Antibiotic Activity of Actinomycetes Isolated from Young Tectona Grandis (L.) Wood and Pith

Rio Risandiansyah 1,*, Arif Yahya 1

1 Faculty of Medicine, Universitas Islam Malang, Malang, Indonesia
* Correspondence: riorisandiansyah@unisma.ac.id (R.R.);

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Abstract: Actinomycetes are a source for novel bioactive compounds and justify obtaining new species from various sources. Hardwoods such as Tectona grandis (L.) have not been studied for actinomycete isolation. We aim to isolate endophytic actinomycetes from young Tectona grandis wood and pith and screen for antibiotic activity. Five young wood were cut and surface sterilized using ethanol and hypochlorous acid. The wood and pith of each sample are placed in eight plates of Humic acid-vitamin B (HV), Tap water Yeast extract (TWYE), and Yeast Extract Casein Digest (YEDC) medium and incubated at 27°C for four weeks. Actinomycetes were isolated from such medium, observed every week, and transferred to an International Streptomyces Project-2 (ISP-2) medium for identification and antibiotic production tested against Staphylococcus aureus, Helicobacter pylori, and Escherichia coli using the Kirby-Bauer method. Seven actinomycetes were isolated from the wood, primarily from YECD and TWYE media, with varying morphological characteristics. One isolate having maroon-colored aerial and vegetative mycelium with grey spores showed moderate antibacterial activity against S. aureus and H. pylori (13.49±1.03 mm and 14.9±0.7 mm, respectively), while two other actinomycete showed weak activity against these bacteria. However, none of the actinomycetes show any activity against E. coli. Tectona grandis (L.) is a potential source for novel actinomycetes with an antibiotic activity which warrants further exploration.

Keywords: actinomycetes isolation; antibacterial activity; drug discovery.

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1. Introduction

Novel antibiotics are currently in demand due to the increasing amount of antibiotic resistance. In 2050, it is predicted that death due to antibiotic resistance will reach 10 million people [1,2]. One solution for curbing antibiotic resistance is the discovery of novel antibiotics having unique and novel structures that may have different bacterial targets or modes of action or be used in conjunction with currently available antibiotics to increase their activity [3,4]. Therefore, drug discovery studies, particularly for antibiotics, are research being conducted in many research centers across the globe.

A promising source of antibiotics is actinobacteria [5,6], with its largest group consisting of Streptomyces. Streptomyces and other actinomycetes are the sources of most antibiotics currently used today and other types of bioactive compounds, including the antibiotics discovered after the 2000s [4,7]. These compounds usually have complex structures and are not easily synthesized, and have various medical uses and bioactivities [7,8]; thus, the discovery of novel compounds from actinomycetes is a goal of interest in drug discovery [9].

The isolation of actinomycetes from various and unique sources is an essential step to achieving that goal [4,10]. Actinomycetes have been isolated from multiple sources, with novel
species discovered from unlikely sources or niches. Isolation of actinomycetes has been conducted on soil samples from various geographical locations [11–13], medicinal plants [14–16], and even from the guts of termites or other insects [17,18]. In nature, actinomycetes are soil-dwellers and endophytic bacteria, occupying the roots, leaves, or bark of various plants or trees [19,20]. In regards to that, Indonesia is researched to be rich in biodiversity and plant species, although it is said to be in decline [21]. However, isolation of actinomycetes from these sources has seldom been conducted, resulting in a mostly untapped natural source for novel species as well as novel bioactive compounds.

Although many studies have successfully isolated actinomycetes from different old and young trees, whether from the roots, leaves, or barks, the isolation of actinomycetes from hardwood trees has been difficult. Novel actinomycetes have been discovered to also dwell inside the wood, even those already used as wooden walls [22]. However, the problem arises in the pre-treatment of the wood. In most studies, to obtain sufficient actinomycetes, the samples must be surface sterilized and ground into 1 – 2 mm fragments in sterile conditions [19,23]. While a blender may be used in leaves or roots dried overnight, only an industrial-grade blender would be able to cut hardwoods. This may result in hardwood isolation studies being currently sparser compared to other parts of the tree.

This study aims to isolate actinomycetes from the wood of *Tectona grandis* (L.), also known as ‘Jati’, from Dau region, Malang, Indonesia, a hardwood usually used in the manufacture of expensive and durable wooden furniture. To somewhat alleviate the difficulty of wood pre-treatment in isolation, the samples used are branches from one-year-old trees, mostly 3 – 5 cm in diameter. After surface sterilization, these would be fragmented using a sterilized carving tool. Due to the relatively softer wood of the younger trees, it would be possible to do it manually. Following this, the actinomycetes would be grown in a production medium and screened for antibacterial activity against Gram-positive and Gram-negative bacteria.

2. Materials and Methods

2.1. General background of research.

This research is an *in vitro* laboratory study conducted at Laboratorium Pusat Riset Kedokteran, Universitas Islam Malang, Indonesia, between January to July 2021.

2.2. Sample of research.

Young *Tectona grandis* (L.) trees were obtained from Dau, Malang, and identified based on morphological characteristics of the leaves, whereas the trees fit the description published in the literature. Leaves were ovate-elliptic to ovate approximately 20 – 30 cm in length and 10 – 20 cm in width and petioles at 2 – 4 cm long. The trees used in the samples were 1.5 – 2 m tall and 10 – 15 cm thick, with grey or grayish brown branches. No flowers or fruits were observed. This study also uses *Escherichia coli*, *Helicobacter pylori*, and *Staphylococcus aureus* to represent Gram-positive and Gram-negative bacteria. *E. coli* and *S. aureus* bacteria were Certified Reference Microorganism (CRM) cultures, with a catalog number for *E. coli* of VT000122-10EA and *S. aureus subspecies aureus* of VT000322-10EA, while *Helicobacter pylori* were obtained from the Microbiology Laboratory, Universitas Muhammadiyah Malang, Indonesia.
2.3. Surface sterilization of wood samples.

A single branch was collected from five different trees. Surface sterilization of the obtained branches was done following previous literature with some modifications [23]. The branches were submerged in 99% ethanol for 60 seconds, transferred in a 3.125% NaOCl solution for 6 minutes, in a different batch of 99% ethanol for 30 seconds, and finally rinsed in distilled water and left overnight to dry. Using a sterilized carving tool and aseptic conditions, the branch was broken open to reveal the wood and piths. The wood was then shaved into thin, 0.5 – 1 cm pieces and collected. Using sterile tweezers, the pith was also broken into the same size pieces and collected in a different container. This was repeated for all five branches.

2.4. Sample inoculation and isolation of actinomycetes.

Three different isolation media were prepared, following previous literature [23]. Fragments of wood and pith were separately inoculated (at eight to ten fragments per plate) in eight taps of water-yeast extract agar (TWYE, 0.25 g yeast extract [Oxoid], 0.5 g K$_2$HPO$_4$, 18 g agar [Himedia], and one liter of tap water), eight yeast extract-casein hydrosylate agar (YECD, 0.3 g yeast extract [Oxoid], 0.3 g glucose anhydrous, 2 g K$_2$HPO$_4$, 18 g agar [Himedia], and one liter of distilled water), and eight humic acid-vitamin B agar (HV, 0.5 g Humic Acid, 0.5 g K$_2$HPO$_4$, 1.71 g KCl, 0.5 g MgSO$_4$.7H$_2$O, 0.01 g FeSO$_4$.7H$_2$O, 0.02 g CaCO$_3$, 0.5 mg Vitamine B, 18 g agar, and one liter of distilled water). After inoculation, the media were incubated in a 27°C incubator for four weeks, checking every three days. Actinomycete cultures were transferred into an International Streptomyces Project 2 medium (ISP2, 4 g yeast extract [Oxoid], 10 g malt extract (Sigma), 4 g glucose anhydrous, 15 g agar [Himedia]) for morphological identification. All media was first adjusted for pH to 7.2 ± 0.2 and sterilized using an autoclave at 121°C for 15 minutes.

2.5. Morphological observation and putative identification of obtained cultures.

Cultures were coded based on the isolation, tree number, plate number, and isolate number. After transferring actinomycetes to an ISP2 medium, as previously described [24], the plates were incubated at 27°C. After five days, the morphological characteristics were noted. After incubation for ten days, a sample of a well-sporulated culture was obtained and observed under a light microscope at 400x or 1000x magnification to note spore formation.

2.6. Extraction of active compounds from obtained cultures.

For antibiotic production, the cultures were streaked in a lawn fashion in ISP2 medium to achieve sufficient biomass. After fourteen days of incubation in ISP2 medium, a small-scale extraction was performed on the plates, following previous literature [25]. The ISP2 medium was cut up (along with the lawn bacteria after sporulation was observed) in 1 cm cubes. These were then submerged in 20 ml methanol and shaken at ±120 rpm for 24 hours for the extraction process. The cubes and bacteria were filtered out using filter paper. Then, these extracts were then used for antibacterial screening.

2.7. Antibacterial assay from extracts.

The antibacterial assay was conducted using a Kirby-Bauer method, following guidelines from the American Society of Microbiology (ASM) [26]. The bacteria used were
first grown in a nutrient agar medium [Himedia] (NA, 5 g peptone, 5 g NaCl, 1.5 g HM peptone B, 1.5 g yeast extract, 15 g agar, and 1 l distilled water) into separate, identifiable colonies were observed. From each plate, colonies were transferred into a 10 ml 0.9% NaCl solution and were compared to a 0.5 McFarland standard. The colonies were homogenized using a vortex and added until the bacterial solution had an approximate density similar to the McFarland standard. Also, a sample of the already homogenized bacterial solution was checked using a spectrophotometer at a wavelength of 600 nm to ensure that the optical density was between 0.08 – 0.1. Using a sterile cotton swab, the suspension was inoculated onto a Mueller-Hinton Agar plate [Himedia] (MHA) in a zig-zag manner, according to the guideline above. The blank antibiotic testing discs were then submerged into the previously obtained extracts and left for 10 minutes. Then, the discs were carefully placed on top of the MHA agar inoculated with bacteria. The plate was then incubated at 37°C for 18 – 24 hours. A clear zone signified antibacterial activity and was measured using a caliper and reported in millimeters. This assay was replicated three times for each bacterium.

2.8. Data analysis.

Statistical data on the zone of inhibition was analyzed using multiple T-test to determine significant differences. Each antibacterial assay was conducted in triplicates.

3. Results and Discussion

3.1. Morphological characteristics of isolated actinomycetes from different media.

Isolation efforts resulted in a low number of actinomycetes due to an overabundance of fungal cultures, both from the wood and the pith, in all media tested. The pith of the wood seldom resulted in any actinomycetes but was a novel source of possibly novel fungal microorganisms. Furthermore, actinomycetes cultures were isolated in the 1st and 2nd week, and no new cultures emerged after the 3rd or 4th week of examination. Overall, seven actinomycetes morphologically different cultures are reported in this study. The morphological and microscopic characteristics of actinomycete cultures are summarized in Table 1 and can be evaluated in Figure 1.

Most actinomycetes isolated from this study originated from the wood, in which only one, T4P11, was found from pith samples. Based on its mycelium characteristics, seven isolates were isolated and tested for actinomycetes, although some found to have had similar mycelium characteristics were collated into one sample. However, although the coloring of the aerial mycelium was the same between one culture and the other, the coarseness of the cultures and overall surface characteristics was taken into account when determining different species. Most show good sporulation after 10 days of incubation, except Y4K23, which had poor sporulation in ISP2 even after 14 days. It is suspected that most of the actinomycetes discovered were Streptomyces based on the observation of its mycelium or spores.

In this study, several issues were met that may have lowered the number of cultures obtained. While HVA medium used in this study is known to be selective for actinomycetes [19,27], no actinomycetes were successfully cultivated from this medium. On the contrary, while TWYE and YECD medium used in this study have also previously been successful in isolating actinomycetes [23,28], however, aside from the actinomycetes obtained from these plates, many plates were overgrown with different and various types of fungi, in which its inclusion was not within the scope of this paper.
Figure 1. Images of macroscopic and microscopic evaluation of isolated actinomycetes. Microscopic observation was obtained using light microscopy at 400x magnification, except for T4P11, which was observed at 1000x magnification. (a) Y4K15, (b) Y3K21, (c) Y3K13, (d) Y4K23, (e) T3K24, (f) T3K11, (g) T4P11.
Table 1. Summary of morphological and microscopic characteristics of isolated actinomycetes.

<table>
<thead>
<tr>
<th>Culture Code</th>
<th>Media source</th>
<th>Sample source</th>
<th>Aerial mycelium color</th>
<th>Vegetative mycelium color</th>
<th>Spore color</th>
<th>Sporulation after 10 days</th>
<th>Microscopic observation of mycelium or spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y4K15</td>
<td>YECD</td>
<td>Wood</td>
<td>Maroon</td>
<td>Maroon</td>
<td>Grey</td>
<td>Good</td>
<td>Rectiaculiaperti</td>
</tr>
<tr>
<td>Y3K21</td>
<td>YECD</td>
<td>Wood</td>
<td>Colorless</td>
<td>Cream</td>
<td>Creamy-white</td>
<td>Good</td>
<td>Rectiaculiaperti</td>
</tr>
<tr>
<td>Y3K13</td>
<td>YECD</td>
<td>Wood</td>
<td>Colorless</td>
<td>Cream</td>
<td>Creamy-white</td>
<td>Poor</td>
<td>Rectiflexibles</td>
</tr>
<tr>
<td>Y4K23</td>
<td>YECD</td>
<td>Wood</td>
<td>Colorless</td>
<td>Colorless</td>
<td>Creamy-white</td>
<td>Poor</td>
<td>Not observable</td>
</tr>
<tr>
<td>T3K24</td>
<td>TWYE</td>
<td>Wood</td>
<td>Pink-red</td>
<td>Colorless</td>
<td>White</td>
<td>Good</td>
<td>Rectiflexibles</td>
</tr>
<tr>
<td>T3K11</td>
<td>TWYE</td>
<td>Wood</td>
<td>White</td>
<td>Cream</td>
<td>White</td>
<td>Good</td>
<td>Globose</td>
</tr>
<tr>
<td>T4P11</td>
<td>TWYE</td>
<td>Pith</td>
<td>Maroon</td>
<td>Maroon</td>
<td>Grey</td>
<td>Good</td>
<td>Rectiflexibles</td>
</tr>
</tbody>
</table>

Fungal growth is rapid and expansive, making it difficult to obtain pure, uncontaminated actinomycete cultures. In this research, several attempts and reculturing had to be done to obtain a final pure actinomycete culture. Endophytic fungi are also a potential source of novel antibiotics [4]; thus, isolation of fungi using a medium optimized for fungal growth should be attempted from the same source.

Considering the status of Indonesia as one of the most biodiverse geographical regions in the world [21], actinomycetes isolation from Indonesian plants is currently under-utilized or undocumented. While the global number of actinomycetes is still undetermined, each plant species is found to have at least hundreds of endophytic actinomycetes species [14]. Indonesia is considered a hotspot with a large variety of plants [14]; however currently, there are only around 590 cultures currently collected in the Indonesian Culture Collection (InaCC) [29]. As stated earlier, the successful discovery of novel actinomycetes, and in turn, novel compounds, is achieved by exploring unique and difficult regions or sources [4]. As actinomycetes are also known to be endophytes, isolation of actinomycetes from plants is a potential source. However, it should be noted that differences in seasons, plant age, and other multiple environmental factors would influence the types of cultures obtained in an isolation project [30–33].

In this study, we report one culture of actinomycete found from the pith samples of the plants, encoded T4P11. However, we cannot cross the possibility that its true origins might have been from the inner wood surface; especially, considering that the morphological characteristics seem similar to another culture obtained from wood (Y4K15). A specific method to isolate actinomycetes or other endophytic samples from the pith of the plant should be developed, particularly considering that no other studies report actinomycetes nor fungal isolation from pith samples. Several research reports the effect of pathogenic bacteria that target plant pith [34,35]; therefore, it is probable that endophytic bacteria or fungi may exist in the pith of the plants, as shown in this study. Thus, further exploration studies targeting pith for the isolation of endophytic fungi and actinomycetes may be warranted.

3.2. Antibiotic screening from isolated actinomycetes.

Only two of the discovered actinomycetes showed any antibacterial activity. Antibiotic production from each culture against *Staphylococcus aureus*, *Helicobacter pylori*, and *Escherichia coli* (Figure 2). Against *S. aureus*, Y4K15 showed moderate antibacterial activity (13.49±1.03 mm), while Y3K21 and T4P11 showed weak antibacterial activity (7.07±0.32 mm and 7.55±0.15 mm, respectively). Similarly, these actinomycetes also showed activity against *H. pylori*, where again Y4K15 showed moderate activity (14.9±0.7 mm), and Y3K21 and T4P11 showed weak activity (9.3±1.25 mm and 9.8±0.15 mm, respectively). However, none of the actinomycetes showed any antibacterial activity against *E. coli*, despite both *E. coli* and *H. pylori* being Gram-negative bacteria.
Three actinomycetes isolated in this study showed some antibacterial activity against *S. aureus* and *H. pylori*, but not *E. coli*, indicating the production of a narrow-spectrum antibacterial agent. Y4K15 was shown to have a moderate to strong activity against these two bacteria; however, the mechanism of action cannot be determined. While *H. pylori* and *E. coli* were both Gram-negative bacteria, some natural compounds have been shown to differentially affect these two bacteria even with their similarities [36–38]. On the contrary, some compounds are known to affect similarly against both *S. aureus* and *H. pylori* [37], despite their difference in cell wall type. Morphological and physiological differences and similarities between the tested bacteria are suspected to be a determining factor in antibiotic activity [39], which requires further studies in the isolation and identification of the bioactive compound.

Another factor to be considered in its mechanism of action is the possibility of more than one bioactive compound being produced. ISP2 medium has been known to be a good production medium in general [40–42]; however, the production of antibiotics from actinomycetes is influenced by many factors. Production of bioactive secondary metabolites is also preceded by sporulation [43–47], in which most of the actinomycetes had good sporulation formed at the time of harvesting and extraction. Aside from the metabolic and genomic capacity of each culture to produce bioactive compounds, certain studies have successfully induced bioactive compound production by adjusting the carbon and nitrogen source of the production medium, the inorganic compositions, or by adding certain trigger compounds to activate antibiotic production metabolic pathways [48–54]. However, these optimization studies are both time-consuming and expensive [55-56] and outside the scope of this paper.

Future directions for this research should include a genetic determination for Y4K15 and other species found in this study using 16S rRNA sequencing for species identification before conducting any optimization studies for antibiotic production and NMR identification of the bioactive compound itself. While laboratory findings do not always translate to clinical application, further characterization and optimization of the lead compounds discovered from Y4K15 may unearth novel antibiotic compounds with a different mode of action.

4. Conclusions

Isolation of actinomycetes from *Tectona grandis* (L.) wood and pith discovered seven actinomycetes with varying morphology, in which three had moderate to low activity against
This study highlights the potential of Indonesian plants in the discovery of endophytic actinomycetes species, particularly for the discovery of antibiotic compounds.

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Conflicts of Interest

The authors declare no conflict of interest.

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