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Antibacterial and Anti-Biofilm Activities of Culture Filtrates from Schizophyllum commune, Coniothyrium sp., and Fusarium sp.

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Antibacterial and Anti-Biofilm Activities of Culture Filtrates from *Schizophyllum commune*, *Coniothyrium* sp., and *Fusarium* sp.

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Abstract

Bacterial infections are usually caused by biofilms that are resistant to extreme conditions. Studies have shown that fungal extracts have the potential to inhibit biofilm formation. The aim of this study was to examine the activity of several fungal culture filtrates in inhibiting bacterial growth and biofilm formation. The fungi were identified by molecular method, and the effects of different concentrations (45%, 75%, and 90%), medium pH (6, 7, and 9), temperatures (30 and 37 °C), and incubation times (24, 48, and 72 h) of the filtrates on their anti-biofilm formation properties were investigated. Anti-biofilm assay was performed using the diffusion test and microtiter assay. The parameters examined included the diameter of the inhibition zone of each well and the optical density of the filtrate solution. Molecular identification based on ITS rDNA regions showed that the fungal isolates in this study were *Schizophyllum commune* (strain JSB2), *Coniothyrium* sp. (strain JB1-3), and *Fusarium* sp. (strain JBB2). Antibacterial assay showed that, among the culture filtrates obtained from all fungi, that from *Fusarium* sp. strain JBB2 exhibits the highest inhibitory activity. Biofilm inhibition assay further revealed optimum fungal culture filtrate activities at pH 7.

Keywords: antibacterial, anti-biofilm, culture filtrate, fungi, inhibition

Introduction

A biofilm is a structured colony of microorganisms that attach to a surface by producing an extracellular polymeric substance (EPS) [1]. Biofilms are also a form of microbial defense against environmental stresses. Pathogenic bacteria, such as *Escherichia coli, Pseudomonas aeruginosa*, *Bacillus cereus,* and *Enterobacter cloacae* are known to form biofilms and cause persistent infections [2,3,4,5]. *Enterobacter cloacae*, one of the main causes of infection, has been reported to harbor a highly resistant to the antibiotic colistin treatment [5], while *B. cereus* is a pathogen responsible for emetic and diarrheal food poisoning [4]. Both biofilm-forming pathogenic bacteria show strong antibiotic resistance [6,7].

Several compounds with anti-biofilm activity have been discovered from natural resources, and extracts from several species of fungi have shown promising anti-biofilm and antibacterial activity against Gram-positive and negative pathogenic bacteria [8,9]. Wardani *et al.* [10] reported that *Lentinula edodes* extract shows antibacterial activities against *E. coli*

and *Staphylococcus aureus*. Boshle *et al*. [11] found that *Ganoderma applanatum* extract shows antibacterial activities against *B. cereus*, *S. aureus*, and *P. aeruginosa*. *Ganoderma* sp. generally produces protease to degrade the polysaccharides in EPSs. Several anti-microbial compounds, such as lectins, triterpenoids, polysaccharides, alkaloids, phenols, and sterols, have also been reported in fungal extracts [12]. The diverse natural compounds obtained from fungi are promising targets in the discovery of novel biological compounds to combat bacterial biofilm resistance. The purpose of this study is to examine the antibacterial and anti-biofilm activities of culture filtrates from three fungal strains and identity these fungal strains using a molecular phylogenetic method. The discovery of antibacterial metabolites, including fungal secondary metabolites, from various sources is important in efforts to seek new antibiotics that can combat the antibiotic resistance of several bacterial diseases.

Materials and Methods

Microorganisms. Three strains of biofilm-forming bacteria, namely, *B.* cereus strain SA 1–2, *B.* cereus strain

SA 1–3, and *Enterobacter cloacae* strain SA 1–5, were obtained from the collection of the Health Microbiology Laboratory, Microbiology Division, Research Center for Biology (LIPI), and three strains of fungal isolates (JBB2, JSB2, and JB1–3) were used in this experiment. This study was conducted at the Health Microbiology La–boratory, Microbiology Division, Research Center for Biology (LIPI), Cibinong, West Java.

Fungal identification by DNA isolation, polymerase chain reaction (PCR) amplification, and sequencing. Genomic DNA was extracted from fungal mycelia grown for 7 d in 5 mL of potato dextrose broth (Difco, USA) using a Plant Genomic DNA Mini Kit (GP100) (Geneaid, Taiwan) following the manufacturer's protocol. Genomic DNA was amplified by the PCR method; here, amplification was performed in a 25 μL reaction volume containing 10 μL of nuclease-free water, 12.5 μL of GoTaq® Green Master Mix (Promega, USA), 0.5 μL of the forward and reverse primers, 0.5 μL of DMSO, and 1 μL of the DNA template. The primer pair of ITS5 (forward) (5′-TCCTCCGCTTATTGATATGC-3′) and ITS4 (reverse) (5′-TCCGTAGGTGAACCTGCGC-3′) [13] were used to amplify both the ITS I and ITS II rDNA along with the 5.8S region. The PCR conditions were set as follows: initial denaturation for 90 s at 95 °C, followed by 35 cycles of 30 s at 95 °C for denaturation, 30 s at 55 °C for annealing, 90 s at 72 °C for extension, and 5 min at 72 °C for the final extension. All PCR procedures were performed in a T100 thermal cycler (Bio–Rad, USA). The PCR products were electrophoresed in 1% (w/v) agarose gel soaked in 1× TAE buffer at 100 V for 30 min. The gel was soaked in ethidium bromide for 30 min prior to UV light examination using a Gel Doc XR system (Bio–Rad.). Finally, the purified PCR products were sent to 1stBASE (Malaysia) for sequencing.

Phylogenetic analysis. Nucleotide sequences obtained from the primer pair (ITS5 and ITS4) were examined and refined using ChromasPro 1.41 software (Technelysium Pty Ltd., Australia). New ITS sequences were submitted to the GenBank database (Figures 1–3). The new sequences were aligned with homologous DNA sequences retrieved from the GenBank database (https://www.ncbi. nlm.nih.gov/) using MUSCLE [14] implemented in MEGA 7 [15]. Phylogenetic analysis was conducted using the neighbor-joining (NJ) method in MEGA 7. The strength of the internal branches of the phylogenetic tree was tested by bootstrap (BS) analysis using 1000 replications. BS values higher than 50% were analyzed.

Fungal Culture Filtrate Preparation. Fungal culture filtrates were prepared using the method of Rossa *et al.* [16] with a modification. Fungal colonies were inoculated onto PDA medium in a Petri dish, and incubated for 7 d. The 7–day mycelium of the fungi was then cut,

transferred into 200 mL of PDB medium, and incubated on a shaker (120 rpm) at 28 °C for 5 d. Next, the fungal inoculum was aseptically transferred to 450 mL of ethanol and incubated on a rotary shaker (120 rpm) for 24 h at 28 °C. The fungal culture filtrate was filtered through sterile gauze, and the resulting supernatant was evaporated using a vacuum evaporator at 45 °C. The solid form of the fungal culture filtrates was dissolved to concentrations of 45%, 75%, and 90% using 20% DMSO.

Diffusion assay. Bacterial isolates were prepared in 5 mL of Luria Bertani (LB) medium and incubated at 37 °C for 24 h. After incubation, each bacterial suspension was swabbed on the surface of Mueller–Hinton Agar (MHA) and then incubated for 10 min. Three holes (diameter, 3 mm) were prepared in the MHA medium, and three concentrations of fungal culture filtrates (45%, 75%, and 90%) from each fungal strain (JBB2, JSB2, and JB1-3) were prepared for 14 d. The fungal culture filtrate obtained from each concentration was placed into the holes of the MHA medium and incubated at 37 °C for 24 h. Thereafter, the optimum concentration of fungal culture filtrate showing maximum inhibitor activity was examined. The same method was applied to qualitatively examine the antibacterial activity of the filtrates at different temperatures and medium pH. Chloramphenicol was used as the positive control, and distilled water was used as the negative control. This experiment was conducted in three replicates. The inhibition zone was determined according to Ulyah [17] as follows:

Inhibition zone (mm) = clear zone diameter (mm) – diameter of the hole (mm).

The inhibition effectiveness parameter was set according to the standards described by Nazri *et al.* [18] as follows:

Diameter of inhibition zone > 20 mm: very strong inhibition activity
Diameter of inhibition zone > 10–20 mm: strong inhibition activity
Diameter of inhibition zone > 5–10 mm: moderate inhibition activity
Diameter of inhibition zone > 0–5 mm: weak inhibition activity.

Microtiter assay. Biofilm inhibition assay was performed using the microtiter assay method [17]. A total of 0.05 mL of bacterial suspension from the LB medium was placed in 4.95 mL of trypticase soy broth (TSB) at different pH (6, 7, and 9). Then, 50 μL of the fungal culture filtrate and 50 μL of the bacterial suspension from the TSB medium were added to the wells of a microplate and incubated at different temperatures (30 and 37 °C) and incubation times (24, 48, and 72 h).

The microplate was rinsed thrice with distilled water, added with 125 µL of 0.1% crystal violet, and then incubated at room temperature for 15 min. Thereafter, the microplate was rinsed thrice using distilled water and dried. A total of 200 µL of ethanol was added to the wells of the microplate, which was subsequently incubated at room temperature for 15 min. After incubation, the absorbance of the solution in each well was measured by a microplate reader at $\lambda = 550$ nm.

The optical density of the solutions was calculated using the following formula:

% biofilm inhibition = 1− (corrected sample/corrected blank) × 100 %.

Notes:
Corrected sample = fungal culture filtrate + bacterial suspension
Corrected blank = DMSO 20% + bacterial suspension

Results and Discussion

Fungal identification. The phylogenetic tree generated from the NJ analysis of JSB2 and its homologous sequences retrieved from GenBank showed that the JSB2 sequence (GenBank Accession No. MN460362) was nested in the same clade as the sequences of *S. commune* with 100% BS (Figure 1). Thus, the JSB2 sequence belongs to *S. commune*. The type sequence of *S. commune* is not available in GenBank. Therefore,

reanalysis of the identity of fungal strain JSB2 is necessary once the type sequence of *S. commune* becomes available. *S. commune* is a basidiomycetous fungus and plant pathogen that colonizes rotten wood [19]. Extracts of *S. commune* have been reported to display antioxidant and anti-microbial activities [20–22].

Phylogenetic analysis of the JB1–3 sequence (GenBank Accession No. MN460360) with closely related sequences showed that this fungus belongs to Ascomycota. The JB1–3 sequence formed a monophyletic clade with *Coniothyrium* sp. strain HKA14 (DQ092505) and *Coniothyrium* sp. strain HKB31 (DQ092521) with 91% BS (Figure 2). Therefore, the JB1-3 sequence was tentatively named *Coniothyrium* sp. strain JB1-3. Members of the genus *Coniothyrium* are plant endophytes, associated with green algae and other marine organisms that act as mycoparasites producing several anti-microbial compounds [23,24].

Phylogenetic analysis of the JBB2 sequence (GenBank Accession No. MN460361) showed that this sequence belongs to the genus *Fusarium* (Figure 3). In the phylotree, the JBB2 sequence formed an independent lineage separate from other *Fusarium* sequences. Further multigene analysis involving translation elongation factor 1-α (TEF 1-α) and the second largest subunit of the RNA polymerase II (RPB2) gene sequence is necessary to determine *Fusarium* sp. strain JBB2 up to the species level.
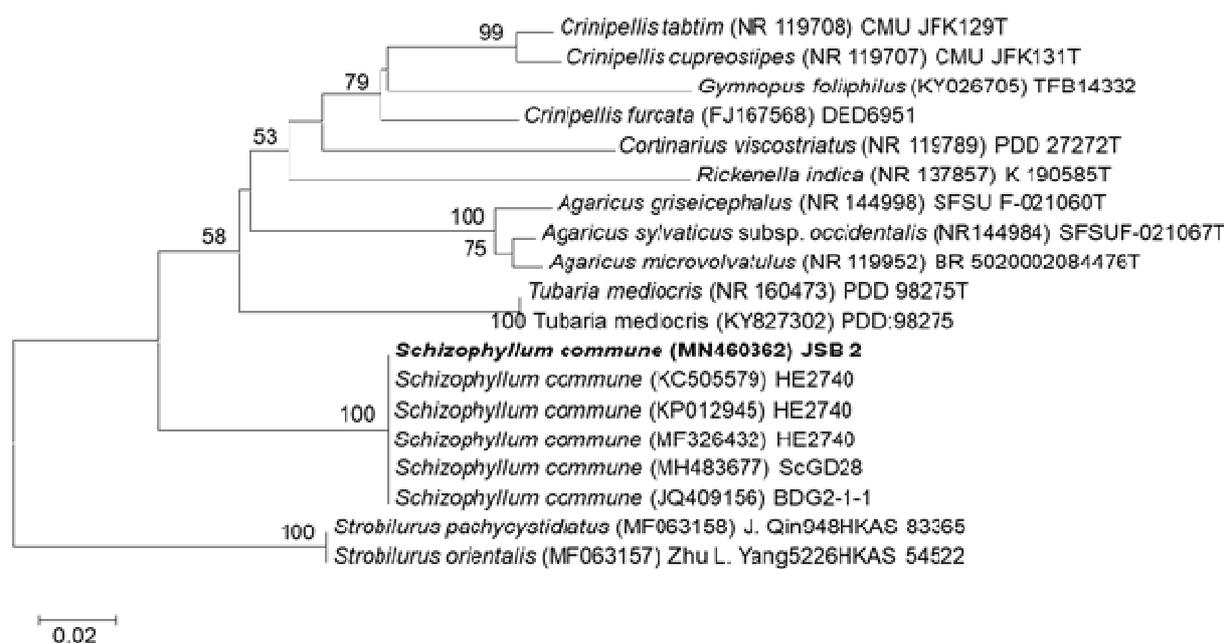
Figure 1. Neighbor-joining Tree Generated from the ITS rDNA Sequences of JSB2 (bold) and Related Sequences with 1000 Bootstrap Replications

Antibacterial assay. Qualitative antibacterial activity was analyzed by examining the ability of the fungal culture filtrates to inhibit the activity and growth of three isolates of biofilm-forming bacteria. All three fungal culture filtrates exhibited antibacterial activity against *B. cereus* strain SA 1–2, *B. cereus* strain SA 1–3, and *E. cloacae* strain SA 1–5 at 30 and 37 °C (Table 1), as demonstrated by their formation of an inhibition zone during the experiment. The culture filtrate from *Fusarium* sp. strain JBB2 at a concentration of 90% could inhibit the growth of *B. cereus* strain SA 1–2 (inhibition zone diameter = 33 mm) at 30 °C and that of *B. cereus* strain SA 1–3 (inhibition zone diameter = 32 mm) at 37 °C. In addition, the culture filtrate from *S.*

commune strain JSB2 could inhibit the growth of *B. cereus* strain SA 1-3 at 30 °C and concentration of 75% (inhibition zone diameter = 30 mm) and *E. cloacae* strain SA 1–5 at 37 °C and concentration of 75% (inhibition zone diameter = 31 mm). The culture filtrate of *Coniothyrium* sp. strain JB1-3 inhibited the growth of *B. cereus* strain SA 1–3 (inhibition zone diameter = 32 mm) and *E. cloacae* strain SA 1–5 (inhibition zone diameter = 31 mm) during incubation at 30 °C and concentration of 90%. The culture filtrate of *Coniothyrium* sp. strain JB1-3 could inhibit the growth of *B. cereus* strain SA 1-2 (inhibition zone diameter = 32 mm) and *E. cloacae* strain SA 1–5 (inhibition zone diameter = 32 mm) at 37 °C and concentration of 90%.
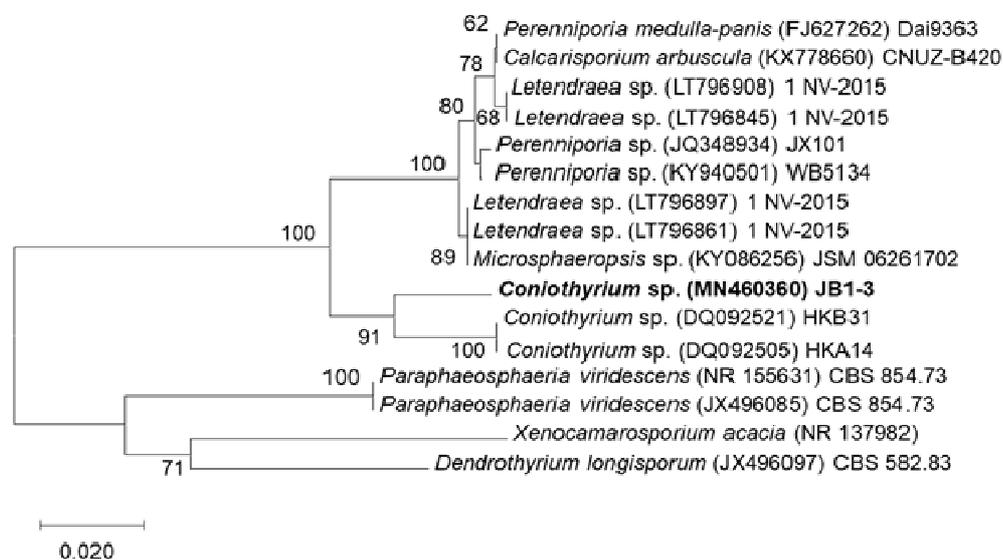
Figure 2. Neighbor-joining Tree Generated from the ITS rDNA Sequences of JB1-3 (bold) and Related Sequences with 1000 Bootstrap Replications
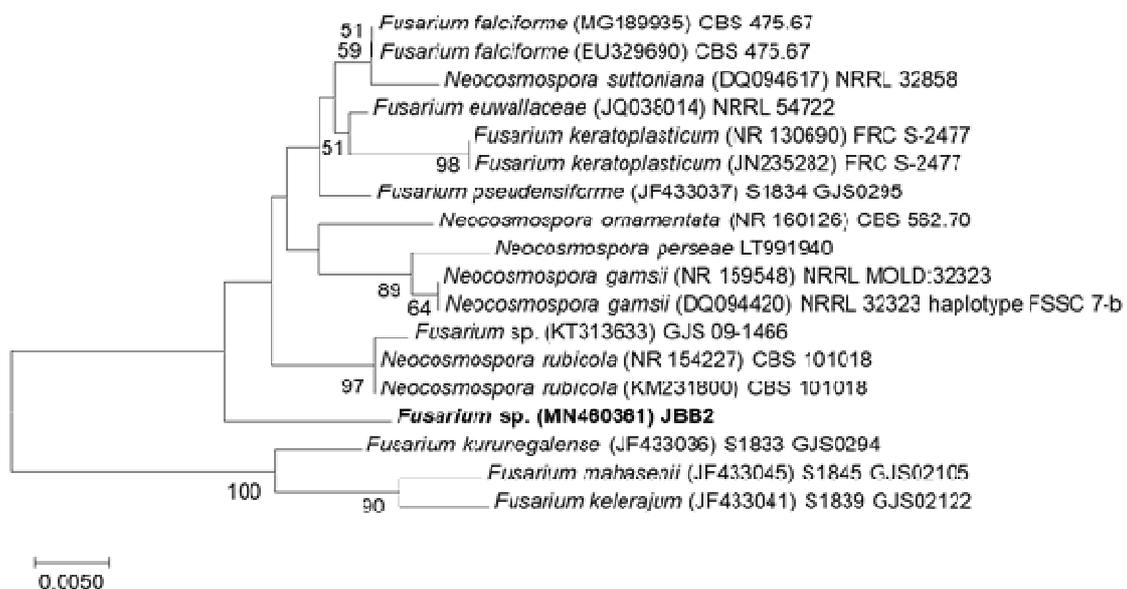
Figure 3. Neighbor-joining Tree Generated from the ITS rDNA Sequences of JBB2 (bold) and Related Sequences with 1000 Bootstrap Replications

Schizophyllum commune, *Fusarium* spp., and *Coniothyrium* spp. are endophytic fungi commonly used in pharmaceutical studies [20–27]. An important bioactive compound obtained from many species of *Fusarium* is equisetin [28]. Members of *Schizophyllum* produce antibacterial polysaccharides called schizophyllan and its oxidized form, schizophyllan (scleraldehyde) [20]. This compound is effective against a broad range of bacterial species. *Coniothyrium* is frequently reported in marine-derived species [24]. Members of *Coniothyrium* have been reported to produce phenalenone derivatives, lactone, sclerodin, lamellicolic anhydride, scleroderolide, and sclerodione [24] a macrolide antibiotic macrosphelide A [23], and hydroxyanthraquinones [25]. Thus, *S. commune*, *Fusarium* spp., and *Coniothyrium* spp. are important fungi in the discovery of new compounds to combat the emergence of antibiotic resistance in several bacterial diseases.

Effect of pH on the antibacterial assay. This assay showed that the growth of all biofilm-forming bacteria was inhibited by all fungal culture filtrates at pH 6, 7, and 9 and 37 °C (Table 2). Culture filtrates from *Fusarium* sp. strain JBB2, *S. commune* strain JSB2, and *Coniothyrium* sp. strain JB1–3 effectively inhibited the growth of Gram-positive (*Bacillus* sp.) and negative (*Enterobacter* spp.) bacteria. The highest inhibitory activities of *Fusarium* sp. strain JBB2, *S. commune* strain JSB2, and *Coniothyrium* sp. strain JB1–3 against *B. cereus* strain SA 1–2, *B. cereus* strain SA 1–3, and *E. cloacae* strain SA 1–5 were found at pH 6 and 9, pH 9, and pH 7, respectively (Table 2). Malik *et al.* [30] noted that several anti-microbial peptides are pH dependent.

Table 1.   Inhibitory Activity of Fungal Extracts Against Biofilm-forming Bacteria at Different Temperatures and Concentrations

Fungal strain	Fungal extract	Diameter of Inhibition Zone					
		Temperature 30 °C			Temperature 37 °C		
		B. cereus strain SA 1-2	*B. cereus* SA 1-3	*E. cloacae* strain SA 1-5	*B. cereus* strain SA 1-2	*B. cereus* SA 1-3	*E. cloacae* strain SA 1-5
Fusarium sp. strain JBB2	45%	20 mm	22 mm	21 mm	22 mm	23 mm	23 mm
	75%	25 mm	26 mm	23 mm	25 mm	27 mm	28 mm
	90%	33 mm	31 mm	27 mm	26 mm	32 mm	30 mm
S. commune strain JSB2	45%	21 mm	23 mm	22 mm	21 mm	23 mm	21 mm
	75%	28 mm	30 mm	30 mm	28 mm	30 mm	31 mm
	90%	28 mm	28 mm	28 mm	29 mm	30 mm	28 mm
Coniothyrium sp.strain JB1–3	45%	28 mm	27 mm	28 mm	29 mm	27 mm	28 mm
	75%	28 mm	30 mm	30 mm	29 mm	28 mm	30 mm
	90%	32 mm	30 mm	31 mm	32 mm	30 mm	32 mm

Table 2.   Inhibitory Activity of Fungal Extracts Against Biofilm-forming Bacteria at Different Temperatures and Concentrations

Biofilm-forming bacteria	Fungal Extract		Temperature	Diameter of Inhibition Zone		
	Strain	Concentration		pH 6	pH 7	pH 9
B. cereus strain SA 1-2	*Fusarium* sp. strain JBB2	90%	30 °C	29 mm	28 mm	28 mm
			37 °C	31 mm	30 mm	31 mm
	S. commune strain JSB2	75%	30 °C	28 mm	28 mm	29 mm
			37 °C	31 mm	30 mm	31 mm
	Coniothyrium sp. strain JB1-3	90%	30 °C	28 mm	29 mm	30 mm
			37 °C	33 mm	32 mm	33 mm
B. cereus strain SA 1-3	*Fusarium* sp. strain JBB2	90%	30 °C	29 mm	28 mm	31 mm
			37 °C	31 mm	33 mm	33 mm
	S. commune strain JSB2	75%	30 °C	30 mm	29 mm	31 mm
			37 °C	32 mm	32 mm	33 mm
	Coniothyrium sp. strain JB1-3	90%	30 °C	29 mm	29 mm	29 mm
			37 °C	31 mm	31 mm	33 mm
E. cloacae strain SA 1-5	*Fusarium* sp. strain JBB 2	90%	30 °C	32 mm	30 mm	29 mm
			37 °C	33 mm	33 mm	30 mm
	S. commune strain JSB 2	75%	30 °C	30 mm	29 mm	28 mm
			37 °C	32 mm	32 mm	30 mm
	Coniothyrium sp. strain JB1-3	90%	30 °C	29 mm	32 mm	30 mm
			37 °C	31 mm	33 mm	32 mm

According to Agustina [31], the potential anti-microbial activity of a substance can be determined if it shows remarkable inhibitory activity against bacterial growth at low concentrations. Harvey *et al*. [28] also reported that, the higher the concentration of antibacterial substances, the stronger the strength of their antibiosis activity. Therefore, the diameter of the inhibitory zone formed around antibacterial substances can be used as a parameter to examine the strength of these compounds in fungal extracts [29]. Anti-microbial substances are divided into two types on the basis of their activity, namely, bacteriostatic and bactericidal anti-microbial substances. Ulyah [17] noted that an anti-microbial substance with strong inhibitory power produces an inhibition zone, of > 20 mm. Therefore, since each of the fungal culture filtrates obtained from *Fusarium* sp. strain JBB2, *S. commune* strain JSB2, and *Coniothyrium* sp. strain JB1–3 produced >20 mm inhibition zones, these fungi show strong inhibitory activity against biofilm-forming bacteria.

The highest antibacterial activities in the culture filtrates of *Fusarium* sp. strain JBB2 against *B. cereus* strain SA 1–2, *B. cereus* strain SA 1–3, and *E. cloacae* strain SA 1–5 were found at concentrations of 90% (37 °C, pH 7), 75% (37 °C, pH 7), and 90% (30 °C, pH 7), respectively (Table 2). The peak antibacterial activity of *S. commune* strain JSB2 extract against *B. cereus* strain SA 1–2, *B. cereus* strain SA 1–3, and *E. cloacae* strain SA 1–5 was observed at concentrations of 90% (37 °C, pH 7), 75% (30 °C, pH 9), and 75% (37 °C, pH 7), respectively. Finally, the peak antibacterial activity of *Coniothyrium* sp. strain JB1–3 extract against *B. cereus* strain SA 1–2, *B. cereus* strain SA 1–3, and *E. cloacae* strain SA 1-5 was found at concentrations of 90% (37 °C, pH 9), 90% (30 °C, pH 7), and 90% (30 °C, pH 7), respectively. Fungi are known to produce various types of primary and secondary metabolites, some of which present antibacterial and anti-biofilm formation activities [32,33]. Members of Ascomycota and Basidiomycota, in particular, can produce EPS to defend themselves against environmental stress factors and during interactions with other microorganisms. EPSs from Ascomycota and Basidiomycota are known for their antioxidant, immune-stimulating, and anti-microbial properties [34].

Anti-biofilm formation assay. In the anti-biofilm formation assay at 30 °C (Figures 4A–4C) among the culture filtrates obtained, that of *S. commune* strain JSB2 exhibited the highest anti-biofilm formation activity against *B. cereus* strain SA 1–2 (73.21%), while those of *Fusarium* sp. strain JBB2 showed the highest anti-biofilm against *E. cloacae* strain SA 1–5 (72.61%) and *B. cereus* strain SA 1–3 (67.59%). At 37 °C (Figures 5A–5C), the highest anti-biofilm activity against *B. cereus* strain SA 1–2 was found in the culture filtrate from *S. commune* strain JSB2 (88.04%). In addition,
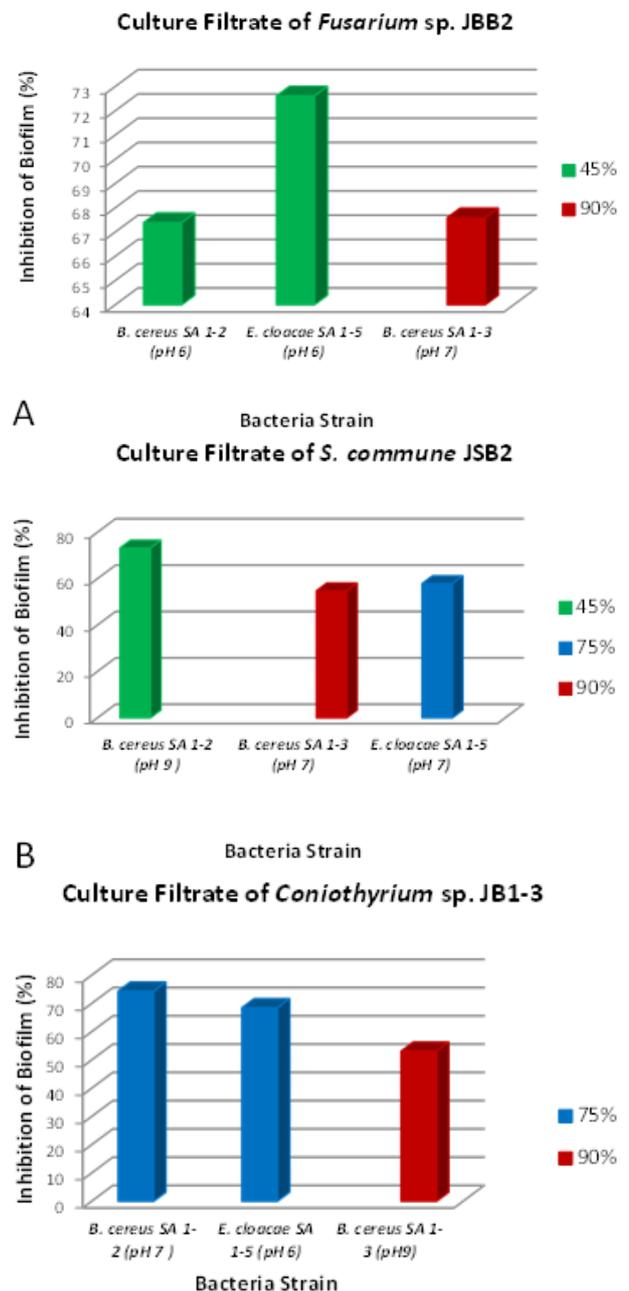
Figure 4. **Anti-biofilm Activity of Fungal Culture Filtrates at 30 °C: A.** *Fusarium* **sp. strain JBB2, B.** *S. commune* **strain JSB2, and C.** *Coniothyrium* **sp. strain JB1-3**

among the culture filtrates, that of *Coniothyrium* sp. strain JB1–3 exhibited the highest anti-biofilm activity against *E. cloacae* strain SA 1-5 (77.22%) and *B. cereus* strain SA 1-3 (74.83%).

Many microbes, especially pathogenic bacteria, adhere to surfaces and produce a complex matrix of polymers surrounding their cells to form a biofilm. This biofilm formation protects the microbes against hostile

Culture Filtrate of *Fusarium* sp. JBB2

A

Culture Filtrate of *S. commune* JSB2

B

Culture Filtrate of *Coniothyrium* sp. JB1-3

C
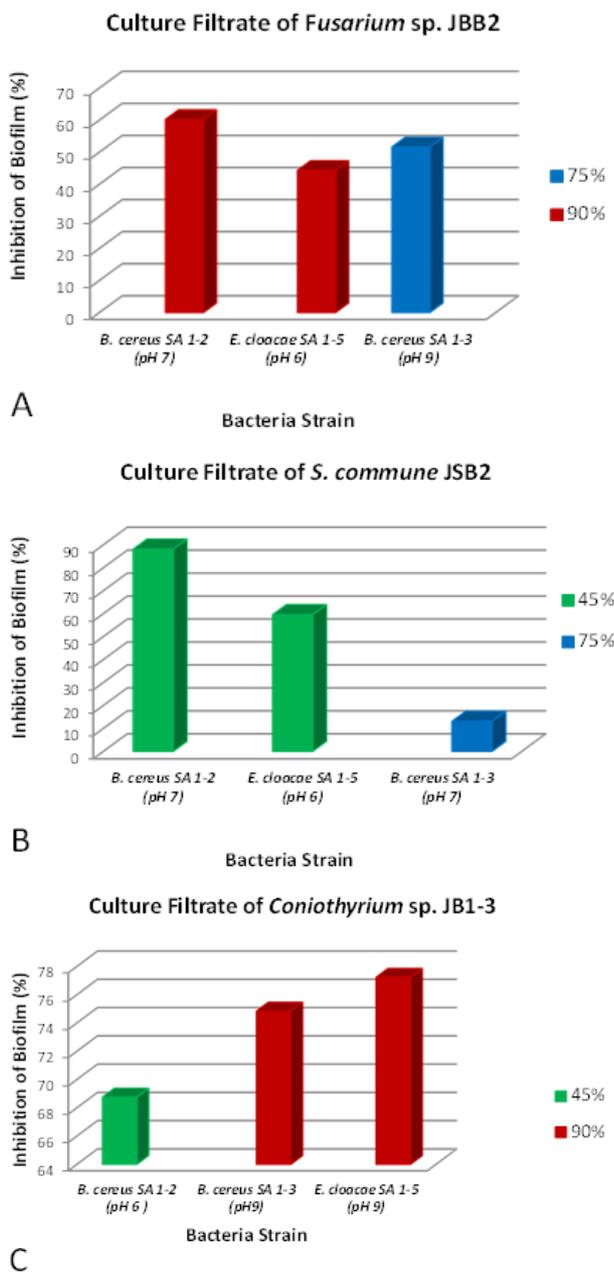
Figure 5. Anti-biofilm activity of fungal culture filtrates at 37 °C: A. *Fusarium* sp. strain JBB2, B. *S. commune* strain JSB2, and C. *Coniothyrium* sp. strain JB1-3

environments and most antibiotics [32]. Therefore, the discovery of new therapeutic compounds against infections due to microbial biofilms is necessary. This study showed that fungal culture filtrates from *Fusarium* sp. strain JBB2, *S. commune* strain JSB2, and *Coniothyrium* sp. strain JB1-3 strongly inhibit the biofilm formation of Gram-positive and negative bacteria. Several mechanisms, such as blockage of the cell communication process essential for biofilm formation (quorum sensing) and degradation of the bacterial cell wall via enzymatic processes, underlie the

observed anti-biofilm formation activity. Further studies on metabolite identification and analysis of the mechanism underlying the anti-biofilm formation activity of *Fusarium* sp. strain JBB2, *S. commune* strain JSB2, and *Coniothyrium* sp. strain JB1-3 are necessary to optimize their production and application.

Acknowledgments

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