Isolation and antagonism of chitinolytic bacteria from *Ipomea pes caprae* against *Lasiodiplodia pseudotheobromae*

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**Abstract.** Chitin is the main component of fungal cell walls. The growth of fungi can be inhibited by chitinolytic bacteria because they can produce chitinase enzymes which play a role in degrading cell walls. The purpose of this study was to test the activity of bacteria isolated from the roots of *Ipomea pes caprae* in inhibiting the growth of the fungus *Lasiodiplodia pseudotheobromae*. The research method was carried out by isolating as much as 5 g of *I. pes caprae* roots using specific media and testing for bacterial antagonism using a dual culture method. The results showed that there were four isolate codes obtained namely IPaR1, IPaR4, IPPA2, and IPPA3 and could inhibit the growth of *Lasiodiplodia pseudotheobromae*.

1. **Introduction**

The role of fungal pathogens has a global significant impact of yield losses. One type of Botryosphaeriaceae fungi that have been reported to infect crops is *Lasiodiplodia pseudotheobromae* which causes grape stem disease [1], *Dimocarpus longan* fruit rot disease [2], and associated with cocoa disease [3,4]. The latest report that dieback caused by *Lasiodiplodia* species was indicated on cocoa cultivation in Indonesia [3]. Pathogenic *Lasiodiplodia* species cause blight, stem cancer, fruit rot, dieback, grape stem disease and gummosis [5].

Chitin is the main component of fungal cell walls [6] including fungal plant pathogens. In the context of biological control agents, therefore, focusing on cell wall degradation of fungal pathogens is necessarily undertaken. A group of microbial communities having ability to produce chitinase is widely known [7, 8]. It is reported that chitinolytic bacteria can produce chitinase enzymes which can inhibit the growth of fungi. Several types of rhizobacteria such as *Bacillus subtilis* which can not only play a role in increasing plant growth [9] can also protect plants from infection of foliar disease pathogen such as *Fusarium oxysporum* [7] and other soil-borne pathogens [10]. Most of the chitinolytic activity is present in several species such as *Streptomyces, Serratia, Vibrio* and *Bacillus* and also the cell walls of various types of fungi [7, 11, 12]. Based on the above background, it is necessary to carry out further analysis regarding the potential of chitinolytic bacteria isolated from *Ipomes pes caprae* root to inhibiting the growth of *L. pseudotheobromae*. 
2. Methods

2.1. Colloidal chitin preparation
Colloidal chitin was prepared following a modified method [11] which provided as much as 10 g of chitin powder were added 200 ml of concentrated HCl (stirred for 2 hours). After that, the chitin was incubated in a refrigerator at 4°C for 24 hours. The chitin that has been incubation filtered using glass wool, and then filtrate was added a cold sterile distilled water and neutralized by sodium hydroxide until the colloidal chitin became neutral (pH 7.0). The precipitate in the form of colloidal chitin is stored in the refrigerator [11].

2.2. Isolation of chitinolytic bacteria
Chitinolytic bacteria isolated from root samples I. pes caprae took from the coast of Tanjung Bayan, the city of Makassar. Five grams of I. pes caprae root were added 10 ml of sterile distilled water. The mixture of aquades and roots of I. pes caprae was crushed using a mortar then left to stand for a few minutes at room temperature [11]. The bacterial suspensions formed were made of a series of dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ and each dilution contained 90 ml of sterile distilled water [13]. From each series of dilutions, 1 ml of the suspension was taken and 500 µl was spread on CCA medium consists of (g / L): Na$_2$HPO$_4$ (6); KH$_2$PO$_4$ (3); NH$_4$Cl (1); NaCl (0.5); yeast extract (0.05); agar (15) and colloidal chitin 0.5% (w/v) in petri dish. Incubation was carried out at 33°C for 72 hours. Chitinolytic isolates are characterized by the presence of halo areas (clear zones) around the colony [13, 14].

2.3. Interaction of chitinolytic bacteria and L. pseudotheobromae in dual culture method
Observations were made every 24 hours after the isolates were paired. Chitinolytic bacteria were scratched on the surface of NA and PDA media then incubated for 48 hours, and then the test fungi were taken using a cork borer and inoculated on the surface of NA and PDA media which had previously been scratched by chitinolytic bacteria. The percentage of inhibition (IH) was calculated using the formula reported by [15] which has been modified: IH = (R1-R2)/R1 x 100%, represented R1 width of L. pseudotheobromae mycelia towards sterile distilled water (control) and R2 L. pseudotheobromae mycelial width towards bacteria.

Antagonism interaction used a completely randomized design with three replications. All data were analyzed by standard analysis of variance, followed by Tukey’s range test (SPSS version 23). Differences were considered significant at the $\alpha = 0.05$ probability level.

3. Results and discussion

3.1. Selection of chitinolytic bacteria
The results of the isolation showed that four bacterial isolates had chitinolytic activity (figure 1). Bacteria that produce chitinolytic enzymes are characterized by the presence of a clear zone in the colony area (halo). Bacteria that have been isolated are purified and given isolate codes, namely IPaR1, IPaR4, IPPA2 and IPPA3. Chitin can be used for the qualitative determination of chitinase namely to identify certain microorganisms as chitinase producers. Chitinases from various microbes have been isolated and characterized [16].

3.2. Bacterial antagonism against L. pseudotheobromae by dual culture method
Test for bacterial antagonism against L. pseudotheobromae fungi using two media, namely NA media and PDA media. Each media resulted in quite a far average percentage (table 1 and 2) difference where on NA media the inhibition percentage was relatively higher in the treatment compared to PDA media.
Figure 1. Isolation of chitinolytic bacteria for 72 hours.

Table 1. The percentage of chitinolotic bacterial antagonism against L. *pseudotheobromae* fungi on PDA.

| Treatment | Days to - | Days to - |
|-----------|-----------|-----------|
|           | 1         | 2         |
| Control   | 0 a       | 0 a       |
| IPaR1     | 7.02 ab   | 8.84 ab   |
| IPaR4     | 14.33 ab  | 22.56 ab  |
| IPpA2     | 19.79 b   | 31.76 b   |
| IPpA3     | 19.98 b   | 31.02 b   |

Note: the numbers followed by the same letters (a, b, c) are not significantly different according to Tukey's test at the 5% level.

The results of the analysis (table 1) showed IPpA2 and IPpA3 were the best isolates to inhibit the fungus growth of visualization interactions between the fungus *L. pseudotheobromae* and bacteria chitinolytic can be observed in figure 2.

Figure 2. Bacterial antagonism against *L. pseudotheobromae* fungi on PDA media; a) Control, b) IPaR1, c) IPaR4, d) IPpA2, e) IPpA3.
Table 2. The percentage of chitinolytic bacterial antagonism against *L. pseudotheobromae* fungi on NA.

| Isolate code | Days to - |
|--------------|-----------|
|              | 1         | 2         | 3         | 4         |
| Control      | 0.00 a    | 0.00 a    | 0.00 a    | 0.00 a    |
| IPaR1        | 13.69 ab  | 3.03 a    | 5.55 a    | 8.82 a    |
| IPaR4        | 37.02 ab  | 49.13 b   | 62.75 b   | 66.01 b   |
| IPpA2        | 50.95 b   | 70.90 c   | 69.81 b   | 72.40 b   |
| IPpA3        | 37.62 ab  | 61.48 bc  | 75.84 b   | 79.67 b   |

Note: the numbers followed by the same letters (a, b, c) are not significantly different according to Tukey’s test at the 5% level.

The results of the observations in table 2 show that the IPaR4, IPpA2 and IPpA3 isolate codes were the best isolate codes in inhibiting the growth of *L. pseudotheobromae*. The visualization of the interaction between *L. pseudotheobromae* and chitinolytic bacteria can be observed in figure 3.

Figure 3. Bacterial antagonism against *L. pseudotheobromae* fungi on NA media; a) Control, b) IPaR1, c) IPaR4, d) IPpA2, e) IPpA3.

From the experimental results of chitinolytic bacterial antagonism against *L. pseudotheobromae* on different media, namely NA and PDA media, each of which resulted in different inhibitions. Fungal growth on PDA media is relatively fast and produces dense hyphae and inhibition by bacteria tends to have no effect.

3.3. General discussion

Chitinase is an extracellular enzyme that plays an important role in hydrolyzing chitin. Chitinase is produced naturally in various organisms such as bacteria, arthropods, vertebrates, and plants. The physiological function of chitinase depends on the source. In plants, chitinase is generally induced by stress factors such as infection with chitin-containing pathogens. In organisms that contain chitin in their cell walls or other structures such as fungi, chitinase is known to play a role in the spore germination process, hyphae growth and branching and mycelium development [17, 18]. According to [19], fungal cells generally consist of chitin, glucans, mannans and glycoproteins.

In the dual culture method experiment, the antagonism of bacteria to the fungus on PDA media did not show any significant inhibition. This is because the condition of the medium is not suitable because it contains high glucose for bacterial growth. Previous study was conducted an experiment on the effect of a medium containing glucose which in the experiment showed that the test bacteria did not produce chitinolytic enzymes [20]. Glucose is the most efficient carbon source for bacteria [21] so that the isolates grown on these substrates use glucose for growth and metabolism [22,23]. This results in the production of other carbohydrate-degrading enzymes such as chitinolytic enzymes [24], which are not reduced. According to [21], this is called catabolite repression. Catabolite repression is a process by which gluconeogenesis is inhibited when glucose or other carbohydrate carbon sources are
available. This is also supported by the opinion [22] which states that when sugars consisting of different monomers are exposed to acid or enzyme activity, the simple monomers are released into the medium which is then used for physiological needs.

The antagonism mechanism in the four bacterial isolates is likely to act as highly active mycoparasites. Mycoparasitic consists of four stages, namely chemotropic growth leading to a chemical stimulus, the introduction of specific chitinolytic bacterial strains and host plant pathogens, attachment and binding of bacteria to the target pathogenic hyphae, and the last process is degradation of the pathogenic cell wall [18].

Chitinolytic bacteria that produce chitinase which was reported to be useful in dealing with waste, especially waste containing chitin [16], biocontrol agents [18], and useful in medical and industrial needs [16]. In agriculture, chitinolytic bacteria are used as an antagonistic agent for pathogenic fungi and it was reported [8] that chitinolytic bacteria can also be used as a biocontrol agent against insect pests. Chitinolytic bacteria have strong antagonistic activity against pathogenic fungi with hyperparasitic mechanisms and their antibiotics. This is supported by the opinion [17, 21, 22], which states that some of the bacterial chitinolytic enzymes are toxic to pathogenic fungi that cause diseases of cultivated plants, but not other microorganisms in soil and host plants.

4. Conclusions

The isolation of chitinolytic bacteria on the roots of *Ipomea pes caprae* resulted in four isolate codes, namely IPaR1, IPaR4, IPpA2, and IPpA3. The best isolate codes to inhibit the growth of *L. psudotheobromae* based on dual culture tests were IPpA2 and IPpA3.

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