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 (*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 leaf 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 (Capsicum 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:

\[
\text{Fungal growth inhibition} = \frac{Rc - Ri}{Rc} \times 100\%
\]

\(Rc\) = 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 \((\alpha= 0.05)\).

**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...
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).

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).

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).
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.

| 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. gloeosporioides (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.

ACKNOWLEDGMENTS
This research was funded by DIPA Research Center for Biology, LIPI (2019). The author also would like to thank Mrs. Mia Kusmiati, Mrs. Yeni Yuliani, and Ms. Gita Azizah Putri for the help in laboratory activity and during the research process.

CONFLICT OF INTEREST
The authors state no conflict of interest from this manuscript.

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