Antagonistic activity screening of *Bacillus siamensis* LDR against fungal associated with crop contamination

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**Abstract.** *Bacillus* spp. are potential biocontrol agent that commonly used to reduce contamination on crop caused by microorganism, especially fungi. The purpose of this research is to investigate the antagonistic activity of *B. siamensis* LDR towards fungal crop contaminant (*Aspergillus flavus* AHM, *Aspergillus clavatus* ABH, and *Aspergillus tamarii* KCP). Antagonist assay was conducted using the dual culture method, streak and pour plate technique. The streak technique showed *B. siamensis* LDR can inhibit the *A. flavus* AHM, *A. clavatus* ABH, and *A. tamarii* KCP with percent inhibition of 42.75 %, 58.89 %, and 28.46 %, respectively. Meanwhile, the pour plate technique represented higher percent inhibition of *A. flavus* AHM, *A. clavatus* ABH, and *A. tamarii* KCP, 89.28 %, 87.32 %, and 66.35 %, respectively.

**Keywords:** Antagonistic activity, *Bacillus siamensis* LDR, dual culture

1. **Introduction**

Filamentous fungi have been contaminating crops across worldwide, become harmful since they produce mycotoxins and threat the food safety [1]. Among those filamentous fungi, *Aspergillus* spp. have high occurrence of contamination such as *Aspergillus flavus* which is able to contaminate various beans [2] and *Aspergillus clavatus* which contaminates barley [3]. It can also be found on grains that are widely intended for human staple food and animal feed [2, 4, 5]. *Aspergillus flavus* has the ability to produce aflatoxin A1, which is mutagenic for human consumption and hepatotoxic to poultry [2]. *Aspergillus clavatus* produces patulin which is assumed to have neurotoxic effect on cattle [4]. Meanwhile, *Aspergillus tamarii* can produce toxic compounds, including cyclopiazonic acid, and kójic acid, a less toxic compound [6].

*Bacillus* is known as potential biocontrol for various microorganism, including filamentous fungi. Biocontrol agent has specific antagonistic mechanism against certain species or strain microorganism and it is considered as environmentally friendly. Potential biocontrol agent is commonly found in the surrounding area of the crops, usually has a plant-growth inducing properties, and biodegradable [7, 8]. *Bacillus siamensis* LDR is indigenous isolate which isolated from cocopeat. *Bacillus siamensis* LDR, one of *Bacillus* species reported has antagonistic activity towards *Aspergillus niger* ART and ABP [9]. Previous study [10] also reported antifungal production of *B. siamensis* LDR in various substrate
fermentations. There were only few studies of \textit{B. siamensis} until this paper was written. Therefore, in this study, the antifungal activity of \textit{B. siamensis} LDR as biocontrol agent against several fungal associated with crop will be explored. The antagonistic and antibiosis activity which reflect the antifungal activity of \textit{B. siamensis} LDR were evaluated towards another fungal associated with crop contamination, \textit{A. flavus} AHM, \textit{A. clavatus} ABH and \textit{A. tamarii} KCP.

2. Materials and method

2.1. Microorganisms
\textit{Bacillus siamensis} LDR and fungal crop contaminant, \textit{Aspergillus flavus} AHM, \textit{Aspergillus clavatus} ABH, and \textit{Aspergillus tamarii} KCP were obtained from Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. All microorganisms were purified using quadrant streak method on Potato Dextrose Agar (PDA) medium. The pure isolates were preserved on PDA slant medium.

2.2. Morphological characterization of fungal crop contaminant
Characterization of fungi was examined according to the macroscopic and microscopic characters. Macroscopic characters were observed by appearance (color and texture) of fungi colony within 7 days on PDA. Microscopic characters, such as conidiophore and vesicle were observed using slide culture method [11, 12] under microscope. The microscopic characters were determined with Leica LAS EZ 3.0 software programm.

2.3. Antagonistic activity assay
Antagonist assay was conducted by dual culture method with streak and pour plate technique on PDA. The streak technique was performed according to Djellel et al. [13]. \textit{Bacillus siamensis} LDR was streaked on PDA, then fungal contaminant, \textit{A. flavus} AHM, \textit{A. clavatus} ABH, or \textit{A. tamarii} KCP was stabbed 2 cm opposite from the bacteria. Meanwhile, pour plate technique was carried out according to Einloft et al. [14]. Suspension cell of \textit{B. siamensis} LDR (200 µL) was inoculated into molten PDA and then poured into petridish. Spore of fungi were suspended then inoculated to paper disc. The paper disc was placed on the center of plate. Moreover, the paper disc containing spore of fungi was also placed on PDA without \textit{B. siamensis} LDR as control.

The positive result of antagonistic activity assay was shown by inhibition growth of fungi after 6 days incubation. The inhibition was represented as Percent Inhibition of Radial Growth (PIRG) according to Malleswari [15]. The radius of fungi colony which opposite from the bacteria colony on streak plates was measured, meanwhile for pour plates diameter of inhibited fungi colony was measured using caliper. The radius or diameter colony was calculated to obtain PIRG value, where $A_1$ and $A_2$ are the radius or diameter of fungi colony in control plate and treatment plate, respectively.

$$PIRG (\%) = \frac{A_1-A_2}{A_1} \times 100 \%$$

3. Results

3.1. Morphological characterization of fungal crop contaminant
Macroscopic characters of \textit{Aspergillus flavus} AHM was exhibited granular texture, earth green yellow surface colony, and earth green reversed colony on PDA (figure 1a). Microscopic characters of \textit{A. flavus} AHM that were observed including long conidiophore, sub globose to globose shaped vesicles (20.96–13.14 µm x 18.62–14.18 µm), uniseriate conidia with sub globose to globose with echinulate ornamentation (figure 1b). According to literature, most of \textit{A. flavus} strain has biseriate conidia and rarely uniseriate [16].
Aspergillus clavatus ABH on PDA has pine green colony color, hyaline reverse colony, velvety texture, and radial furrow (figure 2a). Microscopic characters observed in A. clavatus ABH including vesicle with clavate-shaped (7.76–13.10 µm x 6.56–10.99 µm), uniseriate conidia and produced conidiospores with ellipse-shaped and smooth texture which sized 3.60–5.08 µm x 3.24–4.39 µm (figure 2b). According to the literature, those characters were similar to A. clavatus. Conidial head of A. clavatus was commonly found in columnar shaped and when the conidiospores has released, the shape of vesicle revealed as clavate (figure 2b) [16, 17].

Macroscopic morphology of Aspergillus tamarii KCP which cultured on PDA showed yellow brown which became dark green-brown surface colony, and hyaline reversed colony (figure 3a). Microscopic characters observation on A. tamarii KCP showed rough cell wall of conidiophore, vesicle globose to sub globose (17–23 µm), with uniseriate or biseriate. Conidial head is radiate (41–67 µm) with conidiospore (4–6 µm) ornamented with warts (figure 3b). According to Samson et al. [16], those characters were similar to A. tamarii.

![Figure 1](attachment:image1.png)

**Figure 1.** Morphological observation of A. flavus AHM: (a) fungi colony and (b) conidial head.

![Figure 2](attachment:image2.png)

**Figure 2.** Morphological observation of A. clavatus ABH: (a) fungi colony and (b) conidial head.

![Figure 3](attachment:image3.png)

**Figure 3.** Morphological observation of A. tamarii KCP: (a) fungi colony and (b) conidial head.
3.2. Antagonist assay of Bacillus siamensis LDR

Bacillus siamensis LDR was capable to deter the growing of Aspergillus flavus AHM, Aspergillus clavatus ABH, and Aspergillus tamarii KCP. Result of antagonist assay using streak technique is shown in table 1. Colony of fungal crop contaminant were inhibited when faced with B. siamensis LDR (figure 4). The highest percent inhibition of radial growth (PIRG) was shown by A. clavatus ABH with 58.89 %. Meanwhile, PIRG value of A. tamarii KCP only 28.46 %.

Result of antagonist assay using pour plate technique is shown in table 2. Bacillus siamensis LDR can also inhibit A. flavus AHM, A. clavatus ABH, and A. tamarii KCP (figure 5). The PIRG value of A. flavus AHM, A. clavatus ABH, and A. tamarii KCP were 89.28 %, 87.32 %, and 66.35 %, respectively.

Based on PIRG value of streak (table 1) and pour plate technique (table 2), A. tamarii KCP has higher resistance towards B. siamensis LDR, compared to A. flavus AHM and A. clavatus ABH. The PIRG value of A. tamarii KCP was 28.46 % and 66.35 % in streak and pour plate technique, respectively.

Table 1. Result of antagonist assay of B. siamensis LDR using streak technique.

| Fungi isolate          | Replication | Control (mm) | Treatment (mm) | PIRG (%) |
|------------------------|-------------|--------------|----------------|----------|
| Aspergillus flavus AHM | 1           | 33.36 ± 0.32 | 18.48          | 44.60    |
|                        | 2           | 19.66        | 41.07          |          |
|                        | 3           | 19.16        | 42.57          |          |
| average                |             | 19.10 ± 0.59 | 42.75 ± 1.77   |          |
| Aspergillus clavatus ABH | 1         | 39.39 ± 0.92 | 15.75          | 60.02    |
|                        | 2           | 16.41        | 58.34          |          |
|                        | 3           | 16.42        | 58.31          |          |
| average                |             | 16.19 ± 0.38 | 58.89 ± 0.98   |          |
| Aspergillus tamarii KCP | 1          | 37.79 ± 0.50 | 27.22          | 27.97    |
|                        | 2           | 27.23        | 27.94          |          |
|                        | 3           | 26.65        | 29.48          |          |
| average                |             | 27.03 ± 0.33 | 28.46 ± 0.88   |          |

Figure 4. Result of streak antagonist assay of B. siamensis LDR towards (a) A. flavus AHM, (b) A. clavatus ABH, and (c) A. tamarii KCP.
Table 2. Result of antagonist assay of *B. siamensis* LDR using pour plate technique.

| Fungi isolate       | Replication | Control (mm) | Treatment (mm) | PIRG (%) |
|---------------------|-------------|--------------|----------------|----------|
| *Aspergillus flavus* AHM | 1           | 80.14 ± 0.14 | 8.35           | 89.58    |
|                     | 2           | 9.00         | 88.77          |          |
|                     | 3           | 8.43         | 89.48          |          |
| average             |             | 8.59 ± 0.35  | 89.28 ± 0.44   |          |
| *Aspergillus clavatus* ABH | 1           | 47.33 ± 0.18 | 6.00           | 87.32    |
|                     | 2           | 6.00         | 87.32          |          |
|                     | 3           | 6.00         | 87.32          |          |
| average             |             | 6.00 ± 0.00  | 87.32 ± 0.00   |          |
| *Aspergillus tamarii* KCP | 1           | 37.79 ± 0.50 | 12.03          | 68.17    |
|                     | 2           | 13.16        | 65.18          |          |
|                     | 3           | 12.96        | 65.71          |          |
| average             |             | 12.72 ± 0.60 | 66.35 ± 1.60   |          |

**Figure 5.** Pour plate antagonist assay of *B. siamensis* LDR towards: (a) *A. flavus* AHM, (b) *A. clavatus* ABH and (c) *A. tamarii* KCP.

4. Discussion

Bioactive compounds are able to be produced by many microorganism including bacteria. The genus *Bacillus* is known to produce antifungal compound which can inhibit the growth of fungi causing plant diseases and spoilage of post-harvest crops. Gordillo et al. [18] reported *Bacillus* sp. strain IBA 33 can inhibit the growth of *A. flavus* and *A. clavatus*. Another research by Zhang et al. [19] notified the growth of *A. flavus* was inhibited by *B. subtilis* B-FS06. Our study also showed, *B. siamensis* LDR can inhibit the growth of *A. flavus* AHM and *A. clavatus* ABH.

*Bacillus siamensis* LDR was also capable to inhibit the growth of *A. tamarii* KCP 28.46 % in streak technique and 66.35 % in pour plate technique. Nevertheless, the PIRG value of *A. tamarii* was lower than *A. clavatus* ABH and *A. flavus* AHM. The results indicated that *A. tamarii* KCP seem to be more resistant than *A. flavus* AHM and *A. clavatus* ABH. So far, it was difficult to find the data of antagonistic effect of *Bacillus* spp. against *A. tamarii*. It is due to the fact that *A. tamarii* is known as food fermentation microorganisms than as storage fungi. *Aspergillus tamarii* produce toxic compounds, including cyclopiazonic acid and kojic acid, less toxic compounds [6]. The existence of *A. tamarii* in high concentration in foodstuffs is obviously undesired. *Aspergillus tamarii* is also considered to be an emerging aetiological agent of human keratomycoses in South India [20].
The percent inhibition growth of fungi tested in pour plate technique was higher than streak technique. It might be due to antifungal compound from *B. siamensis* LDR and nutrition or space competition in plate assay. The cell of *B. siamensis* LDR spread evenly in the media, causing an increase in the occupied surface area. In turn, more nutrients utilized along with antifungal compound were produced. Therefore, the growth of fungi was more inhibited because double effect in pour plate technique. Meanwhile, in streak technique, the growth of fungi can be inhibited only which facing the bacteria colony.

5. Conclusion

*Bacillus siamensis* LDR has an antagonistic activity against *Aspergillus flavus* AHM, *Aspergillus clavatus* ABH, and *Aspergillus tamarii* KCP. Meanwhile, *A. tamarii* KCP was more resistance towards *B. siamensis* LDR. The percent inhibition value from pour plate technique (66.35 % to 89.28 %) are higher than streak technique (28.46 % to 58.89 %).

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