolated from *B. ghymnorrhiza* were identified as *P. aeruginosa*, while isolate 6-10 isolated from *Avicenia lanata* was identified as *Serratia marcescens*. This finding was report of bacterial endophyte from mangrove forest of Surabaya, Indonesia with antibacterial and enzyme activity.

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