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Research Article

Antifungal Activity of Bacterial Isolates from Straw Mushroom Cultivation Medium against Phytopathogenic Fungi

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ABSTRACT

Several bacteria were isolated from straw mushroom (*Volvariella volvacea*) cultivation medium. There were three potential isolates previously characterized and had a growth inhibition effect against *V. volvacea*. This screening result leads to further study of the inhibition activity against phytopathogenic fungi. This research aimed to investigate the antifungal activity of three bacterial isolates against three phytopathogenic fungi and identification of the bacteria. The methods used in this study were antifungal assay using co-culture method and disk diffusion assay using the filtrate of each bacteria. The profile of the antifungal compound was identified using ethyl acetate extract followed by evaporation and gas chromatography (GC-MS) analysis. Identification of each isolate was performed using 16S rDNA amplification and sequencing. Three phytopathogenic fungi *i.e Cercospora lactucae* (InaCC F168), *Colletotrichum gloeosporioides* (InaCC F304), and *Fusarium oxysporum* f.sp. *cubense* (F817) were co-cultured with bacterial isolates C2.2, C3.8, and D3.3. The C3.8 isolate has the highest average inhibition activity either using isolate and filtrate. The result is relatively consistent against three phytopathogenic fungi. The metabolite profile of the C3.8 isolate showed the Bis(2-ethylhexyl) phthalate as the main compound with 97% similarity. Bis(2-ethylhexyl) phthalate had a potential effect as an antibacterial and antifungal compound. According to EzBioCloud and GeneBank databases, the C2.2 isolate was identified as *Bacillus tequilensis*, C3.8 as *Bacillus siamensis*, and D3.3 as *Bacillus subtilis subsp. subtilis*. This study also showed the potential of *Bacillus siamensis* C3.8 as biocontrol against phytopathogenic fungi.

Keywords: antifungal, biocontrol, plant pathogen, bioactive compound, identification

INTRODUCTION

Mushroom cultivation is a process to grow fungus in the artificial cultivation medium to produce fruiting bodies. The main process is based on the solid fermentation of several substrates under controlled conditions. The bacteria and fungi have the major roles in converting raw materials into ready-to-use substrates, minimizing the contaminants, and inducing the development of fruiting bodies (Kertesz & Thai 2018; McGee 2018; Vieira & Pecchia 2018). The microbes present in the cultivation medium strongly influence the

fungal growth and the development of fruiting bodies (Carrasco & Preston 2020). The varieties of bacteria-fungal interaction in mushroom cultivation have been described as either positive or negative for the fungal growth, depend on the bacterial characteristics and the growth stage of the fungus (Frey-Klett et al. 2011)

The beneficial microbes in the mushroom cultivation medium can promote mycelial growth even increasing the yield of fruiting bodies. *Bacillus cereus* W34 previously reported has a growth-promoting ability and increases the yield of fruiting bodies in straw mushroom cultivation (Jemsi & Aryantha 2017). Several other bacteria from genera *Alcaligenes, Lysinibacillus, Paenibacillus, Pandorea, Pseudomonas*, *and Streptomyces* also were reported as potential mushroom growth-promoting bacteria (Xiang et al. 2017). However, several bacteria also have a detrimental effect on cultivated mushrooms. Some *Pseudomonas* species are the causal agents of blotch diseases in *Agaricus bisporus* fruiting body, which decrease the mushroom productivity. Those detrimental effects are depending on the fungal developmental stages (Frey-Klett et al. 2011).

Several bacteria and actinobacteria have the inhibition activity against fungi. *Streptomyces* is one of the common actinobacteria which produce antifungal compounds against plant pathogenic fungi, whether it is isolated from agricultural soil, desert soil, or marine sediment (Audinah & Ilmi 2019; Smaoui et al. 2012; Usha Nandhini & Masilamani Selvam 2013). *Bacillus subtilis* also was reported to have antifungal activity against several plant pathogenic fungi such as *Alternaria*, *Fusarium,* and *Colletotrichum* species by producing hydrolytic enzyme and antimicrobial peptides (AMPs) *i.e* iturin, bacillomycin, fengycin, surfactin, and mycosubtilin (Desmyttere et al. 2019; Mardanova et al. 2017).

According to previous research, 25 bacterial isolates from straw mushroom cultivation medium were screened for antifungal activity. The C3.8 has the highest inhibition activity in-vitro against *Volvariella volvacea* followed by C2.2 and D3.3 respectively (Masrukhin & Saskiawan 2020). As the prospect for future application, these three selected bacterial isolates will be tested against phytopathogenic fungi that causing major disease in Indonesia's important horticultural crops. Several major fungal diseases such as anthracnose in chili, leaf spots in cabbages and lettuce, and Panama disease (*Fusarium* wilt) in banana. *Cercospora lactucae* is the causal agent of cercospora leave spot disease in the lettuce which has wide geographic distribution (Nguanhom et al. 2015). *Colletotrichum gloeosporioides* is the major fungal pathogen in pepper which causing anthracnose disease in several important crops such as chili (*Capsium* spp.), black pepper (*Piper nigrum*), and grapefruit (*Citrus paradisi*) (Kurian et al. 2008; Than et al. 2008; Cruz-Lagunas et al. 2020). The third pathogen is *Fusarium oxysporum* f.sp. *cubense* that causing Panama disease, the most detrimental disease in banana (Dita et al. 2018). Therefore, this research aimed to characterize the antifungal activity of these three bacterial isolates from straw mushroom cultivation medium against plant pathogenic fungi *Cercospora lactucae* (InaCC F168), *Colletotrichum gloeosporioides* (InaCC F304), and *Fusarium oxysporum* f.sp. *cubense* (F817). In addition, we also conducted a profiling of its bioactive compound and molecular identification of the bacteria using 16S rDNA.

MATERIALS AND METHODS

Materials

The bacterial isolates used in this study were previously screened from 26 bacterial isolates isolated from *Volvariella volvacea* cultivation medium. There are three potential isolates that have growth inhibition activity against

Volvariella volvacea i.e C2.2, C.38, and D3.3. The three phytopathogenic fungi used in this study were collected from Indonesia Culture Collection (InaCC) fungal collection *i.e Cercospora lactucae* (InaCC F168), *Colletotrichum gloeosporioides* (InaCC F304), and *Fusarium oxysporum f.sp. cubense* (InaCC F817).

Methods

Antagonism Assay against Phytopathogenic Fungi

Antagonism assay was performed according to Oh and Lim (2018) with few modifications. The bacterial isolates were co-cultured with phytopathogenic fungi in Potato Dextrose Agar (PDA) medium. The phytopathogenic fungi were grown in PDA medium prior to antagonism assay and incubated at 30° C for 5 days. The phytopathogenic fungi were taken using cork borer 5 and placed in an 80 mm Petri dish containing PDA medium. Bacterial isolates were inoculated onto PDA-containing phytopathogenic fungi by streaking with a sterile 1 µL inoculating loop along a 30 mm line with a 20 mm distance. The radial growth of mycelium was measured using ImageJ (Schneider et al. 2012) and compared with the control treatment (without bacterial isolates). The mycelial growth inhibition was measured using the formula as follows:

$$Fungal\ growth\ inhibition = \frac{Rc - Ri}{Rc} X\ 100\%$$

Rc = Mycelial growth of control (phytopathogenic fungi without bacterial inoculation)
Ri = Mycelial growth of phytopathogenic fungi co-cultured with bacteria (Narayanasamy 2013).

Antagonism Assay Using Filtrate of Potential Isolates

Bacterial isolates were grown in Nutrient Broth (NB) medium and incubated for 2x24 hours to obtain the optimal growth for bacteria. The bacterial suspension was then centrifuged at 14000 rpm at 4°C temperature for 10 minutes and filtered using cellulose acetate membrane 0.2 µm. Antagonism assay was performed with a similar method and substitute the bacterial isolates with 6 mm sterile paper disk- containing 25 µL filtrate. The mycelial growth was measured using ImageJ software and growth inhibition was calculated similarly as above.

Data Analysis

Data was collected from the antagonism assay and calculated using Ms. Excel. Statistical analysis of the percentage of inhibition was calculated using ANOVA single factor and continued using LSD (least significant differences) with a 5% level of significance (α= 0.05).

Profiling of Antifungal Compound

The bacterial isolates were grown in Luria Bertani Broth and incubated for 48 hours. The bacterial suspension then was centrifuged at 14000 rpm for 10 minutes to precipitate bacterial cells. The supernatant was taken and syringe-filtered through a 0.2 µm membrane filter to make sure there were no bacterial cells were involved. Extraction of the potential antifungal compound was performed three times using ethyl acetate 1:1 (V/V) and shook vigorously at 120 rpm for two hours. Ethyl acetate was then evaporated using a rotary evaporator at 40°C. About 15 mg of evaporated samples were dissolved in 1 mL ethyl acetate. The concentrated samples were then analyzed for their metabolite profile using gas chromatography-mass spectrophotometry GCMS-QP 2010 Ultra (Shimadzu- Japan) with Rtx-5MS

column. The mass spectra of the compound then were compared with the National Institute of Standards and Technology database version 11 (NIST 11).

Molecular Identification of Potential Isolates

Identification of bacterial isolates was conducted by amplification of 16S rRNA using universal primer 27F/1492R (Jiang et al. 2006). Total Genomic DNA was extracted using a boiling method at 80°C for 10 minutes and was precipitated using DNA spin for 5 minutes. As much as 2-3 µL DNA genomic DNA was used as a DNA template for Polymerase Chain Reaction (PCR) amplification. DNA sequencing was conducted using Sanger sequencing through an outsourced sequencing service laboratory. The sequences obtained were analyzed using ChromasPro (Technylesium- AU) for quality checking and trimming process. The processed DNA sequences used for identification through BLAST-N in Genebank with restriction is set on sequences from type material (Altschul et al. 1997) and 16S-based ID in EzBioCloud (Yoon et al. 2017).

RESULTS AND DISCUSSION

Identification of the potential isolates

The identification was performed using two online databases *i.e* Genebank and EzBiocloud. The usage of the GeneBank database because GeneBank contains a huge number of 16s rDNA sequences, however, the status of the strain sequences in GeneBank is often not known (Christensen & Olsen 2018). Therefore, the EzBiocloud database was used as complementary and confirmation for all sequences previously identified using GeneBank. As mentioned by Yoon et al. (2017) the EzBiocloud contains quality controlled 16s rDNA sequences and genomes of type strain bacteria and archaea.

The identification result (table 1) shows that two online databases generate similar for 16S-based identification with close similarity. Isolate C2.2 was identified as *Bacillus tequilensis*, C3.8 as *Bacillus siamensis*, and D3.3 as *Bacillus subtilis subsp. subtilis*. The usage of two or more online databases including GeneBank is recommended for 16s RDNA identification because the interpretation of 16S rDNA sequences depends on the program used by the database provider (Park et al. 2012).

Co-culture of potential isolates with phytopathogenic fungi

Co-culture of potential isolates was conducted as the first antagonism assay against phytopathogenic fungi. All isolates had an inhibition activity against *Cercospora lactucae* (InaCC F168). However, when it was conducted against

Table 1. Identification of three potential isolates based on Genebank and 16S-based ID- EzBioCloud.

Isolate code	GeneBank (NCBI)			EzBiolab (ChunLab)		
	Identification	Similarity	Accession	Identification	Similarity	Accession
C2.2	*Bacillus tequilensis* strain KCTC 13622	99.57	MN543830.1	*Bacillus tequilensis*	99.64	AY-TO01000043
C3.8	*Bacillus siamensis* KCTC 13613	99.71	KT781674.1	*Bacillus siamensis*	99.64	AJVF01000043
D3.3	*Bacillus subtilis subsp. subtilis* Str 168	99.42	CP053102.1	*Bacillus subtilis subsp. subtilis*	99.64	ABQL01000001

Colletotrichum gloeosporioides (InaCC F304) and *Fusarium oxysporum f.sp. cubense* (InaCC F817) only *Bacillus siamensis* C3.8 which significantly inhibited InaCC F304 (Figure 1).

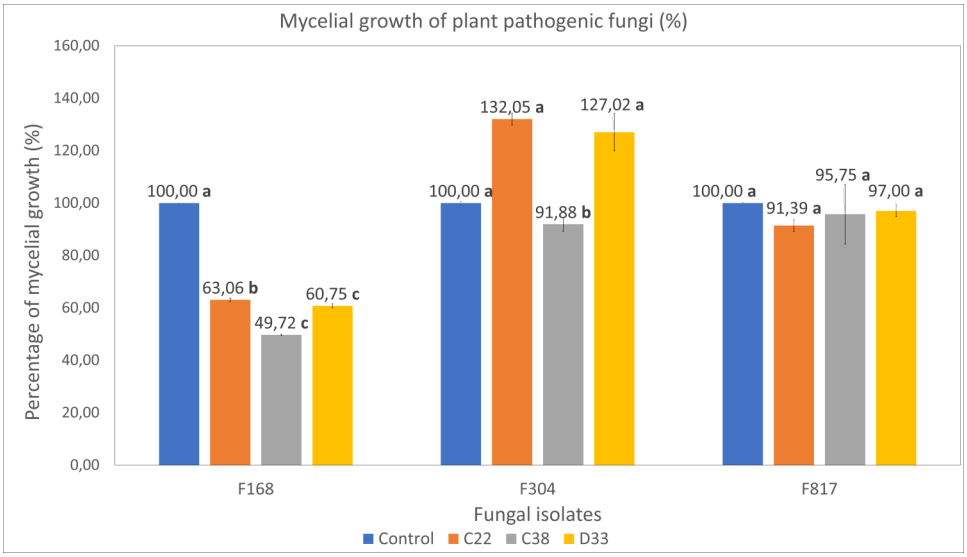

Figure 1. Mycelial growth of plant pathogenic fungi co-cultured with bacterial isolates. Bacteria: C2.2 (*Bacillus tequilensis*), C3.8 (*Bacillus siamensis*), D3.3 (*Bacillus subtilis subsp. subtilis*). Fungi: F168 (*Cercospora lactucae*), F304 (*Colletotrichum gloeosporioides*), and F817 (*Fusarium oxysporum* f.sp. *cubense*).

Many biological control agents have been developed through the screening of potential microbial isolates either from prokaryotes such as bacteria and actinobacteria or eukaryotes such as yeast and fungi. The screening of antifungal compounds can be performed through the co-culture method. This method is applied under the presumption that microbes interact with each other in a natural environment and compete for space and resources (Li et al. 2020; Oh & Lim 2018). The co-culture method can be complemented with the disk diffusion method to determine the active antifungal compound. The disk diffusion assay shows *Bacillus siamensis* C3.8 has the highest average inhibition activity among three isolates, either applied as whole isolates or filtrate followed by C2.2 and D3.3 respectively. However, in the antagonism assay using filtrate, the average is not significantly different among the three isolates tested (Figure 2).

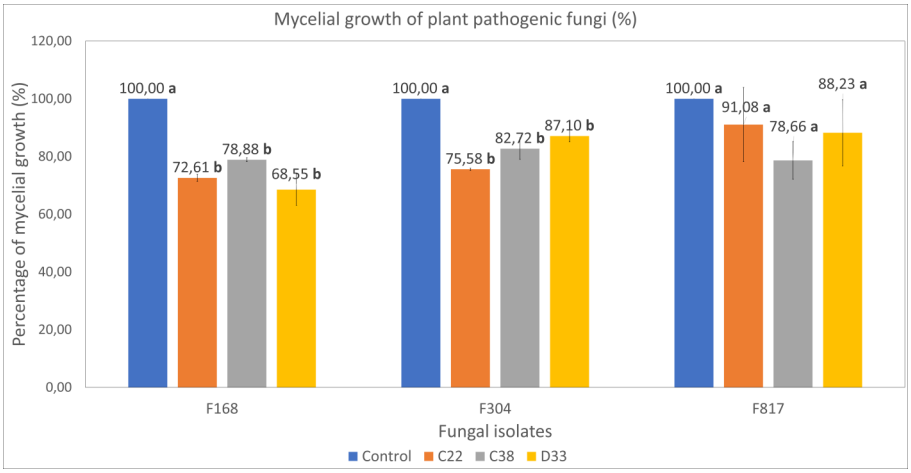

Figure 2. Mycelial growth of plant pathogenic fungi co-cultured with bacterial filtrate. Bacteria: C2.2 (*Bacillus tequilensis*), C3.8 (*Bacillus siamensis*), D3.3 (*Bacillus subtilis subsp. subtilis*). Fungi: F168 (*Cercospora lactucae*), F304 (*Colletotrichum gloeosporioides*), and F817 (*Fusarium oxysporum* f.sp. *cubense*).

Bacillus species are known as producers of a wide array of antagonistic compounds against other bacteria, fungi even viruses. Generally, the most important bioactive molecules are from non-ribosomal peptides, lipopeptide, polyketide compounds, bacteriocins, and siderophores (Fira et al. 2018). In this research, *B. siamensis* C3.8 has the highest and stable antifungal activity against three phytopathogenic fungi among three selected isolates. It is also supported by previous research that C.38 has the highest inhibition activity against *Volvariella volvacea* mycelial growth (Masrukhin & Saskiawan 2020). Previously Zhang et al. (2020) reported that *Bacillus siamensis* was able to inhibit *Botrytis cinerea* and *Rhizopus stolonifer* by producing volatile organic compounds (VOC) 2, 6-di-*tert*-butyl-4-methylphenol (BHT), and 2,4-di-*tert*-butylphenol (2,4-DTBP). Other *Bacillus* species, such as *B. amyloliquefaciens*, *B. tequilensis,* and *B. subtilis* were reported also have antagonism activity against *Candida albicans Magnaporthe oryzae* and *Penicillium roqueforti* by producing cyclic lipopeptide 6-2, iturin-like compound (Chitarra et al. 2003; Li et al. 2018; Song et al. 2013).

Profiling of active compound

Profiling of bioactive compounds showed that there were 16 active compounds detected in *B. siamensis* C3.8 with 91- 97 % similarity (data was not shown). However, there were four major bioactive compounds with the highest percentage of peak area. Bis(2-ethylhexyl) phthalate was the main major compound detected followed by 1-Heptacosanol, 1-Nonadecene, and E-15-Heptadecenal respectively (Table 2). Those bioactive compounds were previously described as antimicrobe and antifungal compounds. However, it needs further purification and assays to confirm that those bioactive compounds were responsible for *B. siamensis* C3.8 antifungal activity.

Bis(2-ethylhexyl) phthalate is an ester of phthalic acid which was widely used as a plasticizer in many materials. This compound is mostly considered as a pollutant due to the persistent characteristic and often found in the environment as the effect of extensive usage (Ortiz & Sansinenea 2018). Instead of environmental pollution, several researches have shown that Bis(2-ethylhexyl) phthalate is produced by microorganisms such as *Bacillus subtilis*, *Aspergillus awamori,* and crown flower (*Calotropis gigantea*). The bis(2-ethylhexyl phthalate has antimicrobe and antifungal characteristic against bacteria such as *Bacillus subtilis, Escherichia coli, Sarcina lutea, Shigella dysenteriae, Shigella sonnei, Staphylococcus aureus,* and *Aspergillus flavus* fungus (Habib & Karim 2009; M. M. Lotfy et al. 2018; W. A. Lotfy et al. 2018). According to this research, the isolate *B. siamensis* C.38 could produce Bis(2-ethylhexyl) phthalate which previously known has antifungal activity. This isolate is the potential to be applied as a biocontrol agent against phytopathogenic fungi, however, the other characteristics and the mode of action should be further studied.

Table 2. GC-MS profile of major active compound identified in *B. siamensis* C.38.

No	Retention time	% Area	Identified compound	Similarity (%)	Formula	Function
1	17.154	6.24	E-15-Heptadecenal	96	C17H32O	Antimicrobe (Abdel-Wahab et al. 2017)
2	18.634	9.02	1-Nonadecene	96	C19H38	Antimicrobe and Antifungal (Smaoui et al. 2012)
3	21.209	5.33	1-Heptacosanol	94	C27H56O	Antimicrobe (Chowdhary & Kaushik 2019)
4	22.337	22.39	Bis(2-ethylhexyl) phthalate	97	C24H38O4	Antimicrobe (M. M. Lotfy et al. 2018; W. A. Lotfy et al. 2018). Antifungal and antibacterial (Ortiz & Sansinenea 2018)

CONCLUSION

All bacterial isolates were identified through two online databases *i.e* Genebank and EzBioCloud. The identification result showed that C2.2 was identified as *B. tequilensis*, C3.8 as *B. siamensis,* and D3.3 as *B. subtilis subsp. subtilis*. All isolates had antifungal activity against *C. lactucae* (InaCC F168), *C. gloeosporides* (InaCC F304), and *F. oxysporum* f.sp. *cubense* (InaCC F817). The *B. siamensis* C3.8 had the highest and stable antifungal activity among three bacterial isolates. The Bioactive compound profile showed that Bis(2-ethylhexyl) phthalate was the main major compound detected in *B. siamensis* C3.8 and needs further purification and assays to confirm that this compound is responsible for the antifungal activity of C3.8.

AUTHORS CONTRIBUTION

All authors have reviewed the final version of the manuscript and approved it for publication. M and ALP were designed the study; M, ALP, IP, and MYN performed research and collected the data; M, ALP, TRS, MI, IP, IS and MYN analysed the data and wrote the paper. M and ALP are the main contributor of this manuscript.

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CONFLICT OF INTEREST

The authors state no conflict of interest from this manuscript.

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