Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2020.25274

188 (2020) 282–287
June

Endophytic bacteria of *Anisophyllea disticha* (Raja Berangkat) from tropical lake environment in Malaysia

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Received 17 June 2019; Accepted 22 December 2019

ABSTRACT

Lake Kenyir is the largest artificial lake in South East Asia and serves as habitats for diverse flora and fauna. Moreover, Lake Kenyir is a rich resource of medicinal plants which has been used by local community surrounding the lake. Endophytic bacteria are able to colonize plants without causing harm and they can be highly beneficial towards their plant hosts. The aim of this study is to isolate and identify endophytic bacteria from the leaves and roots of a medicinal plant, *Anisophyllea disticha* (Raja Berangkat), and to evaluate their antimicrobial capabilities. Twenty isolates each from the leaves and roots were randomly selected for molecular identification using 16S rRNA gene. Partial 16S rRNA gene sequences revealed that genus *Bacillus* dominated the endophytic bacteria from the leaves with two isolates belonging to *Paenibacillus* and *Aeribacillus*. Five genera namely, *Bacillus*, *Staphylococcus*, *Lysinibacillus*, *Paenibacillus*, and *Mycobacterium* were successfully obtained from the roots of *A. disticha*. Nine endophytic bacteria—five *Bacillus* species (R3, R11, R13, R26, R37), *Lysinibacillus* (R9), two *Staphylococcus* species (R6, R27) and *Mycobacterium* species (R33) from roots were found to show weak antibacterial and/or antifungal activities. Findings from this study provide an insight to endophytic bacteria inhabiting *A. disticha* and the role they may play in their plant host.

Keyword: Endophytic bacteria; *Anisophyllea disticha*; 16S rRNA; Antimicrobial

1. Introduction

Lake Kenyir is the largest man-made lake in South East Asia and one of the important tropical freshwater environment in Malaysia. Lake Kenyir was formed to supply the Sultan Mahmud Hydroelectric Power Plant. The lake is surrounded by more than 40 rivers and streams, and this lake is considered one of the oldest tropical forest in the world and holds a diverse array of flora and fauna. Lake Kenyir is also home to many unique medicinal plants such as Tongkat Ali (*Eurycoma longifolia* Jack), Kacip Fatimah (*Labisia pumila*), and Mahkota Dewa (*Phaleria macrocarpa*).

Endophytic bacteria is a term referring to bacteria which are able to colonize the interior parts of plants such as root, stem, and seed without harming their host. Endophytic bacteria and medicinal plants are believed to have a mutualistic relationship which means both parties benefit from each other. Endophytic bacteria usually benefit from the host by gaining protection from extreme environmental stresses such as temperature fluctuation, changing in osmotic pressure, and also exposure to ultraviolet radiation. The plant host, in turn, gaining benefits from the endophytic bacteria by getting bioactive compounds namely secondary metabolites such as antibiotics and hydrolytic enzymes, that are produced by the endophytic bacteria upon stresses like pathogenic infections, viral diseases, and against predators like insects that keeps

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the longevity and survival of the plant [1]. The unique ability of endophytic bacteria in producing novel bioactive compound has drawn great interests among the natural product discovery researches. Examples of novel antibiotics produced by endophytic bacteria include Munumbicins synthesized by *Streptomyces* NRRL 30562 isolated from *Kennedia nigrican* [2]; Kakadumycins produced by *Streptomyces* NRRL 30566 which colonized *Grevillea pteridifolia* [3]; Xiamycin B obtained from *Streptomyces* HKI0595 that lived within *Bruguiera gymnorrhiza* [4]; and diketopiperazines produced by *Streptomyces* SUK 25 isolated from *Zingiber spectabile* [5]. Moreover, the presence of endophytic bacteria can promote the growth and development of the plant host by stimulating the physiological process and induce phytohormone production [6].

Anisophyllea disticha also locally known as kayu pacat or raja berangkat, grows as a shrub or small tree with a height reaching to about 8 m and commonly found in Malaysia, Thailand, Indonesia, and Brunei. Traditionally, the plant has been used as walking sticks, and as therapeutic in libido stimulants and anti-aging therapies. The leaves can be used as herbal tea drink for the treatment of dysentery and diarrhea. Besides being used as energy booster, it can also be used as an alternative to treat jaundice in new born baby [7]. Local people of Borneo have been reported to use the roots and leaves of this plant and ground them with other ingredients to be taken as concoction for weariness [8]. Other than being popular for its benefits in enhancing the sexual ability of a woman, the roots have been used as an anti-aging as it is believed to contain tannins that function to shrink the skin tissues [7]. Recent studies reported that extracts from the leaves of *A. disticha* showed strong anti-leishmanial activity [9] and anti-influenza activity [10]. Despite the medicinal potential of *A. disticha*, the status of endophytic bacteria inhabiting this plant is relatively unknown. Hence, findings from this study will certainly provide the insight of endophytic bacteria associated with *A. disticha*.

2. Materials and methods

2.1. Collection of plant materials

A. disticha (Raja Berangkat) was collected from Kenyir Lake, Terengganu and was taxonomically identified followed by deposition of specimen voucher in Kulliyyah of Science Herbarium. *A. disticha* was processed immediately upon collection.

2.2. Surface-sterilization of leaves and roots

The collected leaves and roots of *A. disticha* were washed in running tap water and those with superficial injury that was visible to the naked eye were excluded. Surface-sterilization technique was performed on each leaf and root samples to avoid contamination. Both leaves and roots were immersed in 70% ethanol for 30 s. After that, they were treated with sodium hypochlorite (5%) solution for 3 min. Finally, the leaves and roots were dipped in absolute ethanol for about 10 s before last rinsing with autoclaved distilled water. The rinsing with autoclaved distilled water was repeated at least three times to remove any ethanol and sodium hypochlorite residue.

2.3. Isolation of endophytic bacteria from leaves and roots

Each isolation procedure was conducted in triplicate. The outer surface of the sterilized leaves and roots was trimmed with sterile razor blade. The pieces were then further macerated in phosphate buffer saline (PBS) solution (g/L—sodium chloride 8, potassium chloride 0.2, disodium hydrogen phosphate 144 and potassium dihydrogen phosphate 0.24, pH 7.4) and were serially diluted up to 10^{-3} dilution. Next, 0.1 mL of each triplicate was plated onto nutrient agar plates. All plates including the control were incubated at 37°C for 5 d and observed periodically for bacterial growth. Morphologically distinct colonies as identified by colony characteristics were selected, isolated, and used for further studies.

2.4. PCR amplification of 16S rRNA gene on selected endophytic bacteria

In total of 40 isolates from leaves (L1–L20) and roots (R1–R20) were selected randomly for molecular identification using 16S rRNA gene. Genomic DNA of selected endophytic bacteria from leaves and roots were extracted using GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies, USA) as per the manufacturer's protocols. Genomic DNA obtained were then subjected to PCR amplification of 16S rRNA gene sequences using the following primers: 27F 5′-AGAGTTTGATCCTGGCTCAG-3′, 1492R 5′-GGTTACCTTGTTACGACTT-3′ [11]. PCRs were performed in a final volume of 50 μl, consisting, if 200 ng DNA template, 25 μl MyTaq Mix 2X (Bioline, UK), and 0.4 μm primers under the following temperature profile: Initial denaturation at 94°C, 5 min, followed by 30 cycles of 94°C, 30 s, 55°C for 60 s, and 72°C for 1 min, and final extension step of 72°C for 5 min. The presence of the expected size of PCR product (~1,500 bp) was confirmed by agarose gel electrophoresis and sent to first Base Laboratory, Malaysia for purification and sequencing. The resultant 16S rRNA gene sequences were manually verified and edited using the BioEdit Sequence Alignment Editor. The partial 16S rRNA gene sequences were compared with the available sequences in the GenBank database from the National Center for Biotechnology Information (NCBI) by using nucleotide blast (BLASTn) tool. Partial 16S rRNA gene sequences of 20 isolates from roots of *A. disticha* were deposited to GenBank for accession numbers (MF102108–MF102127).

2.5. Evaluation of antimicrobial activity using disc diffusion method

Antimicrobial activity was conducted on 20 isolates obtained from the root part of *A. disticha*. The bacterial strain was inoculated in 50 mL of nutrient broth (NB) in a 250 mL Erlenmeyer flask. The inoculated flask was incubated in a rotary shaker of 150 rpm at 37°C for 48 h. After incubation, 1 ml of the culture broth was centrifuged at 13,000 rpm, 15 min to obtain the supernatant followed by filter-sterilization using 0.2 μm membrane filter. Endophytic bacteria were tested against six test microorganisms—4 gram-negative bacteria; *Salmonella typhi*, *Escherichia coli*, *Klebsiella pneumonia*, and *Proteus vulgaris*, a gram-positive bacterium; *Staphlococcus aureus*, and a fungus; *Candida albicans* using agar well-diffusion method. Wells were made using sterile cylinder borer

measuring 6 mm in diameter on Mueller Hinton agar (MHA; Oxoid, UK) that was previously seeded with test microorganisms. Then, 100 µl of cell-free supernatant was dispensed into each well. Sterile NB was used as negative control. The agar plates were then kept at 4°C for 4 h to facilitate the diffusion of the supernatant in the culture medium. The assay was conducted in triplicate and the agar plates were incubated for 24 h (37°C) and 48 h (30°C) for bacteria and fungus, respectively. Zone of inhibition around each well was measured and recorded in the following manner: weak activity (5–9 mm), moderate activity (10–20 mm), and good activity (>20 mm).

2.6. Biochemical characterization

Endophytic bacteria displaying antibacterial and/or antifungal activity were subjected to the following biochemical tests—Oxidase test, Catalase test, Sulfur, Indole, Motility test, Indole test, Methyl Red-Voges Proskuer test, and Citrate utilization test according to the manufacturer's instructions (Oxoid, UK).

3. Results and discussion

In total of 47 and 52 isolates were successfully recovered from leaves and roots of *A. disticha*, respectively. All isolates were morphologically recorded and subjected to Gram staining. Following this, 20 isolates from leaves (L1–L20) and 20 isolates from roots (R1–R20) were selected randomly for molecular identification using 16S rRNA gene. Table 1 depicts the results obtained from comparison of the partial sequences of 16S rRNA gene for isolates from leaves of *A. disticha* with NCBI GenBank Database. Majority of the isolates were found to belong to the genus *Bacillus* with high similarity of 99%–100%. Findings of this study suggested that genus *Bacillus* dominating the settlement within the leaves of *A. disticha*. Similar findings were reported by Pisarska and Pietr [12] and Yaish et al. [13] indicating *Bacillus* as one of the dominant genus of bacteria inhabiting in plant parts.

Isolate L12 showed high similarity to *Paenibacillus dendritiformis* (97%). The genus of *Paenibacillus* has been isolated from many other medicinal plants such as *Plectranthus tenuiflorus* [14], *Pteris vittata* and *Pteris multifida* [15], and from leaves of *Gynura procumbens*, one of the important medicinal plants in Malaysia and other Asian countries [16]. Interestingly, isolate L14 was highly similar to *Aeribacillus pallidus*. Genus *Aeribacillus* belongs to the phylum Firmicutes, order Bacillales, and family Bacillaceae, and is most closely related to the genera *Geobacillus* and *Anoxybacillus*. Genus *Aeribacillus* currently has only two members—*A. pallidus* [17] and *A. composti* [18]; and they are usually related to thermophilic environment such as hot springs [19] and sand dunes [20] but never from plants. Thus, this is the first report of the genus *Aeribacillus* as endophytic bacterium in plant. The association of *Aeribacillus* as endophyte in *A. disticha* and how this plant benefited the *Aeribacillus* is yet to be explored.

There are five major genera of endophytic bacteria found in roots of *A. disticha* namely, *Bacillus*, *Staphylococcus*, *Lysinibacillus*, *Paenibacillus*, and *Mycobacterium* (Table 2). The most abundant genus was *Bacillus* (65%) followed by *Staphylococcus* (15%), *Lysinibacillus* (10%), *Paenibacillus* (5%), and *Mycobacterium* (5%). Generally, all of the bacterial genera belong to Firmicutes phylum except for *Mycobacterium* which belongs to Actinobacteria phylum. *Bacillus* sp. and *Bacillus subtilis* are commonly found endophytes in various plant species [21]. Besides being the most commonly isolated taxa, *Bacillus* species had shown significant role in promoting plant growth and biomass, where their secondary metabolites have been examined in various researches [22,23]. Isolates R6, R27, and R28 belonged to the genus *Staphylococcus*. This genus was identified within endophytic bacteria isolated from ginger [24], rice [25], and this genus was also reported to be the majority endophytes colonizing sweet pepper [26].

Isolate R33 is highly similar (99%) to *Mycobacterium* sp. A molecular study conducted by Conn et al. [27] to detect the diversity of actinobacteria in the root of wheat which resulted in *Mycobacterium* being the most predominant genus. In recognition of *Mycobacterium* endophytes abundancy in plants, Quambusch et al. [28] did a quantitative PCR to confirm the dynamics of this genus in *Prunus avium* (sweet cherry) plant. Isolate R7 showed 99% similarity with *Paenibacillus aestuarii*. Several novel *Paenibacillus* species were successfully isolated from the root nodules of *Periandra mediterranea* [29] and root of *Oenothera biennis* (evening primrose). This proves the familiarity of *Paenibacillus* as endophytic bacteria colonizing plant roots. Isolate R8 and R9 are closely related to *Lysinibacillus*

Table 1
Comparison of partial 16S rRNA gene sequences of isolated endophytic bacteria from the leaves of *A. disticha* with NCBI GenBank Database

Isolate	Closest match	Similarity (%)	Isolate	Closest match	Similarity (%)
L1	*Bacillus* sp.BK-L40	99	L11	*Bacillus megaterium* H10	100
L2	*Bacillus* sp. FJAT-4468	99	L12	*P. dendritiformis* PP	97
L3	*Bacillus* sp. BCH439	99	L13	*Bacillus oryzaecorticis* WJB116	99
L4	*Bacillus* sp. S1M4	97	L14	*A. pallidus* WJB147	100
L5	*Bacillus aryabhattai* strain N2-2	100	L15	*Bacillus toyonensis* CSR_25	99
L6	*Bacillus oleronius* strain M1/25	99	L16	*Bacillus thuringiensis* strain O43	100
L7	*Bacillus thuringiensis* Eca12	99	L17	*Bacillus megaterium* JF-O3	99
L8	*Bacillus cereus* SCD10	99	L18	*Bacillus thuringiensis* J1	100
L9	*Bacillus ginsengisoli* A1Cr	99	L19	*Bacillus thuringiensis* KWA	99
L10	*Bacillus* sp. sFJAT-43194	99	L20	*Bacillus megaterium* VNRL1	100

Table 2
Comparison of partial 16S rRNA gene sequences of isolated endophytic bacteria from the roots of *A. disticha* with NCBI GenBank Database

Isolate	Closest match	Similarity (%)	Isolate	Closest match	Similarity (%)
R1	*Bacillus* sp. RKND-0553	99	R11	*Bacillus* sp. DB14705	100
R2	*Bacillus* sp. strain ARB- SM12	99	R12	*Bacillus* sp. JCM 28843	100
R3	*Bacillus cereus* p6	100	R13	*Bacillus subtilis* ABS1	98
R4	*Bacillus* sp. DB14709	100	R26	*Bacillus subtilis* Lmb042	100
R5	*Bacillus* sp. DB14832	100	R27	*Staphylococcus hominis* subsp. *novobiosepticus* GTC 1228	99
R6	*Staphylococcus aureus* subsp. *anaerobius* IIF8SW-P2	100	R28	*Staphylococcus* sp. ST5-08	100
R7	*P. aestuarii* CJ25	99	R29	*Bacillus* sp. SBI-13	99
R8	*L. sphaericus* A348	100	R30	*Bacillus altitudinis* RmL5	100
R9	*Lysinibacillus xylanilyticus* PT26	99	R33	*Mycobacterium* sp. C48	99
R10	*Bacillus* sp. DB14859	100	R37	*Bacillus tequilensis* BK206	100

Table 3
Antimicrobial activity of isolates from roots of *A. disticha*

		Inhibition zone (mm)					
Isolate	Genus	*S. aureus*	*S. typhi*	*E. coli*	*K. pneumoniae*	*P. vulgaris*	*C. albicans*
R3	*Bacillus*	–	–	–	–	–	1.0 ± 0.0
R6	*Staphylococcus*	–	–	–	–	3.33 ± 0.47	4.0 ± 0.82
R9	*Lysinibacillus*	–	–	–	–	–	1.0 ± 0.0
R11	*Bacillus*	2.67 ± 0.47	–	–	–	–	–
R13	*Bacillus*	–	–	1.33 ± 0.47	–	–	–
R26	*Bacillus*	1.0 ± 0.0	–	–	–	–	2.0 ± 0.82
R27	*Staphylococcus*	–	–	3.0 ± 0.6	–	–	–
R33	*Mycobacterium*	–	2.0 ± 0.0	–	–	–	–
R37	*Bacillus*	–	–	–	–	–	–

sphaericus and *Lysinibacillus xylanilyticus*. Several studies had demonstrated the presence of endophytic *Lysinibacillus* in number of plants such as *Theobroma cacao* [30] and *Panax notoginseng* [31].

Endophytic isolates from roots were then subjected to antimicrobial test on the basis that root endophytes showed more diversity than the leaf endophytes as well as more medicinal potentials are usually associated with the root part of *A. disticha*. Only 9 isolates displayed antibacterial and/or antifungal activity (Table 3). Generally, isolates were demonstrating weak antibacterial and/or antifungal activity. Majority showed only antibacterial to one test bacterium and none were found to be able to inhibit the growth of *K. pneumoniae*. Isolates R6 which was closely related to *S. aureus* showed antifungal activity against *C. albicans* and antibacterial activity against *P. vulgaris*, while isolate R26 (closely related to *B. subtilis*) was able to inhibit *S. aureus* and *C. albicans*. *Bacillus* is a known microbe which can produce various kinds of proteins that act as repressive compounds [32]. Hence, this might explain the antimicrobial activity showed by isolates R3, R11, R13, R26, and R37. Isolate R9 belonging to genus *Lysinibacillus* displayed weak antifungal activity against *C. albicans* while isolate R33 (genus *Mycobacterium*) was able to inhibit pathogenic bacterium, *S. typhi*. Although these endophytic bacteria showed weak antibacterial and/or antifungal activity, findings from this study exemplified the potential application of these bacteria in natural product discovery.

Endophytic isolates exhibited antibacterial and/or antifungal potential were the subjected to several biochemical tests for further characterization (Table 4). R3, R11, R13, R26, and R37 belong to the genus *Bacillus* with R13 and R26 most similar to *B. subtilis*, R3 highly similar to *B. cereus*, and R37 closely related to *B. tequilensis*. These isolates gave negative results to indole and H_2S gas production while positive results for catalase, oxidase, MR/VP, citrate, and glucose, all of which are common biochemical characteristics for *Bacillus*. When comparing some biochemical characteristics of R9 to *Lysinibacillus xylanilyticus* [33], R9 gave negative results for MR/VP, H_2S gas production, and indole, all of which were similar to that of *L. xylanilyticus*. However, results for citrate, catalase, glucose, and lactose/sucrose showed otherwise whereby these characteristics were positive for *L. xylanilyticus* but not for R9. Biochemical characteristics of R3 are similar to that of *S. aureus* which further confirmed the identity of R3 as *S. aureus*. Some biochemical test results of R27 were similar

Table 4
Biochemical traits of endophytic isolates exhibiting potential antimicrobial activity

Isolate	Biochemical traits	Isolate	Biochemical traits
R3	Indole(–), MR(+), VP(+), Oxidase (+), Catalase(+), Citrate(+), H_2S(–) Glu(+), Lac/Suc(–)	R26	Indole(–), MR(+), VP(+), Oxidase (+), Catalase(+), Citrate(+), H_2S(–), Glu(+), Lac/Suc(+)
R6	Indole(–), MR(+), VP(–), Oxidase (–), Catalase(+), Citrate(–), H_2S(–) Glu(+), Lac/Suc(+)	R27	Indole(–), MR(+), VP(–), Oxidase (–), Catalase(–), Citrate(–), H_2S(–) Glu(+), Lac/Suc(+)
R9	Indole(–), MR(–), VP(–), Oxidase (+), Catalase(–), Citrate(–), H_2S(–) Glu(–), Lac/Suc(–)	R33	Indole(–), MR(+), VP(–), Oxidase (–), Catalase(+), Citrate(–), H_2S(–) Glu(+), Lac/Suc(+)
R11	Indole(–), MR(+), VP(–), Oxidase (+), Catalase(–), Citrate(+), H_2S(–) Glu(+), Lac/Suc(+)	R37	Indole(–), MR(+), VP(+), Oxidase (+), Catalase(+), Citrate(+), H_2S(–) Glu(+), Lac/Suc(+)
R13	Indole(–), MR(+), VP(+), Oxidase (+), Catalase(+), Citrate(+), H_2S(–) Glu(+), Lac/Suc(+)		

Glu, Glucose utilization. Lac/Suc, Lactose/Sucrose utilization.
The presence of an activity is indicated by "+", and the absence is indicated by "–."

to *S. aureus* except for catalase test. However, more biochemical tests are required to further confirm R27 as *S. hominis*. Similarly with isolate R33, the current biochemical tests conducted might not be definitive for R33 to confirm its identity as *Mycobacterium* sp. although molecularly R33 was closely related to *Mycobacterium* sp. Thus, a different set of biochemical tests may be required to further ascertain its identity as *Mycobacterium* sp.

4. Conclusion

In conclusion, the present findings from this study demonstrate the diversity and biosynthetic capabilities of endophytic bacteria inhabiting the plant host, *A. disticha*. Endophytic bacteria may also be valuable resources of interesting compounds and proteins for biotechnological exploitation. Further investigation on these endophytic bacteria may provide understanding on their association and relationship with plant host.

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