Screening of Antagonistic Activity of *Bacillus siamensis* LDR Against *Fusarium oxysporum, Aspergillus flavus* AHM and *Aspergillus clavatus* ABH

Mahardika Pertiwi[1], Iman Santoso[2*], Qonita Gina Fadillah[1]

[1]*Departement of Biology, Faculty of Mathematic and Natural Science Universitas Indonesia, Kampus UI Depok, 16424, Indonesia*
[2]*Center of Excellence for Indigenous Biological Resources-Genome Studies* (*CoE IBR-GS*), *Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia*

[*]*Corresponding author*: *imans121260@gmail.com*

Abstract. *Bacillus* spp. is a common potential biocontrol agent as an effective alternative to reduce crop contamination by microorganism. The aim of this research is to screen antagonistic activity of *B. siamensis* LDR cells and filtrate against few species of mold, in vitro. Purification and morphology characterization of fungi were done on PDA, which then identified as *Fusarium oxysporum*, *Aspergillus flavus* AHM and *Aspergillus clavatus* ABH. Screening of antagonistic activity was done by antagonistic assay using dual culture method and antibiosis assay using 12 and 14 days fermentation filtrate of *B. siamensis* LDR. Percentage of inhibition were determined by radial growth and biomass of threaten fungi, compared to untreated fungi as control. Result of antagonistic assay showed *F. oxysporum, A. flavus* AHM *and A. clavatus* ABH were inhibited for 26.08%, 42.74% and 27.24% by using dual disc technique and 92.94%, 87.15% and 85.48% by using pour plate technique, respectively. Result of antibiosis assay showed the highest inhibition activity in 14 days fermentation filtrate, which were 41.89% to *F. oxysporum*, 33.22% to *Aspergillus flavus* AHM, 63.54% to *Aspergillus clavatus* ABH on PDA and 80.42%, negative, 89.57% on PDB, respectively. Therefore, *B. siamensis* LDR cell and filtrate has high antagonistic activity against *F. oxysporum, A. flavus* AHM *and A. clavatus* ABH, increasing as domination of the cell in the competition as shown by pour plate and more production antifungal substances in secondary metabolite as predicted in 14 days filtrate.

Keywords : Antagonistic, *Bacillus siamensis*, dual culture, *Aspergillus*, *Fusarium*

Introduction

Mold have been contaminating crops in Indonesia, causing economic loss and could be harmful to health by causing mycosis and produced mycotoxin [6]. Among these molds some of them are *Fusarium oxysporum* which has wide varieties of specific host including tomatoes, bananas and many other fruits [1,5]; *Aspergillus flavus* and *Aspergillus clavatus* also contaminate grains and beans [6,11]. *Bacillus* are known as potential biocontrol of various microorganism, including mold. Biocontrol agent has specific antagonistic mechanism to certain species or strain of other microorganism. Biocontrol agent commonly found in the surrounding area, it usually has a plant-growth inducing property and environmentally friendly [9, 14]. Unfortunately, there were only few research of *B. siamensis* as biocontrol and there was no research of its antagonistic activity against *Fusarium oxysporum*, *Aspergillus flavus* and *Aspergillus clavatus*. Therefore, research of *B. siamensis* antagonistic activity against *F. oxysporum, A. flavus* AHM, *A. clavatus* ABH must be conducted.

Material and Methods

Microorganism

Microorganism were selected from the collection of Applied Microbiology Laboratory, Department of Biology, Faculty of Mathematic and Natural Sciences, Universitas Indonesia. Cultures were purified using quadrant streak method for further treatment and observation.

Morphological Characterization

Morphological characterization of *Bacillus* was observed from its macroscopic and microscopic features. The observed macroscopic characters were color, elevation, margin and texture [3]. On the other hand, the microscopic characters includes size and shape of cell, Gram, endospore and capsule [4] which were observed using one and two days old cultures by using Gram and Schaeffer-Fulton technique [2]. Mold characterization

was carried out by macroscopic and microscopic characterizations. The observed Macroscopic characters were color, texture, radial furrow, zonation and exudate drop. Whereas the microscopic characters were spores, spore bearing structures and some other unique characteristic [10] which were observed using four until six days old slide culture.

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Antagonistic Assay

Antagonistic assay was done by dual culture method, consisting of dual disc and pour plate disc technique on PDA, after six days incubation period with three replication. Both were started by pouring sterilized water to the 5 days old slant culture and the spores were scrapped to make suspension. Dual disc technique was done by adding fungi and bacteria suspension (10 µL), separately on a disc. Disc containing bacteria suspension and disc with fungi suspension was put opposite to each other and placed 3 cm away from the edge of petri dish [7]. Pour plate disc technique was done by inoculating bacteria suspension (200 µL) to an unsolidified PDA media using pour plate method and disc containing fungi suspension was put on top of solidified PDA which was also the center of the plate. Percent inhibition of radial growth (PIRG) of the mold was measured [3].

$$\text{PIRG (\%)} = \frac{(\phi \text{ Control (mm)} - \phi \text{ Threatened (mm)})}{(\phi \text{ Control (mm)})} \; x \; 100\%$$

PIRG = Percent inhibition of radial growth

Antibiosis Assay

Bacillus siamensis LDR was inoculated to PDB and incubated for 2 days to make a fermentation starter (1%, v/v), which was used for still culture fermentation in PDB for 12 and 14 days. To examine antibiosis activity of *B. siamensis* culture filtrate, the cell was separated by centrifugation at 3000 g for 40 minutes. Antibiosis assay was conducted by mixing each of 12 and 14 days *B. siamensis* LDR fermentation filtrate as solvent to each PDA and PDB as fungi growth medium. 10 µL suspension of each *F. oxysporum*, *A. flavus* AHM and *A. clavatus* ABH were inoculated to a 6 mm disc which was put in the center of PDA + filtrate. 100 µL suspension of each fungi were also inoculated to 35 mL of PDB + filtrate. These isolates were incubated in six days and made into three replications. The experiment was repeated three times. Percent inhibition of radial growth (PIRG) on PDA and percent reduction of biomass (PRB) on PDB of the mold were measured [3].

$$\text{PIRG (\%)} = \frac{(\phi \text{ Control (mm)} - \phi \text{ Threatened (mm)})}{(\phi \text{ Control (mm)})} \; x \; 100\%$$

PIRG = Percent inhibition of radial growth

$$\text{PRB (\%)} = \frac{(\text{m Control (g)} - \text{m Threatened (g)})}{(\text{m Control (g)})} \; x \; 100\%$$

PRB = Percent reduction of biomass

Result and Discussion

Morphological Characterization

Bacillus and mold characterization were done in macroscopic and microscopic. Macroscopic and microscopic morphological characterization were done on PDA (Fig. 1 a-c). Macroscopic characteristics of *Bacillus* were translucent, raised, entire to undulate and mucoid. Microscopic morphology (Fig. 1 d & e) were rod shaped, 0.53—0.73 µm x 0,92—2.27 µm, Gram positive and having central to subterminal cell endospore which were 0.3 µm – 0.6 µm. Characters were match to *B. siamensis* [8,9].

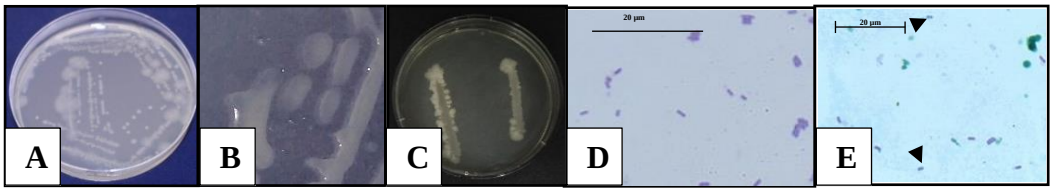
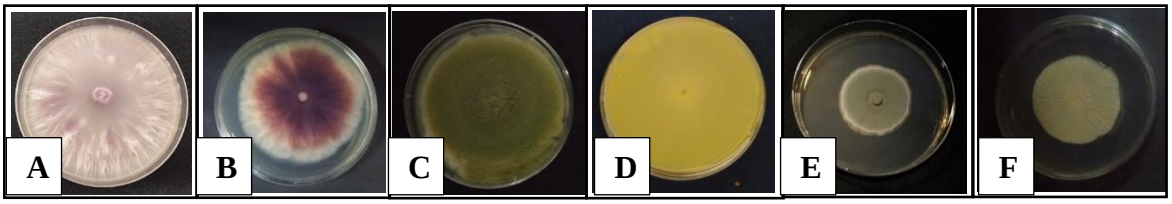
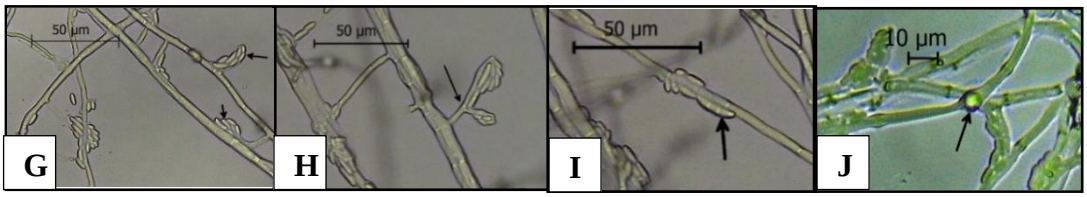


Figure 1. Macroscopic and microscopic morphology observation of *Bacillus siamensis* LDR. (A) pure colony by quadrant streak purification method, (B) mucoid texture and entire margin of colony, (C) undulate margin of colony (slightly inconsistent morphology) (D) Gram positive, (E) central endospore.

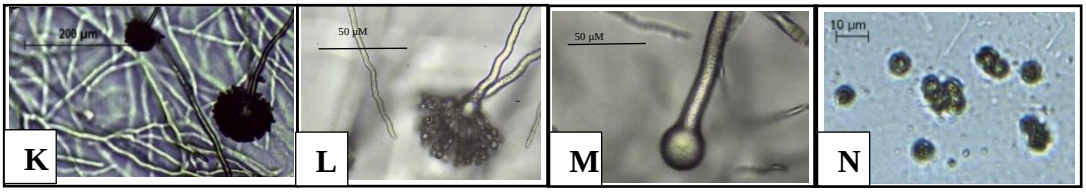
Macroscopic morphology of *Fusarium* observed on PDA (Fig. 2 A & B) were white with light red violet to dark violet pigmented on its surface colony, hyaline reverse colony with light red violet to red violet pigmented, cottony texture and sparse to floccose hyphae. There was no sporodochia observed. Microscopic characteristic (Fig. 2 G—J) which observed were monofialid at lateral conidiophore, microconidia with oval shape which sized 8.53—12.12 µm x 2.66—4.11 µm, macroconidia with fusiform shape which sized 15.48 µm x 3.35 µm (slightly curved, two septate, foot-shaped basal and blunt apical) and single intercalary chlamidiospore on conidiophore. According to literature, those characters approximately match to *F. oxysporum*. Macroconidia commonly varied from three to five septates, with foot-shaped basal and tapered to slightly hooked apical. Shape of macroconidia are more persistent and sporodochia may appear on minimal medium [1]. According to literature, chlamidiospore commonly found single or couple on intercalary or terminal of conidiophore. *Aspergillus* sp. AHM on PDA (Fig. 2 C & D) were granular in texture, earth green yellow colony and earth green reversed colony. Microscopic colony (Fig. 2 K—N) that were observed includes long conidiophore up to 89,44 – 354,37 µm x 6,28 -- 9,33 µm and aseptate hyphae, their vesicles has sub globose to globose shape which sized 20,96 – 13,14 µm x 18,62 – 14,18 µm, uniseriate and their conidia were sub globose to globose shape with echinulate ornamentation. According to literature, those characteristic belongs to *A. flavus*. Differ by its darker color than *A. oryzae* and *A. oryzae* has rough-walled ornamentation of conidia [10].



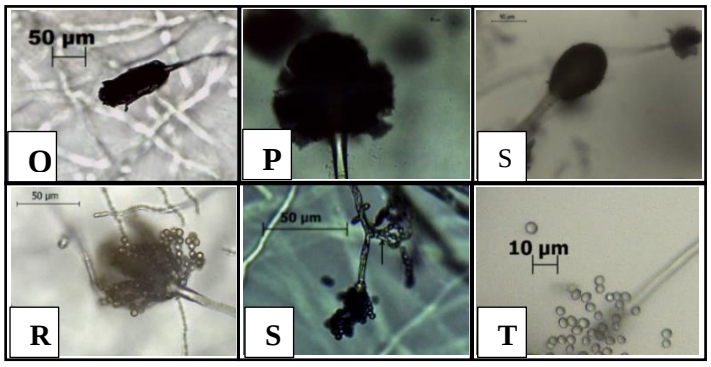
Macroscopic morphology observation *Fusaeium oxysporum* (A) surface (B) reverse, *Aspergillus flavus* AHM (C) surface (D) reverse, *Aspergillus clavatus* ABH (E) surface (F) reverse.



Microscopic morphology observation of *Fusaeium oxysporum* (G) microconidia, (H) monofialid, (I) macroconidia, (J) intercalary chlamidiospore



Microscopic morphology observation of *A. flavus* AHM (K) conidial head with fully covered vesicle, (L) conidial head, (M) vesicle, (N) conidiospore



Microscopic morphology observation of *A. clavatus* ABH (O—R) conidial head, (S) footcell, (T) conidiospore

Figure 2. Macroscopic and microscopic morphology observation of mold.

Aspergillus sp. ABH (Fig. 2 E & F) has pine green colony color, hyaline reverse colony, granular texture and having radial furrow. Microscopic colony observed including vesicle with clavate-shaped in the size of 7,76 – 13,10 µm x 6,56 – 10,99 µm in diameter, uniseriate and produced conidiospores with ellipse-shaped and smooth texture which sized 3,60 – 5,08 µm x 3,24 – 4,39 µm. According to literature, those characteristic were similar to *A. clavatus*. Conidial head commonly found in columnar shape and when conidiospore has released, the shape of the conidial head turn into clavate (Fig. 2 O—Q).

Antagonistic Assay

Bacillus siamensis LDR was able to inhibit the growth of *Fusarium oxysporum*, *Aspergillus flavus* AHM and *Aspergillus clavatus* ABH (Fig. 3). Dual disc technique result shows percent inhibition of radial growth (PIRG) to *F. oxysporum*, *A. flavus* AHM and *A clavatus* ABH were 26,08%, 42,74% and 27,24% respectively. Overall, pour plate disc technique exhibited greater inhibition than 50% PIRG, which were 92,94%, 87,15% and 85,48% to *F. oxysporum*, *A. flavus* AHM and *A clavatus* ABH, respectively. *Bacillus siamensis* LDR was suggested to have ability to compete with *F. oxysporum*, *A. flavus* AHM and *A clavatus* ABH for nutrition and space, also produced secondary metabolite during dual culture. PIRG result was bigger on pour plate disc technique as there was more domination of *B. siamensis* LDR cells that spreads evenly in the media.

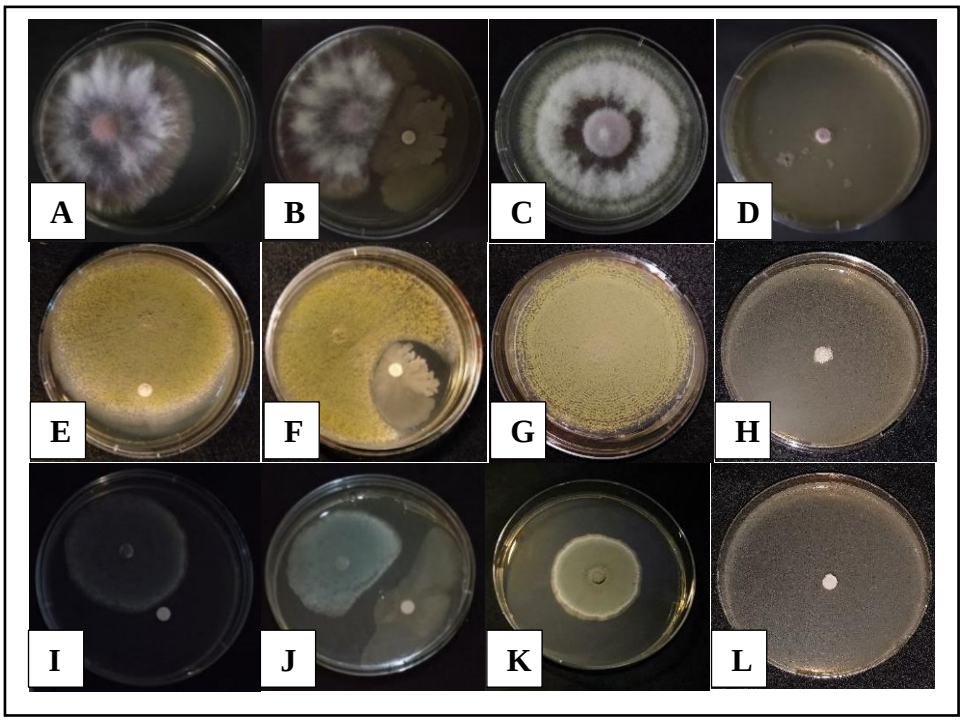


Figure 3. Antagonistic assay (A, B, E, F, I , J) dual disc technique and (C, D, G, H, K, L) pour plate technique of *Bacillus siamensis* LDR against: (A-D) *F. oxysporum*, (E-H) *A. flavus* AHM, (I-L), *A. clavatus* ABH.

Antibiosis Assay

Antibiosis activity of *Bacillus siamensis* LDR (Fig. 4 & 5) occurred on filtrate harvested from 12 days fermentation and much more increased after being extended to 14 days fermentation. Result of treatment by PDA + filtrate 12 days showed percent inhibition of radial growth up to 1.23—7.58%, negative and 20.70% to *Fusarium oxysporum*, *Aspergillus flavus* AHM and *Aspergillus clavatus* ABH, respectively. Result of treatment by PDA + filtrate 14 days showed PIRG was increased up to 41.89%, 33.22% and 63.54% for *F. oxysporum*, *A. flavus* AHM and *A. clavatus* ABH, respectively. Treatment on PDB + filtrate has more significant result. Percent

reduction of biomass (PRB) of PDB + filtrate 12 days were 19.70—33.97%, negative and 28.77% for *F. oxysporum*, *A. flavus* AHM and *A. clavatus* ABH, respectively. While PRB of PDB + filtrate 14 days were 80.42%, negative and 89.57%. Negative result of *A. flavus* AHM inhibition were unstable and the data is not shown. Antibiosis activity was not only reduced the diameter or biomass of fungi, but also gave impact on the morphological characteristic. Therefore, filtrate of *Bacillus siamensis* LDR has antibiosis activity against the three strain of fungi tested.

Antibiosis activity mechanisms are specific. Metabolites which has antibiosis activity were produced after being triggered by the other microorganism existing in the same niche. According to Xu et al. [12] and Meidong et al. [9], *B. siamensis* has ability to produce various hydrolytic enzyme (ribosomal peptide synthesis) and cyclic lipopeptide (non-ribosomal synthesis). Based on Lee at al. [14] and Meidong et al. [9] hydrolytic enzyme yielded by B *siamensis* were protease and cellulase in high concentrations and chitinase in low concentrations. Cyclic lipopeptide protein (CLPs) yielded belongs to Iturin, Fengycin and Surfactin group. Based on research of another species in *Bacillus* genus, Iturin group followed by fengycin plays an important role in antibiosis activity against *Fusarium* and *Aspergillus*. While surfactin triggers any bacteria to produce biofilm and inhibit biofilm formation by other microorganism in the same niche.

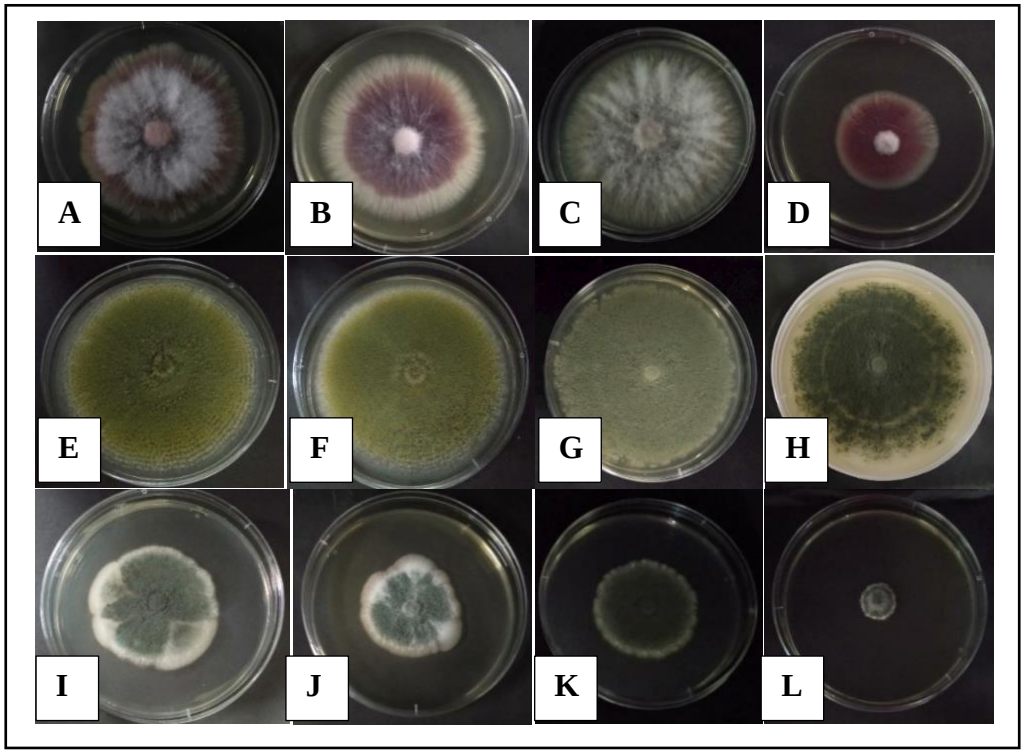


Figure 4. Antibiosis assay (A, B, E, F, I , J) on PDA + 12 days filtrate and (C, D, G, H, K, L) PDA + 14 days filtrate of *Bacillus siamensis* LDR against: (A-D) *F. oxysporum*, (E-H) *A. flavus* AHM, (I-L), *A. clavatus* ABH.

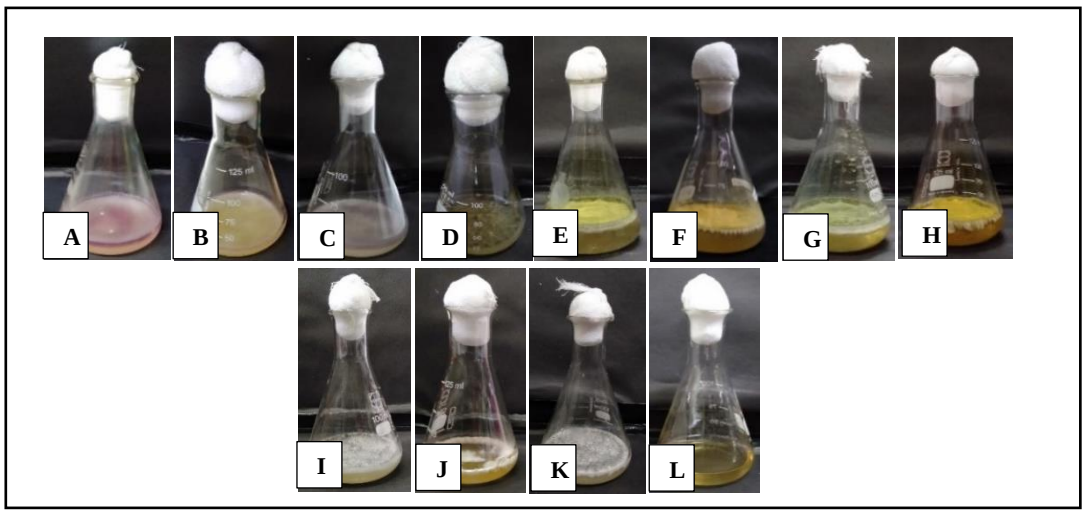


Figure 5. Antibiosis assay (A, B, E, F, I , J) on PDB + 12 days filtrate and (C, D, G, H, K, L) PDB + 14 days filtrate of *Bacillus siamensis* LDR against: (A-D) *F. oxysporum*, (E-H) *A. flavus* AHM, (I-L), *A. clavatus* ABH.

Specifically mentioned on literature, Iturin A prevents spore germination and mycelium forming of *F. oxysporum* by disrupting cell wall integrity and membrane cell structure [13]. Macroscopic morphology appeared to be a thinned aerial hyphae and shortened radial of mycelium. Meanwhile bacillomycin D has the same effect on *A. flavus*. Macroscopic morphology of the fungi appeared to have radial furrow and irregular edge of mycelium. There was no literature about research on the inhibition of *A. clavatus* by *Bacillus* spp., but *A. clavatus* ABH was seen to produce secondary metabolite against *B. siamensis* LDR. The effectivity of filtrate depends to bioactive compound stability and its resistance through enzymatic degradation, extreme temperature and pH. The percent of inhibition to *A. flavus* AHM were unstable might be due to degradation during storage of filtrate which were used to test *A. flavus* AHM inhibition.

Conclusion

Bacillus siamensis LDR has antagonistic activity against *Fusarium oxysporum*, *Aspergillus flavus* AHM and *Aspergillus clavatus* ABH, antibiosis activity was effective on a 14 days fermentation filtrate. Further identification of metabolite produced by *B. siamensis* LDR and in vivo antagonistic assay are recommended.

Acknowledgment

This research was supported by Research Grant awarded from Ministry of Research, Technology and Higher Education basic research funding program to Drs. Iman Santoso, M. Phil. I wish to acknowledge the help provided by Laboratory of Application Microbiology, Department Biology, Faculty of Mathematics and Natural Science for valuable lessons and technical assistance.

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