Eco. Env. & Cons. 27 (February Suppl. Issue) : 2021; pp. (S101-S107)
Copyright@ EM International
ISSN 0971–765X

Antifungal production of *Bacillus siamensis* LDR against *Aspergillus niger* ABP and ART using rice starch as carbon source

Inneke Kusanggraeni[1], Iman Santoso[1,2] and Yasman[1]*

[1]Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok 16424, Indonesia
[2]Center of Excellence for Indigenous Biological Resouces-Genome Studies (CoE IBR-GS), Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok 16424, Indonesia

(Received 26 August, 2020; Accepted 25 September, 2020)

ABSTRACT

Problems can be emerged from contaminated food and agricultural products by *Aspergillus niger*. The growth of *A. niger* can be managed by biocontrol using antifungal compound produced by fermentation process. Nutrient composition of fermentation medium is one of important factors. In this research, production of antifungal compound from *Bacillus siamensis* LDR was carried out against two strains of *A. niger* ABP and ART. Rice starch as carbon source and yeast extract was added into Czapek-Dox medium as a fermentation medium. Fermentation was performed in still culture method within 10, 12, and 14 days. Antibiosis assay was done using filtrate of fermentation medium as solvent for preparing PDB and PDA media. The result was determined based on the biomass growth in PDB and the growth diameter in PDA from *A. niger* ABP and ART. Antibiosis assay using filtrate of 10 days fermentation showed significant effectivity in biomass reduction of *A. niger* ABP (99.20%) and growth diameter inhibition (83.28%). Significant effectivity was shown by filtrate of 12 days fermentation which reduced biomass (89.62%) and growth diameter inhibition (87.95%) of *A. niger* ART. Plate agar antibiosis assay was also done with the crude extract of medium from 12 days fermentation. Filtrate medium was extracted with ethyl acetate using liquid-liquid extraction method. Concentrations of antifungal crude extract were 2,500, 5,000, 10,000, and 20,000 ppm. The inhibition growth of *A. niger* ABP and ART were already observed at 2,500 ppm.

Key words : *Antibiosis, Antifungal, Aspergillus niger, Bacillus siamensis, Rice starch*

Introduction

Agriculture industry is important for Indonesia to supply food demand. One of the problem in agricultural industry is plant-fungal pathogen infection and contamination of agricultural products. One of the fungus that might emerge a problem in agriculture industry is *Aspergillus niger. Aspergillus niger* is commonly attack many fresh fruits, such as apple, tomato, grape, coffee bean, and red bean, and vegetables such as garlic, onion, and potato (Perrone *et al*., 2007; Frisvad *et al*., 2011).

Plant infections and contaminated agricultural products by*A. niger* may reduce economical value and even give health-risk for the consumer. Growth of *A. niger* will decolorize and degrade agricultural product (Perrone *et al*., 2007). On the other hand, *A. niger* also may synthesize a secondary metabolite or mycotoxin, that may cause damages to the internal organ, such as liver, kidney, nervous system,

muscle, skin, respiratory organ, digestive organ; mycotoxin is also carcinogenic (Frisvad *et al*., 2011; Gautam *et al*., 2011; Bui-klimke and Wu, 2015).

Previously, growth of fungal such as *A. niger* can be controlled using synthetic fungicide. Nowdays, the synthetic fungicide are not utilized anymore, because considered as not eco-friendly. Due to the explained reasons, the usage of biocontrol agent that produce natural antifungal compound has been studied intensively (Satish *et al*., 2007).

The use of bacteria as plant pathogen biocontrol agent has been observed extensively (Elkahoui *et al*., 2012; Youcef-Ali *et al*., 2014; Vajpayee *et al*., 2015). Research done by Feio *et al.,* (2004) showed that bacteria from the genus *Bacillus* possess antagonistic and antifungal compounds towards *A. niger*. *Bacillus* bacteria has many advantages, besides thick peptidogly can also grow fast and produce endospores that endure high temperature, making the bacteria ideal candidates as antagonist agent (Grata and Nabrdalik, 2012; Siahmoshteh *et al*., 2016).

Study antagonistic agent of *Bacillus siamensis* LDR by Putri *et al*. (2020) showed that the bacterium produced antifungal compound in PDB medium. One of the factors in fermentation process including producing antifungal agent is the medium composition (Iwai and Omura, 1982). Carbon source and nitrogen are crucial in metabolism. The choice of carbon source is also important in calculating price of fermentation medium, which further determine price of the product (Singh *et al*., 2017). One of the local carbon sources that may abundant and relatively cheap are rice, cassava, and sago starch. Therefore, qualitative study of amylase activity of *B. siamensis* LDR was observed to determine carbon source to be used in fermentation medium for antifungal. Antibiosis test of the antifungal of *B. siamensis* LDR was performed against *A. niger* ABP and ART which have been isolated from garlic and bread respectively.

Materials and Methods

Microorganisms

Bacillus siamensis LDR, *A. niger* ABP, and *A. niger* ART were provided by Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia. All of microorganisms were purified using quadrant streak method and maintained on PDA slant.

Macroscopic and microscopic observation

Identification of *A. niger* ABP and *A. niger* ART isolates were done on the basis of morphological characteristics. Macroscopic features such as colony texture, color, and elevation as well as microscopic such as size of conidia, and their arrangements were examined using slide culture method on PDA medium under microscope (Leica ICC50 HD) and measured with Leica LAS EZ 3.0 software program. *Bacillus siamensis* LDR was also observed for the cell size and endospores.

Qualitative amylase activity

Amylase activity assay of *B. siamensis* LDR was observed qualitatively on plate agar method. The medium used based on Czapek-Dox Agar which consist of 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$, 0.001% $FeSO_4$, and 0,15% agar and as carbon source is 0.1% Rice Starch, 0.1% Tapioca Starch or 0.1% Sago Starch. The plate medium was streaked with *B. siamensis* LDR, then incubated for 7 days at 30 °C. Observation of amylase activity was accomplish by flooding iodine solution for 15 minutes. Formation of clear zone around colonies indicated starch hydrolysis.

Antifungal compound production

Suspension of 1% (v/v) *B. siamensis* LDR was inoculated into 200 mL modified Czapek-Dox Broth Media with composition as follow: 0.2% rice flour, 0.3% yeast extract, 0.3% sucrose, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl and 0.001% $FeSO_4$. Fermentation was performed with still culture method for 10, 12, and 14 days of incubation. Cells were removed by centrifugation at 15,000 g for 25 minutes. Cell-free culture filtrate was collected and used as a solvent for preparing PDA or PDB medium used in antibiosis assay. PDA or PDB-filtrate was sterilized at 121°C for 15 minutes.

Antibiosis assay

PDA-filtrate plate agar

PDA-filtrate medium of antibiosis assay was poured into Petri dish and leaved until it solidified. Prepare the spore suspension of *A. niger* ABP and ART by using sterile aquadest. A paper disc (ø 6 mm) was inoculated with 0.1% spore suspension and aseptically was placed at the center of Petri dish. As a control, inoculated paper disc was placed

on the central of normal PDA plates. Plates were incubated at 30 °C for 5 days and observed for the inhibition of fungal growth. Plate were performed in three replications for each treatment. The growth inhibition of the test fungi was calculated using the following formula (Bharose and Gajera, 2018):

$$Growth\ Inhibition\ Rate\ (GIR)\% = \frac{(C-T)}{C}\ x\ 100\%$$

C = diameter growth of fungal in control PDA
T = diameter growth of fungal in PDA-filtrate

PDB-filtrate medium

About 20 mL PDB-filtrate medium of antibiosis assay was filled into 100 mL Erlenmeyer and inoculated with 100 µL spore suspension of *A. niger* ABP and ART. As a control 100 µL spore suspension was inoculated into normal PDB. After 7 days of incubation, both biomass from treated and control medium were harvested using filter paper (Whatman No. 1). The biomass was dried and weighed. Each treatment were performed in three replications. The percent of weight reduction of fungi was calculated according following formula (Trivedi *et al*., 2008) :

$$\%\ Biomass\ reduction = \frac{(w1-w2)}{w1} x\ 100\%$$

w1 = biomass of fungi in control PDB
w2 = biomass of fungi with PDB-filtrate

Extraction of antifungal compound

Cell-free filtrate of 10, 12 and 14 days fermentation process were extracted with ethyl acetate. Evaporation was done in a rotary evaporator at 40 °C. Mass of crude extract was dried with oven at 40 °C for 48 h, or until the weight was constant. The dry extract was placed in a vacuum desiccator.

Antifungal assay using disc diffusion method

Antifungal activity of crude extract from *B. siamensis* LDR was assayed on PDA plate. Spore of *A. niger* ABP and ART were suspended in sterile aquadest. About 10 µL spore suspension was inoculated into sterile paper disc (ø 6 mm) and then placed into the center of PDA plate. Crude extract of antifungal compound from 12 days fermentation was dissolved using ethyl acetate to prepare concentrations of 2,500, 5,000, 10,000, and 20,000 ppm. Sterile paper disc was placed, approx. 2 cm in the left and right side of paper disc that has been inoculated by *A. niger*, and then filled with 50 µL of each concentration of crude extract. Plates were incubated at 30 °C for 5 days. Plates were perfomed in duplo. Presence of antifungal activity can be observed through the inhibition or formation of hyphal growth.

Results and Discussion

Morphology of microorganisms

Morphology of *A. niger* ABP and ART

Morphological characteristics of *A. niger* ABP and ART on PDA were mostly similar (Fig. 1). Both isolates were grown rapidly over the agar surface within 5 days of incubation. After sporulation, the colony turned into soft black color and its reverse

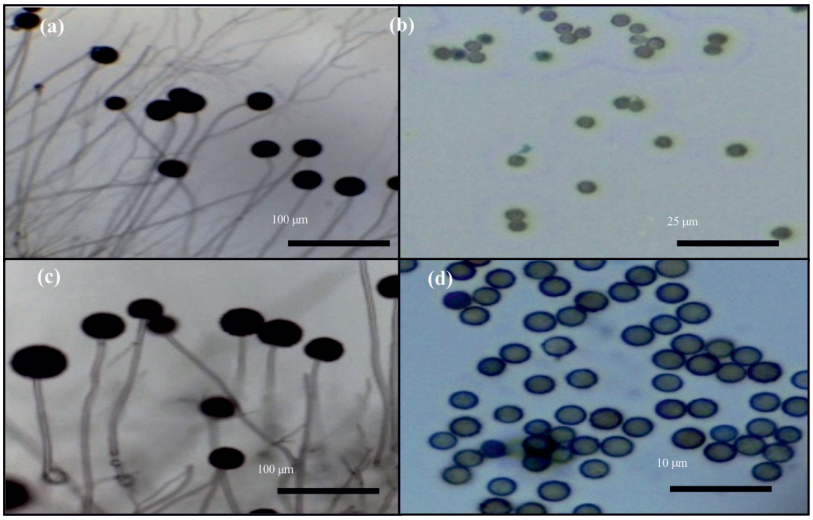

Fig. 1. Microscopic of *A. niger* ABP and ART. *A. niger* ABP: (a) Conidial head (100x); (b) Conidiospore (400x); *A. niger* ART : (c) Conidial head (100×); (d) Conidiospore (1000x).

colony turned into zinc yellow color. Colony of *A. niger* ABP and ART were granular, umbonate elevation, with entire margin. *A. niger* ABP and ART have dark brown to black conidia, with bisseriate conidiophores and globose vesicle. However, the spore ornamentation of *A. niger* ABP was spinulose and for ART was warty.

Morphology of *B. siamensis* LDR

Based on macroscopic observation, colony of *B. siamensis* LDR has circular shape with raised elevation, mucoid texture, with entire margin. Results of microscopic examination showed that isolate has rod-shaped structure with (1.5—3.0) x (0.3—0.5) µm size and belongs to a Gram-positive, spore-forming bacteria (Fig. 2).

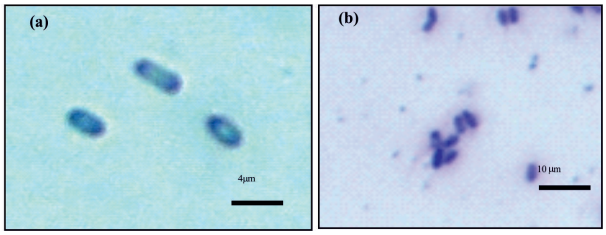

Fig. 2. (a) Endospore of *B. siamensis* LDR (1000x); (b) *B. siamensis* LDR Cell (1000x).

Amylase activity of *B. siamensis* LDR

Bacillus siamensis LDR that was grown on modified Czapek-Dox medium with rice flour, appear to have more clear zone, with the lowest color intensity compare to the other carbon source (Fig. 3). The results indicated that the highest amylase activity was occurred in the medium which contains rice flour. Better growth using yeast extract as nitrogen source might be due to the utilization of yeast extract as a source of B-complex vitamin and growth factor that can optimize bacterial growth (Tortora *et al*., 2010). Gupta *et al*., (2003) also explained, used of organic nitrogen such as yeast extract is more favorable for synthesizing amylase.

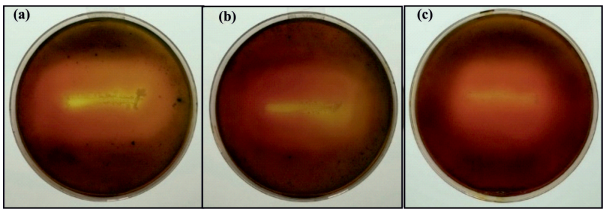

Fig. 3. Results of Amylase Activity Test of *B. siamensis* LDR: (a) Rice Flour; (b). Sago Flour; (c) Tapioca Flour

Antibiosis test using filtrate of fermentation

The result of antibiosis test showed that isolate *B. siamensis* LDR was capable to produce antifungal compound on modified Czapek-Dox medium. Yeast extract and sucrose in the modified Czapek-Dox medium were components that support growth of *B. siamensis* LDR and also the production of antifungal compound. Research done by Hmidet *et al*. (2017) stated that the production of antifungal compound fengycin of *Bacillus* sp. is optimized by using yeast extract as nitrogen source. The research also concluded that adding of sucrose might increase the production of antifungal compound fengycin by *B. subtilis*.

Antibiosis test showed that, filtrate of ten days fermentation has shown positive result. The result in PDA-filtrate plate for *A. niger* ABP and ART can be seen in Fig. 4. The results of PDA-filtrate plate were parallel with antibiosis test in PDB-filtrate. As shown in Fig. 5, the growth of *A. niger* ABP and ART were not as abundant as control, which indicated there was inhibition effect. Data of growth inhibition in Table 1 showed that filtrate of 10, 12, and 14 days of fermentation will inhibit growth of *A. niger* ABP and ART. The highest growth inhibition for *A. niger* ABP was obtained using filtrate of 10 days fermentation (83.28%), while for *A. niger* ART was from filtrate of 12 days fermentation (87.95%).

Data for dry-weight biomass were presented in Table 2. The result of dry-weight biomass of *A. niger* ABP and ART were supported data from Table 1. The highest percentage of biomass reduction of *A. niger* ABP (99.20%) was obtained from filtrate of 10

Table 1. Average of Growth Inhibition Rate (%) of *A. niger* ABP and ART

Incubation Time	*GIR (%) Aspergillus niger*	
	ABP	ART
10 days	83.28%	84.92%
12 days	81.93%	87.95%
14 days	81.51%	85.25%

Table 2. Average of Biomass Reduction (%) of *A. niger* ABP and ART

Incubation Time	*Biomass Reduction (%) Aspergillus niger*	
	ABP	ART
10 days	99.20%	46.64%
12 days	97.05%	89.62%
14 days	92.77%	86.73%

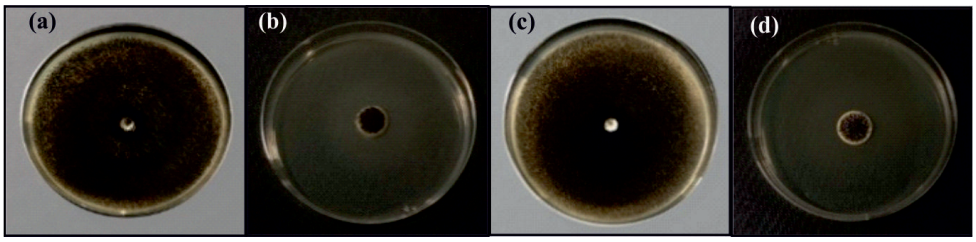

Fig. 4. Antibiosis Test Result Using PDA-filtrate *A. niger* ABP: (a) Control; (b) Filtrate of 10 Days Fermentation. *A. niger* ART :(c) Control; (d) Filtrate of 12 Days Fermentation

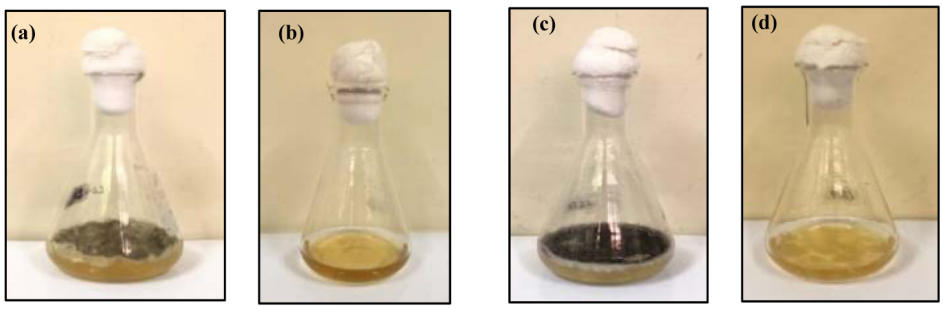

Fig. 5. Antibiosis Test Result Using PDB-filtrate: *A. niger* ABP: (a) Control; (b) Filtrate of 10 Days Fermentation. *A. niger* ART: (c) Control; (d) Filtrate of 12 Days Fermentation.

days fermentation, and for *A. niger* ART (89.62%) was from filtrate of 12 days fermentation. The different results of antibiosis effectivity in *A. niger* ABP and ART may be due to the difference of strain between *A. niger* ABP and ART. The research done by Lahtchey *et al*., (2008) stated that different strain may show different response towards antimicrobial agent.

Antibiosis test using disc diffusion method

50 mL crude extract of 20,000, 10,000, 5,000, and 2,500 ppm was dropped in each treated paper disc. The concentration used was equal to 1, 0.5, 0.25, 0.125 mg. The antibiosis test of crude extract indicated the presence of antibiosis activity. The extracts of antifungal compound with the concentration of 20,000, 10,000, 5,000, and 2,500 ppm inhibited the growth of *A. niger* ABP and ART isolates. The colony of *A. niger* ABP and ART as seen in Fig. 6 will show groove formation around treated paper disc. The groove formation was predicted as an indication that the antifungal compound inhibited the hyphae of *A. niger* ABP and ART. Groove can be seen clearly in the higher concentration, but in lower concentration, the groove was not so clear. Mardanova *et al*. (2017) stated that antifungal compound may cause hyphae growth inhibition.

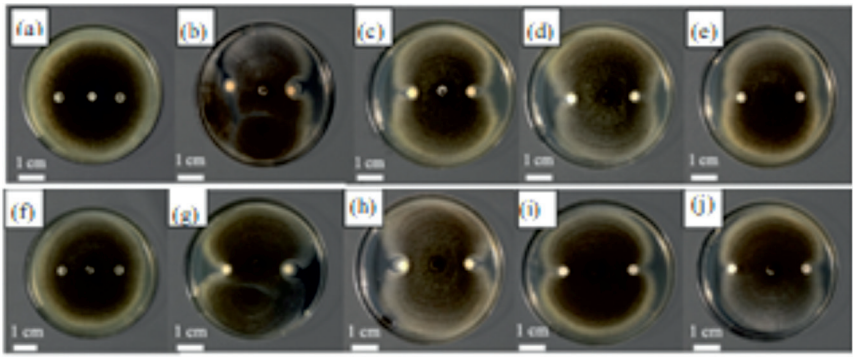

Fig. 6. Antibiosis test of *A. niger* ABP (a-e) and ART (f-j). (a & f): Control; (b & g):20,000 ppm; (c & h): 10,000 ppm; (d & i): 5,000 ppm; (e & j): 2,500 ppm

Conclusion

The best amylase activity of *B.siamensis* LDR was occured in medium containing rice flour as carbon source. *Bacillus siamensis* LDR were able to grow and produce antifungal in the modified Czapek-Dox medium added with yeast and rice starch susbtances. The antifungal compounds was already produced within 10 days fermentation. It seems that there was no significant differences of antifungal activity related to duration of fermentation time. But apparently, strain of *A. niger* ABP was more sensitive than ART, to the antifungal compound produced by *B. siamensis* LDR as revealed in the percentage of biomass reduction.

Acknowledgement

The research was financially supported by Universitas Indonesia of grant research Final Project Indexed International Publication B (PITTA B) to Dr. rer. nat. Yasman, M.Sc with grant number: 476/SK/R/UI/2019.

References

Bharose, A.A. and Gajera, H.P. 2018. Antifungal activity and metabolites study of *Bacillus* strain against aflatoxin producing *Aspergillus*. *J. Appl. Microbiol. Biochem.* 2 : 1-8. https://doi.org/https://doi.org/10.21767/2576-1412.100024

Bui-Klimke, T.R. and Wu, F. 2015. Ochratoxin A and human health risk: A review of the evidence. *Crit. Rev. Food Sci.* 55 : 1860-1869. https://doi.org/https://doi.org/10.1080/10408398.2012.724480

Elkahoui, S., Djébali, N., Tabbene, O., Hadjbrahim, A., Mnasri, B., Mhamdi, R., Shaaban, M. and Limam, F. 2012. Evaluation of antifungal activity from *Bacillus* strains against *Rhizoctonia solani*. *Afr. J. Biotechnol*. 11 : 4196-4201. https://doi.org/https://doi.org/10.5897/AJB11.3354

Feio, S.S., Barbosa, A., Cabrita, M., Nunes, L., Esteves, A., Roseiro, J.C. and Curto, M.J.M. 2004. Antifungal activity of *Bacillus subtilis* 355 against wood-surface contaminant fungi. *J. Ind.Microbiol. Biot*. 31: 199-203.https://doi.org/https://doi.org/10.1007/s10295-004-0133-x

Frisvad, J.C., Larsen, T.O., Thrane, U., Meijer, M., Varga, J., Samson and R.A. and Nielsen, K.F. 2011. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. *PLoS One.* 6: 1-6.https://doi.org/10.1371/journal.pone.0023496

Gautam, A.K., Sharma, S., Avasthi, S. and Bhadauria, R. 2011. Diversity, pathogenicity and toxicology of *A.niger*: an important spoilage fungi. *Res. J. Microbiol.* 6 : 270-280. https://doi.org/https://doi.org/10.3923/jm.2011.270.280

Grata, K. and Nabrdalik, M. 2012. Antifungal activity of *Bacillus* spp. against *Fusarium* spp. *Proc. Ecopole.* 6 : 151-155.https://doi.org/https://doi.org/10.2429/proc.2012.6(1)020

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K. and Chauhan, B. 2003. Microbial α-amylases: a biotechnological perspective. *Process. Biochem.* 38 : 1-18. https://doi.org/10.1016/S0032-9592(03)00053-0

Hmidet, N., Ayed, H.B., Jacques, P. and Nasri, M. 2017. Enhancement of surfactin and fengycin production by *Bacillus mojavensis* A21: application for diesel biodegradation. *Biomed. Res. Int.* 2017: 1-8.https://doi.org/10.1155/2017/5893123

Iwai, Y. and Omura, S. 1982. Culture conditions for screening of new antibiotics. *J. Antibiot.* 35 : 123-141. https://doi.org/https://doi.org/10.7164/antibiotics.35.123

Lahtchev, K.L., Batovska, D.I., St P, P., Ubiyvovk, V.M. and Sibirny, A.A. 2008. Antifungal activity of chalcones: A mechanistic study using various yeast strains. *Eur. J. Med. Chem.* 43 : 2220-2228.https://doi.org/10.1016/j.ejmech.2007.12.027

Mardanova, A.M., Hadieva, G.F., Khilyas, M.T., Minnullina, I.V.E., Gilyazeva, L.F., Bogomolnaya, A.G., Sharipova, L.M. and Lutfullin, M.R. 2017. *Bacillus subtilis* strains with antifungal activity against the phytopathogenic fungi. *Agric. Sci.* 8 : 1-20.https://doi.org/https://doi.org/10.4236/as.2017.81001

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakarnchanakul, W. and Samson, R.A. 2007. Biodiversity of *Aspergillus* species in some important agricultural products. *Stud. Mycol.* 59 : 53-66.https://doi.org/10.3389/fmicb.2014.00377

Putri, B. R., Santoso, I. and Yasman. 2020. Antagonistis potential of *Bacillus siamensis* LDR against *Aspergillus niger* ABP and ART. *AIP Conf. Proc.* 2242: 1—7. https://doi.org/10.1063/5.0012314

Satish, S., Mohana, D.C., Ranhavendra, M.P. and Raveesha, K.A. 2007. Antifungal activity of some plant extracts against important seed borne pathogens of *Aspergillus* sp. *J. Agric. Tech.* 3 : 109-119.http://www.ijat-aatsea.com/pdf/JUN_V3_07/9-IJAT2007_12-R.pdf

Siahmoshteh, F., Hamidi-Esfahani, Z. and Razzaghi-Abyaneh, M. 2016. Antifungal activity, biodegradation and production inhibition of aflatoxins B. *J. Pure App. Microbiol.* 10 : 2541-2549.https://doi.org/https://doi.org/10.22207/JPAM.10.4.08

Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti M. and Tripathi, C.K.M. 2017. Strategies for fermentation medium optimization: an in-depth review.

Front. Microbiol. 7 : 1-16.https://doi.org/https://doi.org/10.3389/fmicb.2016.02087

Tortora, G.J., Funke B.R. and Case, C.L. 2010. *Microbiology: An Introduction. 10th ed.* Pearson Benjamin Cummings, San Francisco.

Trivedi, P., Pandey A. and Palni, L.M.S. 2008. *In vitro* evaluation of antagonistic properties of *Pseudomonas corrugata*. *Microbiol. Res.* 163 : 329-336. https://doi.org/10.1016/j.micres.2006.06.007

Vajpayee, G., Asthana, S., Purwar, S. and Sundaram, S. 2015. Screening of antagonistic activity of bacteria against *Tilletia indica*. *Indian J. Nat. Sci.* 5 : 4302-4303.

Youcef-Ali, M., Chaouche, N.K., Dehimat, L., Bataiche, I., Mounira, K.A.R.A., Cawoy, H.E. and Thonart, P. 2014. Antifungal activity and bioactive compounds produced by *Bacillus mojavensis* and *Bacillus subtilis*. *Afr. J. Microbiol. Res.* 8 : 476-484.