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Characterization of Endophytic Bacteria Isolated from *Avicennia marina*'s Leaf Tissue Collected from Ekowisata Mangrove Wonorejo Surabaya, Indonesia

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Abstract. The previous experiment showed that *Avicennia marina*'s leaf collected from Ekowisata Mangrove Wonorejo, Surabaya, Indonesia contained endophytes. The endophytes were known as bacteria which was demonstrated by biochemical tests based on *Bergey's manual of determinative bacteriology*. This experiment aimed to characterize the three isolates of bacteria by using Deoxyribonucleic acid. (DNA)sequencing. The result showed that isolate 1 and isolate 2 were known as *Bacillus subtilis* with a different strain, whereas isolate 3 was considered as *Bacillus* sp. It was in line with the result of biochemical tests. To know the sensitivity of three isolates against antibiotics, a 30 µg tetracycline disc on Nutrient Agar that was overgrown by the isolate was used. The results showed that halo was observed on three isolates, meaning that all of them were sensitive to 30 µg tetracycline.

Keywords: Deoxyribonucleic acid sequencing, potential endophytic bacteria, grey mangrove

1 Introduction

An endophyte is an organism that lives inside the plant's tissue or organ. Endophytes can be fungi, bacteria, or other microorganisms. Endophytic bacteria can spread in all of the plant's organs, or only can live in a particular organ of the plant. However, they colonize host tissue without creating any harm to the host [1]. The interaction between endophytes with the hosts may vary, *e.g.* commensalism, mutualism, or even parasitism [2]. Reports about the alteration of the role of endophytes to parasites and *vice–versa* are available.

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Some factors determine whether the microorganisms inside the host plant act as an endophyte or a parasite. The key factor is in the balance between the need of the microorganism itself and the response from the host [3].

Beneficial endophyte is often associated with mutualism interaction with the plant. That is, there should be a positive impact on the host plants as the existence of endophyte. In this case, the benefit of endophyte is many, for example, the ability to produce plant hormone [4] and the useful chemical compounds are advantageous to be developed to improve yield [5].

The previous study found that inside the leaf tissue of *A.marina* isolated from Ekowisata Mangrove Wonorejo Surabaya, Indonesia there were three bacterial endophytes. Those three isolates were tested biochemically, and then it was known that they were *Bacillus megaterium* (isolate 1), *Corynebacterium kutscheri* (isolate 2), and *Shigella sonnei* or *Yersinia pestis* (isolate 3).

This research aimed to make sure that the three isolates were the same as expected before, but by using the DNA sequencing technique. After it is confirmed, the experiments will be continued to find out their interaction and their benefit for the host plants. Therefore, these experiments are beneficial to finding out the potential of these endophytic bacteria for agriculture and so on.

2 Materials and methods

The experiments were conducted at the Faculty of Biotechnology, University of Surabaya, Indonesia from January 2017 to October 2017.

DNA genome isolation was performed with Wizard Genomic DNA Purification Kit (Promega). Besides that, the boiling lysis technique was also used to collect the DNA genome. The DNA sequences were analyzed on the NCBI website. The tree was then captured to find out its kinship with other bacteria or other species.

Biochemical tests were once again conducted to reconfirm the species. This biochemical test particularly led to the species name in which the information was obtained from DNA sequencing analysis.

The susceptibility test by using 30 µg tetracycline discs was performed to know the ability of three isolates against the antibiotic. As many as three tetracycline discs were put on the Nutrient Agar (NA) overgrown by the bacteria. This treatment was carried out four times for each isolate. The halo was then measured by calipers.

The interaction test was executed in a Petri disc containing NA. Each, both, or all of the isolates were streaked together to observe whether there is a clear zone (halo) around them.

Reinfection of bacterial isolates on the host's leaf tissue was implemented to know the effect of the endophytes inside the plant. As much as 5 mL of bacterial isolates either one or in the combination of 2 and 3 isolates were injected into the leaf tissue by syringe. The effect on the plant host was observed after 24 h, 36 h, 48 h, and 60 h post-injection.

3 Results and discussion

3.1 Isolation of DNA genome

The boiling lysis method in this experiment was successfully extracted the DNA genome of all the isolates. The electrophoresis result of this technique is as seen in Figure 1.

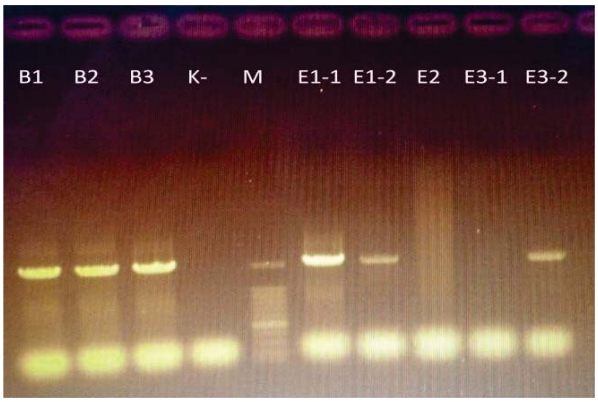

Fig. 1. PCR result of template DNA with boiling lysis technique and with kit. B, boiling lysis; K-, negative control; M, marker; E, extraction using kit; 1, sample1; 2, sample2, 3, sample3.

DNA genome isolation by using boiling lysis technique in principle is to lyse partial bacterial cells so that the plasmid molecule can come out from the cell. Meanwhile, most of the DNA genome is trapped inside cell debris and then bounced out. The rest of the DNA genome is eliminated during the denaturation stage. In this case, we use high temperature rather than high pH. The reannealing stage was conducted after this step. As a result of supercoiling, the plasmid molecule can be reassociated rapidly, where the DNA genome is still denaturated. This technique is more beneficial because can be performed without the ethanol precipitation step [6].

The DNA genome extraction by using Wizard Genomic DNA Purification kit by Promega, initially only successful on isolate 1, yet after the annealing temperature was raised to 60 ºC, the isolation for three isolates was all successful. Annealing is a stage where the primer is attached to the DNA template. So it is understandable that if the annealing temperature is not optimal will cause failure to the attaching step.

The boiling lysis technique is really easy, effective, and cheap. This technique is 100 % successful in this experiment. This method is better than the technique by using the kit.

3.2 DNA sequencing and analysis

The result of DNA sequencing with contig analysis was conducted for further analysis by using BLAST alignment. The result showed that isolate 1 was identified as *Bacillus subtilis* strain HYM07 16s ribosomal RNA gene, partial sequence, with query cover 99 %, E–value 0.0, and identity 98.62 %. Whilst, two was defined as *Bacillus subtilis* strain YRL02 16s ribosomal RNA gene, partial sequence, with query cover 99 %, E–value 6e–106, and identity 99.54 %. Furthermore, alignment was also addressed for isolate 3. The result showed that isolate 3 was defined as *Bacillus* sp. 16s ribosomal RNA with query cover 60 %, E–value 3e–123, and identity 98.10 %. The phylogenetic tree was constructed to see the clustering of those isolates as it is as shown in Figure 2. It was generated by using Unweighted Pair Group Method (UPGMA) method. The closest cluster would be joined [7]. The number was defined as a distance number for clustering. It suggested that isolate 1 and isolate 2 were in the same cluster since they were identified as *Bacillus subtilis*.

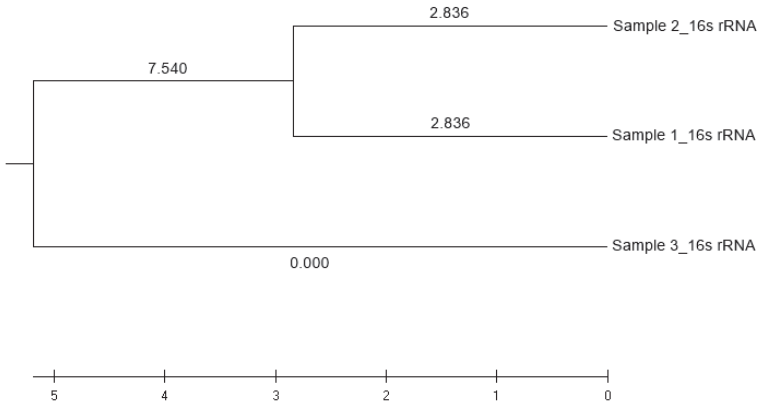

Fig.2. Clustering of three isolates of bacteria sample by using Unweighted Pair Group Method (UPGMA) method

3.3 Biochemical confirmation test

As has been informed from the DNA sequencing analysis result, there are no species that match the previous identification performed by biochemical tests. That is why the confirmation test was conducted once again. The five main confirmation tests were Gram staining, starch, citrate, Voges Proskauer (VP), and 6.5 % NaCl. Isolate 1 and isolate 2 are positive for all the tests, so they really are *Bacillus subtilis*. However, isolate 3 is negative for starch, citrate, and 6.5 % NaCl. So, basically isolate 3 is from the genus *Bacillus*, but there is still no evidence to indicate it as *B. subtilis*.

3.4 Susceptibility against 30 µg tetracycline

All three isolates are sensitive to 30 µg tetracycline. The sensitivity is shown by the clear zone (halo) that appears around the antibiotic discs. Table 1 shows the halo diameter produced by the antibiotic. From the table, we know that isolate 3 is the most sensitive than both isolates because it is significantly (by ANOVA and Tukey test) has the longest halo diameter by 2.44 cm.

Table 1. The Halo Average After Susceptibility Test by 30µg Tetracycline.

Isolate	Halo Average (cm)
1	2.27^{ab*}
2	2.15^{b}
3	2.44^{a}

Remarks: a number followed by different letters shows that there is a significant difference between the treatment

Based on Table 1, all the isolates are susceptible to 30 µg tetracycline. The efforts to produce axenic culture on leaf tissue of *A. marina*'s were previously prohibited due to the endophytes. Tetracycline, maybe in lower concentrations, can be one of the alternatives to

suppress the endophytes' growth in the leaf tissue culture. To be more precise, further experiments are needed to discover the minimal inhibitory concentration (MIC) as well as minimum bactericidal concentration (MBC) of tetracycline against the endophytes.

3.5 Interisolates interaction

Figure 3 indicates the pathway to test isolates' interaction. From the figure, we can conclude that there is no inhibitory activity between the three isolates as no clear zone is observed. The three isolates are fine to culture together, they live in a likely commensalism form with each other inside the Petri dish, but there is still no evidence that they share mutual benefits together (mutualism). The result provides information that a mixed culture of the three isolates can be used for reinfection, without any concern of antagonistic activity against each other. In such a way, observation can be focused only to inspect the plant's response towards the mixed culture as the comparison of the single culture.

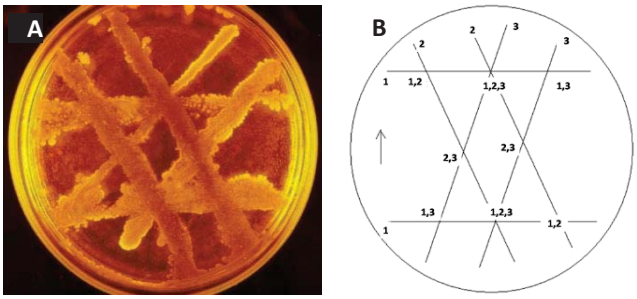

Fig. 3. Interisolates interaction inside the Petri dish. A; Streak result, B; Streak pathway (1: isolate 1, 2: isolate 2, 3: isolate 3)

On the contrary, research conducted by Zalila–Kolsi *et al*. suggested that three strains of *Bacillus* spp. showed inhibitory zones when cultured together [8]. This finding indicates that mixed culture is not recommended prior to application to the plant.

3.6 Reinfection to host plant

The reinfection is conducted to ascertain whether there is benefit or loss the host plant will be experienced after the isolates' inoculation. Each, in two combinations, or in three combinations of isolate(s) are inoculated to the host plant's leaf by syringe. The result is not satisfactory as the leaf is starting to wither and subsequently fall (Figure 6). There is no change happened in the host plant because it is likely that the isolates are unsuccessful to infect the plant. The problem probably comes from the physical and chemical barriers from the host plant. The character of *A. marina* leaf tissue is thick of wax. This is one example of a physical barrier.

It has been stated that there were actually some other techniques to introduce bacteria to the plant, i.e. needle pricking method after suspension penetration, leaf immersion to the bacterial suspension, bacterial suspension pouring into the growth medium, and suspension spraying to the plant's root [9]. However, there are still optimations needed for each technique chosen, because the plant structure condition differs between species.

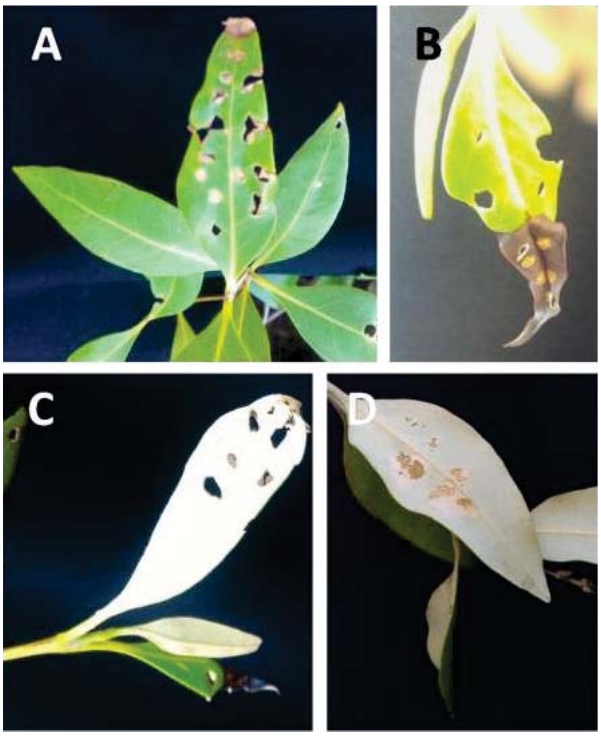

Fig. 4. Reinfection result on *A. marina*'s leaf tissue. A, Isolate 1; B, Mix of isolate 2&3; C, Isolate 2; D, Isolate 3

However, some researchers have been reported the positive impact of *Bacillus* application on the plant. Some species from the *Bacillus* genus were able to decrease the disease severity of infected plants and some were inducing plant growth hormone production [10, 11].

4 Conclusions

Two of the three endophyte isolates are *Bacillus subtilis* but from a different strain, while the third one is *Bacillus* sp. Both identification types, molecular and biochemical tests produced the same result. These three *Bacillus* are sensitive to 30 µg tetracycline. They live in a commensalism way on the Petri dish, however, there is no evidence that they can live in such a mutualistic way. The reinfection method by using a syringe is not successful. Thus, an improvement to this method is needed in order to know the effect of the three isolates on the host plant.

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