Antagonist effect of *Bacillus* spp. against *Aspergillus niger* CP isolated from cocopeat powder

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Abstract. *Bacillus* spp. are known as potential bacteria as biocontrol agent against fungal phytopathogen, such as *Aspergillus niger*. The *Aspergillus niger* can cause many problems, including in agriculture sector. Antagonist activity of 3 bacteria isolates *Bacillus* sp., KRG, KRT and LDR have been carried out against *Aspergillus niger* CP. Dual culture method for antagonist assay was performed using streak, disc, and pour plate technique. Result from the disc technique showed that there is no significant antagonist activity differences between all isolates KRG, KRT, and LDR (51.94 %, 61.24 %, and 54.53 %). The highest inhibition effect was shown clearly in pour plate technique with inhibition value almost 100 %. The LDR isolate was selected for further evaluation based on the physicochemical characters according to strong catalase activity and rapid fermentation. Antibiosis assay was performed in order to evaluate the antifungal compound produced by LDR isolate using agar and broth culture method. The filtrates from growth medium 7, 10 and 12 days fermentation were used for antibiosis assay against *Asp. niger* CP. The growth of *Asp. niger* CP was inhibited by 10- and 12-days filtrate fermentation in all the 2 methods used. The percentage of inhibition of isolates observed in agar culture were 84.41 % and 84.21 %. Biomass of *Asp. niger* CP reduced by 74.55 % and 85.54 %.

Keywords: Antagonistic activity, *Aspergillus niger*, *Bacillus* spp., dual culture, identification

1. Introduction
Indonesia is known as one of the biggest coco industry country in the world with around 3.7 million hectare of plantation area [1]. In the coco industry, there will be a coconut husk waste product which can be processed further to produce cocopeat. This cocopeat has been used in many applications, one of the examples is popular for hydroponic media of various plants including horticultural [2, 3].

One of the problems in horticulture is plant disease caused by fungal phytopathogen. *Aspergillus niger* is one of the most common species of genus *Aspergillus* which is known as fungal phytopathogen. It can infect grapes, maize, peanuts, onions, garlic, and tomatoes [4, 5]. Fungal growth on food or agricultural products may cause problems. Besides spoilage which can reduce economic value of the product, fungal growth can also produce mycotoxin. *Aspergillus niger* can produce toxin such as ochratoxin, fumonisins and aflatoxin [4, 6, 7]. For those reasons, fungal phytopathogen should be controlled to prevent potential loss of economic value and for food safety. Nowadays, the use of
synthetic chemicals pesticide has been replaced by biocontrol agents which is safer and more eco-friendly [8]. Biocontrol by using bacilli bacteria now is more common and are used widely [9].

*Bacillus* spp. are known as potential bacteria to be used as biocontrol agent. Many species of *Bacillus* have been recognized to be able to inhibit certain plant pathogenic fungi. *Bacillus* spp. are being a potential genera for biocontrol agent because they grow rapidly, have simple nutritional requirements, have thick peptidoglycan cell wall and also produce endospore which make the cell more resistant to stress environmental condition [10]. The antagonist effect of *Bacillus* due to its ability to produce various bioactive compounds including antimicrobial [11] or antifungal peptide [9], lytic enzymes [12], and volatile organic compounds [10, 13]. Akocak et al. [14] reported *Bacillus cereus* and *B. thuringensis* can produce chitinase enzyme to inhibit growth of *Asp. flavus*. Research by Li et al. [8] showed *B. amyloliquefaciens* SYBC H47 has known as biocontrol for *Botryosphaeria dothidea* which caused gummosis. *Bacillus siamensis* JFL 15 produces lipopeptide antifungal compound effective against *Magnapothe grisea*, *Rhizoctonia solani*, and *Colletotrichum gloeosporioides* [15].

Assay for controlling the growth of certain pathogenic microorganisms by using microorganisms is known as the antagonistic test. The antagonist assay can be carried out using dual culture method with pour plate technique [16], discs technique [17] and streak culture technique [11, 18].

All microorganisms used in the study were isolated from cocopeat powder. The experiment was carried out to evaluate the antagonist activity of 3 potential bacterial isolates, KRG, KRT and LDR against *Asp. niger* CP using dual culture method. Identification of bacterial genus was done by phenotypic characters according to Barrow et al. [19]. Morphological characterization of *Asp. niger* CP isolate was done according to Samson et al. [20], and molecular identification was analyzed using the ITS region. Filtration of growth media of selected bacterial isolate will be assayed further in the antibiosis test.

2. Materials and Method

2.1. Isolation and characterization of bacteria

Isolation of three bacteria was performed using spread method on Potato Dextrose Agar (PDA) medium. The bacteria were purified using quadrant streak method [21]. Pure isolates were maintained on PDA slant medium.

The characters of bacteria isolates were identified according to Barrow et al. [19] which consist of morphological observation and biochemical tests. Cell morphology was observed under Leica microscope (Leica ICC50 HD), after Gram staining and spores staining. Cells were measured using Leica LAS EZ 3.0 software programme. The bacteria were also checked for their motility, ability to grow in air and anaerobically, catalase, oxidase, glucose and OF.

2.2. Isolation and characterization of *Aspergillus niger* CP

*Aspergillus niger* CP was isolated and purified by quadrant streak method from spores suspension. The pure isolate was maintained in PDA. Characters of macroscopic and microscopic structure of *Aspergillus niger* CP was observed according to Samson et al. [20]. The microscopic structures were observed using slide culture method [21, 22] under Leica microscope (Leica ICC50 HD) and measured by Leica LAS EZ 3.0 software programme.

Molecular identification was performed by checking the homology of ITS 1 and ITS 4 regions [23, 24] with BLAST and constructing the dendrogram using Neighbor-Joining with K2P and 1000 bootstrap in MEGA X [25]. The DNA extraction, PCR amplification, and DNA sequencing were performed using the Macrogen services (Korea). We analyzed the sequences trimming, BLAST, and constructed the dendrogram. We used *Metarhizium anisopliae* (AF218207) as an outgroup and *Asp. flavus* (EU982011) and *Asp. fumigatus* (EU982013) as representatives of Flavi and Fumigati clade of genus *Aspergillus* [26].
2.3. Antagonist assay of bacteria isolates

Antagonist assay of bacteria against *Asp. niger* CP was done qualitatively using dual culture method [10, 17]. Three techniques were used for dual culture method namely streak [8, 18, 27], disc technique [18, 28, 29] and pour plate [16, 27].

Streak technique was done by streaking the bacteria isolates on PDA medium. The *Asp. niger* CP was stabbed 2 cm beside the bacteria. The plates were incubated for 3 days to observe antagonist effect of the bacteria isolates. Dual culture method using disc technique according to Naglot et al. [29] and El-Debaiky [28] were done by inoculated bacteria and spore suspension in paper disc (ø 6 mm). The paper disc was placed on PDA medium for 2 cm in distance. Antagonist assay results were observed after 7 days incubation. Percentage of inhibition value was expressed as Growth Inhibition Rate (GIR) calculated using formula by Li et al. [8].

\[
GIR (\%) = \left(\frac{C - T}{C}\right) \times 100
\]

with 
\( C \): mycelia growth of the control (mm)
\( T \): mycelial growth in treatment (mm)

Antagonist assay using pour plate technique was done by inoculating 0.1 % (v/v) bacterial cell suspension (10⁷ CFU/mL) to melted Potato Dextrose Agar (PDA) medium in a Petri dish. Meanwhile, *Asp. niger* CP spores were suspended and inoculated on to a paper disc (ø 6 mm). Then, the disc was placed on the centre of PDA plate containing bacterial cells and without bacteria as a control plate. Antagonist assay activity was observed after 7 days incubation.

2.4. Antibiosis assay of selected bacteria isolates

Antibiosis was carried out only from growth filtrate of selected bacteria. The selected bacteria was cultured in Potato Dextrose Broth (PDB) for 7, 10 and 12 days incubation in two replications. Growth medium of the bacteria was centrifuged for 5590 x g for 30 minutes to obtain filtrate medium. The filtrate then was used to dissolve PDB powder (24 g/L) and PDA powder (39 g/L). PDB filtrate and PDA filtrate media were sterilized with autoclave at 121°C for 15 minutes.

About 100 µL suspension of *Asp. niger* CP spores (10⁷ CFU/mL) were inoculated in to 25 ml PDB filtrate and incubated for 7 days. Growth of *Asp. niger* CP was observed and weighted for the dry weight of biomass. Meanwhile for PDA filtrate, spore suspension was inoculated to paper disc (10 µL) and put on PDA filtrate plate. The plates were observed within 5 days incubation. As a control, PDA or PDB medium were dissolved in aquadest solution and also inoculated with *Asp. niger* CP.

3. Results and discussion

3.1. Isolation and characterization of bacteria

As many as 3 different morphological characters were observed and isolated. The colonies were labelled as KRG, KRT and LDR according to their colony appearance. Microscopic observation of bacteria morphology showed that the cells are rod shape and produce central spores (figure 1a).

All bacteria gave the same reaction to the biochemical tests as presented in table 1. The LDR isolate has stronger catalase activity and more active metabolism in fermentation condition compared to KRG and KRT (figure 1b and figure 1c). According to Barrow et al. [19] aerobic bacteria with rod shape and endospore are characters of the genus *Bacillus*.

3.2. Isolation and characterization of Aspergillus niger CP

Macroscopic colony of *Aspergillus niger* CP is shown in figure 2a. *Aspergillus niger* CP colony within 4 days incubation was 67.1 mm in diameter. The colony colour of *Aspergillus niger* CP is black with granular texture and white to yellow in the reverse of the colony.
Figure 1. Characterization of bacteria isolates: (a) spore, (b) fermentative test; (c) catalase test

Table 1. Characters of bacteria isolates

| Characters            | Bacillus sp. (Barrow et al., 1993) [19] | Isolates |
|-----------------------|----------------------------------------|----------|
|                       |                                        | KRG      | KRT      | LDR      |
| Shape                 | Rod                                    | +        | +        | +        |
| Spore                 |                                        | +        | +        | +        |
| Motility              | D                                      | +        | +        | +        |
| Growth in air         |                                        | +        | +        | +        |
| Growth anaerobically  |                                        | D        | +        | +        |
| Catalase              |                                        | +        | +        | +        |
| Oxidase               |                                        | D        | -        | -        |
| Glucose               |                                        | +        | +        | +        |
| OF                    |                                        | O / F / -| O / F    | O / F    |

D: different reactions in different species of the genus; d: different reactions in different strains; +: positive reaction; -: negative reaction; O: oxidation; F: fermentation

Figure 2. Characterization of Asp. niger CP isolate, (a) colony, (b) conidial head (young), (c) conidial head (mature), and (d) conidiospores.

In slide cultures, observation of Aspergillus niger CP can be done directly under the microscope so the development of Aspergillus niger CP structures can be followed easily. The method also provides relatively complete whole structure of Asp. niger CP since there is no disruption of the morphological
structures [21]. Results of structure observation after 2 days incubation is presented in figure 2 (b-d) and table 2. According to Samson et al. [20], the structures of Aspergillus niger CP isolate are similar to Asp. niger. Black colony with dark brown to black conidiospores are specific characters of Asp. niger [20].

3.3. Molecular identification of Aspergillus niger CP
We analyzed the full length of ITS 1 and ITS 4 that covered the small and large subunit of ribosome. All 596 base pairs of Asp. niger CP isolate have 100 % similarity with Asp. niger based on BLAST result (table 3). The Aspergillus niger CP also has 100 % similarity with Asp. welwitschiae (MK450668.1). But since Asp. welwitschiae is synonyms of Aspergillus niger by Mycobank [30], the Asp. niger CP is a group of Aspergillus niger. Based on NJ analysis, the molecular identification supported the macroscopic and phenotypic analysis of Asp. niger CP isolates as Aspergillus niger group (figure 3).

| Table 2. Microscopic observation result of Asp. niger CP isolate. |
|---------------------------------------------------------------|
| Characters | Aspergillus niger (Samson et al.) [20] | Asp. niger CP |
| Conidial head | Radiate Black | Radiate Black 113.78–144.72 µm |
| Conidiospore | Globose to subglobe Globose to subglobe | 4.5–6 µm |
| Conidiospore | 3.5–5 µm | Globose |
| Vesicle | Brown with ornamented structure Dark brown with ornamented structure | 50–100 µm 34.03–57.96 µm |
| Phialide | + | + |
| Metulae | Hyaline to brown + | + |
| Conidiophore | Smooth Hyaline to brown | Smooth Hyaline to brown |

+ : present

| Table 3. Top 10 BLAST result of Asp. niger CP isolate query sequence. |
|---------------------------------------------------------------|
| No | Species | Max score | % Identity | Accession number |
|---|---------|------------|-------------|-----------------|
| 1. | Aspergillus niger (MT588793.1) | 1101 | 100.00 % | MT588793.1 |
| 2. | Aspergillus niger (MT530272.1) | 1101 | 100.00 % | MT530272.1 |
| 3. | Aspergillus niger (MT530261.1) | 1101 | 100.00 % | MT530261.1 |
| 4. | Aspergillus niger (MT530227.1) | 1101 | 100.00 % | MT530227.1 |
| 5. | Aspergillus niger (MT529210.1) | 1101 | 100.00 % | MT529210.1 |
| 6. | Aspergillus niger (MT529192.1) | 1101 | 100.00 % | MT529192.1 |
| 7. | Aspergillus niger (MT529154.1) | 1101 | 100.00 % | MT529154.1 |
| 8. | Aspergillus niger (MT316340.1) | 1101 | 100.00 % | MT316340.1 |
| 9. | Aspergillus niger (MT447518.1) | 1101 | 100.00 % | MT447518.1 |
| 10. | Aspergillus welwitschiae (MK450668.1) | 1101 | 100.00 % | MK450668.1 |
3.4. Antagonist assay of bacteria isolates

Antagonist assay was performed using three different techniques to check the consistency of bacterial potency in antagonist effect. The first qualitative antagonist assay was streak technique. The results showed that the growth of *Asp. niger* CP hyphae facing bacterial colony will be inhibited. The inhibition effects were shown as truncated hyphae of *Asp. niger* CP colony (figure 4).

The second antagonist assay using disc technique also showed inhibition of *Asp. niger* CP growth. The hyphae of *Asp. niger* CP which is facing the bacteria were inhibited by bacteria isolates KRG, KRT or LDR. The inhibition was shown as truncated hyphae (figure 5). Percent inhibition data were presented in table 4.

The third antagonist assay was pour plate techniques. Antagonist effect results were showed in figure 6. Observation after 7 days of incubation showed the *Asp. niger* CP was inhibited almost 100%. *Asp. niger* CP grew very restricted, only on the disc and no sporulation can be observed. In contrast, diameter of growth of *Asp. niger* CP in a control plate was almost covering surface of medium.

![Figure 3. The position of Aspergillus niger CP isolate in the dendrogram within Aspergillus group and Metarhizium anisopliae as an outgroup.](image)

![Figure 4. Antagonist assay of bacteria isolates against Asp. niger CP using streak technique, (a) KRG, (b) KRT, and (c) LDR.](image)
Figure 5. Antagonist assay of bacteria isolates against *Asp. niger* CP using disc technique, (a) KRG, (b) KRT, and (c) LDR.

Table 4. Percent inhibition hyphae of *Asp. niger* CP against bacteria isolates.

| Isolate | Treatment (mm) | Control (mm) | GIR (%) | Average GIR (%) |
|---------|----------------|--------------|---------|-----------------|
| KRG     | 39.3           | 42.4         | 53.76   | 51.94           |
| KRT     | 31.1           | 34.8         | 63.41   | 61.24           |
| LDR     | 36.4           | 40.9         | 57.18   | 54.53           |

Figure 6. Antagonist assay of bacteria isolates against *Asp. niger* CP using pour plate technique, (a) control *Asp. niger* CP, (b) KRG, (c) KRT, and (d) LDR.

Data obtained from three different antagonist assays showed that all strains of KRG, KRT and LDR bacterial isolate have antagonist effect against *Asp. niger* CP. Further study will be carried out only for *Bacillus* sp. LDR although all strains bacteria have antagonist effect. Consideration to select *Bacillus* sp. LDR was based on the higher activity of catalase enzyme and also OF ability compared to strain KRG and KRT. Activity of catalase enzyme showed ability of bacteria to hydrolyze hydrogen peroxide or other toxins, therefore higher catalase activity will increase live ability of bacteria in environment. The higher OF ability represented metabolism activity of bacteria either in aerobic or anaerobic condition, therefore it was showed *Bacillus* sp. LDR can growth faster in both condition. Cells of *Bacillus* sp. LDR strain was also smaller, and it means have higher ratio of volume and surface area. Further study then would be carried out to investigate antagonist mechanism of the selected bacterial isolate, *Bacillus* sp. LDR.
3.5. Antibiosis assay of LDR isolate

Antibiosis study was carried out to check the possibility of bacterial isolate to produce antifungal compound. Antibiosis study was performed only with the LDR strain isolate as selected bacterial isolate. Antibiosis assay was performed by growing the Asp. niger CP in PDA filtrate and PDB filtrate.

Results observation of antibiosis assay on PDA filtrate after 5 days incubation was presented in table 5 and figure 7. Data showed that growth of Asp. niger CP in PDA filtrate of 7 days filtrate was not inhibited. Inhibition of Asp. niger CP growth appeared in PDA filtrate of 10 and 12 days filtrate. No significant difference in diameter was observed between filtrate of 10 days (13.09 mm) and 12 days (13.25 mm). Most probably antifungal compound was already produced at 10 days incubation of LDR isolate.

Antibiosis effect of LDR filtrate on growth of Asp. niger CP was also observed in PDB. Effect of antibiosis was measured as dry weight of Asp. niger CP biomass (table 6). The antibiosis effect from dry weight same as growth inhibition of Asp. niger CP on PDA plate. The dry weight of Asp. niger CP was harvested after 7 days incubation from PDB of 10 days filtrate only 0.028 g and reduced to 0.012 g in PDB of 12 days filtrate. These results also suggest that the LDR bacteria was already producing antifungal compound in 10 days incubation.

Based on characters of the 3 bacteria isolates, KRG, KRT and LDR are aerobic, have rod shape, Gram positive, and have central spore which belong to genus Bacillus [19]. Antagonist assay results showed that all 3 bacteria can inhibit the growth of Asp. niger CP using streak, disc and pour plate of dual culture method. The clearest effect of antagonist was obtained from pour plate technique, where the hyphae growth was restricted only on the paper disc or assumed almost 100 %. Sporulation was also not clear observed.

| Replication | Diameter colonies of Asp. niger CP (mm) |
|-------------|----------------------------------------|
|             | Control | 7 days | 10 days | 12 days |
| 1           | 84.36   | 83.26  | 12.51   | 12.37   |
| 2           | 82.95   | 82.92  | 12.87   | 12.90   |
| 3           | 84.50   | 82.40  | 13.90   | 14.49   |
| Average (mm)| 83.94   | 82.86  | 13.09   | 13.25   |
| GIR (%)     | 1.29 %  | 84.41 %| 84.21 % |

![Figure 7](image-url)  
**Figure 7.** Colony of Asp. niger CP within 5 days incubation on (a) normal PDA, PDA filtrate of: (b) 7 days, (c) 10 days, and (d) 12 days incubation.
The Table 6. Biomass dry weight of Asp. niger CP grew in PDB filtrate.

| Biomass dry weight of Asp. niger CP (g) | Filtrate | 7 days | 10 days | 12 days |
|----------------------------------------|---------|--------|---------|---------|
| Control                                |         | 0.060  | 0.110   | 0.083   |
| Replication 1                          |         | 0.235  | 0.030   | 0.012   |
| Replication 2                          |         | 0.213  | 0.025   | 0.012   |
| Average                                |         | 0.224  | 0.028   | 0.012   |
| GIR (%)                                |         |        | 74.55 % | 85.54 % |

The result of pour plate antagonist assay most probably because the Bacillus sp. KRG, KRT, and LDR were directly faced to Asp. niger CP, without any distance of growth. Such condition will make bacteria more competitive in utilizing nutrient in agar plate, growth in abundant cells and produce secondary metabolites which would impact to restricted growth of Asp. niger CP hyphae. In the case of streak and disc technique, the distance of inoculating cell of bacteria and mold, gave more chance to both microorganisms to grow. The distance between the two microorganisms made the secondary metabolite of bacteria was not effective enough yet to inhibit growth of the Asp. niger CP hyphae. That is the reason why the percentage of inhibition in disc technique is only about 51.94 % up to 54.53 %, less than percentage inhibition in pour technique, which assumed almost 100 %.

The result of antibiosis assay from 10 days filtrate fermentation of Bacillus sp. LDR showed percentage inhibition was higher than 7 days fermentation. It was due to accumulation of antifungal compound in fermentation medium. The Bacillus sp. LDR showed antifungal activity against Asp. niger CP. Lee et al. [31] reported Bacillus siamensis H30-3 isolated from from rhizosphere of tomato plants. Bacillus siamensis H30-3 can produce cellulase enzyme and antifungal-producing bacteria towards fungal phytopathogen in Chinese cabbage, Alternaria brassicola and Colletotrichum sp. [31].

4. Conclusion
The three bacteria isolates (KRG, KRT and LDR) have antagonistic effect on Asp. niger CP. The Bacillus sp. LDR isolate produced antifungal compound at 10 days incubation which was exhibited by antibiosis assay.

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