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The potency of *Actinomycetes InaCC* A758 against dual-species biofilms: *Candida albicans* and *Staphylococcus aureus*

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ABSTRACT

Candida albicans and *Staphylococcus aureus* can coexist to form a biofilm, leading to infections associated with biofilms. *Actinomycetes* produce secondary metabolites known as antibiotics, antifungals, antibiofilm, anticancer, and antimalarials. This study was aimed to explore the antibiofilm activity of secondary metabolites of *Actinomycetes InaCC* A758 extracts (*InaCC* A758) against dual-species biofilms, i.e., *C. albicans* and *S. aureus*. Ethyl acetate and chloroform were used as solvents in a maceration extraction technique to isolate the compound designated *InaCC* A758. The fractionation of the *InaCC* A758 extracts was carried out using semi-preparative HPLC. Identification of the compounds from the *InaCC* A758 extracts was performed using gas chromatography-mass spectrometry analysis. Antimicrobial and antibiofilm testing of the *InaCC* A758 extracts was done utilizing the micro broth dilution method. The morphology of the biofilms following treatment with the *InaCC* A758 extracts was visualized using scanning electron microscopy (SEM). The minimum inhibitory concentration of *InaCC* A758 against dual-species biofilms was 400 µg/ml. The concentrations of *InaCC* A758 required to inhibit 50% and 80% of dual-species biofilm formation (BIC_{50} and BIC_{80}) ranged from 3.57–6.66 and 29.25–30.50 µg/ml, respectively. The concentrations needed to reduce formed dual-species biofilms by 50% and 80% (BRC_{50} and BRC_{80}) ranged from 100.62–131.85 and 596.4–849.6 µg/ml, respectively. SEM observation showed a reduced number of cells and disruption of the cell membranes when exposed to the *InaCC* A758 extracts. The *InaCC* A758 contains seven compounds, i.e., dodecanoic acid 3-hydroxy-; 1,3,5-pentanetriol,3-methyl-; 1,3-dioxolane-4-methanol, 2-ethyl-; 2-cyclopropylcarbonyloxytridecane; 2-hepten-1-ol,(E); methyl 6-methyl heptanoate; and 11-octadecenoic acid, methyl ester. The InaCC A758 extract altered cell morphology and exhibited potential as an anti-biofilm agent, particularly against dual-species biofilms.

INTRODUCTION

Multi-species biofilms have a significant role in the pathogenesis of various infections [1] as bacteria grown in multi-species biofilms can interact synergistically, mutually, and antagonistic [2,3]. Infection-related multi-species biofilms caused an increase in mortality rate (70%) than mono-species biofilms (23%). *Candida albicans* (*C. albicans*) and *Staphylococcus aureus* (*S. aureus*) are the pathogens that most often cause increasing morbidity and mortality in hospitalized patients [2]. They are the most common microorganisms found in blood cultures, and both can act as co-infectors in various infections. About 27% of candidaemia cases are also found *S. aureus* in blood preparations [4]. *Staphylococcus aureus* can form microcolonies on the surface of biofilms covered with a matrix released by *C. albicans* in dual-species biofilm architecture [5].

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The interaction between bacteria and fungi is clinically related to increasing the severity of the disease. Co-infections caused by dual-species biofilms are found in burns and cystic fibrosis. Dual-species biofilms are also found on the surface of medical devices such as dentures and prosthetic implants and are most often found on catheters [2].

Antibiotics and antifungals can be used as therapy for biofilm infections, i.e., cephalosporins, fluoroquinolones, amphotericin B, azole groups, and echinocandins. However, these antimicrobials had been reported to become resistant [6,7] and had severe side effects [8–10]. In other cases, *C. albicans* in polymicrobial biofilms can increase vancomycin resistance to *S. aureus* [5]. So far, no adequate and safe drugs for treating biofilm-associated infections have been available. Subsequently, discovering nontoxic, inexpensive, and more potent antibiofilm materials is very important.

Actinomycetes are Gram-positive, anaerobic, or facultative bacteria with a morphological appearance similar to fungi, widespread in water and land. *Actinomycetes* produce a variety of diverse secondary metabolites and functions to defend themselves from their environment. Several new compounds from *Actinomycetes* have been found as antifungal, i.e., Caerulomycin A, which has antifungal activity on *Candida* [11]; kasugamycin from *Streptomyces kasugaensis,* which acts as a protein biosynthetic inhibitor; 2,4-di-tert-butyl-phenol which has antifungal activity in *Candida* and other fungal pathogens [12]. In addition, *Actinomycetes* isolated from coral mucus *Acropora digitifera* had activity as an antibiofilm because it can reduce cell surface hydrophobicity, which is an essential factor in the formation of *Streptococcus pyogenes* biofilms [13]. The active compound of *Actinomycetes* in inhibiting biofilms is by inhibiting proliferation, adhesion, metabolic enzyme activity (protease and phospholipase), hypha formation, and biofilm development [14].

Actinomycetes InaCC A758 isolated from rhizosphere soil in Kepulauan Seribu, Jakarta, Indonesia, reported have wide-spectrum antimicrobial activities against *C. albicans* ATCC 10231, *S. aureus* ATCC 6538, *Escherichia coli* BTCC B 614, *Bacillus subtilis*, and *Pseudomonas aeruginosa* with minimum inhibitory concentration (MIC) ranged at 3.125–50 µg/ml [15]. The *InaCC* A758 has 99.85% similarity with *Streptomyces badius* strain NRRL B-2567 based on 16S rRNA gene identification [15]. In this study, we investigated the potency of *Actinomycetes InaCC* A758 extracts against dual-species biofilms *C. albicans* and *S. aureus*.

MATERIALS AND METHODS

Organisms and antibiofilm agents

This study used *C. albicans* ATCC 10231 and *S. aureus* ATCC 6538, acquired from the Regional Laboratory of Yogyakarta, Indonesia. The stock cultures were stored at −80°C, subcultured on sabouraud dextrose agar medium for *C. albicans* ATCC 10231 and Muller Hinton agar medium for *S. aureus* ATCC 6538.

We used ethyl acetate (EA) and chloroform extract from *Actinomycetes InaCC* A758 for antimicrobial and antibiofilm agents. *InaCC* A758 was obtained from the Indonesian Research Institute (LIPI) collection. Five milligrams of *Actinomycetes* extract was dissolved into 5 ml dimethyl sulfoxide (DMSO) 5% for the stock.

Extraction of *InaCC* A758

The extraction of *InaCC* A758 used the maceration method with EA and chloroform as solvents. Briefly, *InaCC* A758 were inoculated in 10 ml SYP (yeast extract 0.4%, peptone 0.2%, and starch 1%) medium at 30°C for 3 days in an incubator shaker (130 rpm). After incubation, 10 ml precultured of *InaCC* A758 was added into 90 ml SYP medium (1:9) in an Erlenmeyer baffle flask 500 ml and incubated at 30°C for 3 days in an incubator shaker (130 rpm) for cultivation.

The extraction process used a liquid–liquid multi-stage extraction method, which begins with maceration using a nonpolar solvent (chloroform). Two liters cultured of *InaCC* A758 were added to 2 l of solvent (1:1) and incubated for four hours in an orbital shaker (120 rpm). The solution was poured into a separatory funnel, and the organic phase was collected into the receiving flask and evaporated using a rotary evaporator at 40 mPa at 40°C [15]. The dry extract was put into a tube and stored at 4°C until used.

Antimicrobial and antibiofilm testing of *InaCC* A758 extract

Inoculum preparation

Candida albicans ATCC 10231 was inoculated in 5 ml of yeast peptone dextrose (Sigma-Aldrich, USA) medium and incubated in a shaking incubator overnight at 35°C. *Staphylococcus aureus* ATCC 6538 was inoculated in brain heart infusion (BHI; Sigma-Aldrich, USA) medium and incubated in a shaking incubator at 37°C overnight. After overnight culture, the samples were centrifuged at 3,000 rpm for 5 minutes. The cells were washed twice with sterile phosphate buffered saline (PBS; Sigma-Aldrich, USA), and the pellets were resuspended with 5 ml of BHI medium (Sigma-Aldrich, USA). The final concentration of the cell suspension was adjusted at 1×10^8 CFU/ml according to McFarland standard 0.5 [16].

Antimicrobial activity assay of InaCC A758 extract

Antimicrobial activity assay used the micro broth dilution method based on the Clinical and Laboratory Standards Institute 2015 [17]. *Candida albicans* ATCC 10231 (~10^6 CFU/ml) alone, *S. aureus* ATCC 6538 (~10^5 CFU/ml) alone and dual-species *C. albicans* ATCC 10231 (~10^6 CFU/ml), and *S. aureus* ATCC 6538 (~10^5 CFU/ml) were added into microplate 96-wells in serial two-fold dilutions of *InaCC* A758 extracts (1.56–400 µg/ml). Negative controls used yeast or bacteria only, and positive controls used fluconazole alone, gentamicin alone, and fluconazole and gentamycin combined. The plates were incubated at 35°C for 24 hours, and the minimum inhibition concentration (MIC) values were determined by visual observation.

Biofilm inhibitory assay of InaCC A758 extracts against dual-species biofilms

Determination of the minimum biofilm inhibition concentration (MBIC) of dual-species biofilms *C. albicans*

ATCC 10231 and *S. aureus* ATCC 6538 using the method from Kart *et al*. [18] with modification. One hundred microliters of suspension containing *C. albicans* 10^5 CFU/ml were added to each well's microtiter plate and then incubated at 37°C for 90 minutes for the *Candida* biofilm attachment phase. After incubation, the plates were washed three times with 150 µl of sterile distilled water. One hundred microliters of BHI medium containing *S. aureus* 10^6 CFU/ml with various concentrations of test compounds (1.56–400 µg/ml) were put into wells and incubated at 37°C for 48 hours. Medium containing DMSO was used as solvent control, the microbial suspension was used as a negative control, fluconazole (0.39–100 µg/ml) and gentamycin (0.39–100 µg/ml) as a positive control, and BHI medium as a blank. After incubation, the medium was removed, washed with PBS three times, and dried at room temperature for 10 minutes. The biofilm formed was stained with 100 µl 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide solution and incubated at 37°C for 2 hours. The microplate was washed with sterile aquadest three times, and 125 µl of 96% ethanol was added to dissolve the biofilm formed. The optical density (OD) was obtained with an ELISA Reader at 570 nm [19]. The experiments were carried out three times replications. The percentage of biofilm-forming inhibition (% BFI) was calculated using the formula [20]:

$$\% \text{ BFI} = \frac{\overline{X}OD_c - \overline{X}OD_t}{\overline{X}OD_c - \overline{X}OD_{bl}} \times 100\%$$

ODc = optical density (570 nm) of the negative control (medium, yeast cells, and DMSO); ODt = optical density (570 nm) of the test well; ODbl = optical density of the blank.

Biofilm reduction assay of InaCC A758 extract against dual-species biofilms

The determination of minimum biofilm reduction concentration (MBRC) of dual-species *C. albicans* ATCC 10231, and *S. aureus* ATCC 6538 was the same as that of MBIC dual-species. Biofilms were grown together for 24 hours in the microplate, and then test compounds were added. The OD was obtained with an ELISA Reader at 570 nm [19]. The experiments were carried out three times replications. The percentage of biofilm-forming reduction (% BFR) was calculated using the formula [20]:

$$\% \text{ BFR} = \frac{\overline{X}OD_c - \overline{X}OD_t}{\overline{X}OD_c - \overline{X}OD_{bl}} \times 100\%$$

ODc = optical density (570 nm) of the negative control (medium, yeast cells, and DMSO); ODt = optical density (570 nm) of the test well; ODbl = optical density of the blank.

Scanning electron microscopy (SEM)

For SEM observations, dual-species *C. albicans* ATCC 10231 and *S. aureus* ATCC 6538 biofilm were grown on sterile polyvinyl chloride coverslips (with a thickness of 0.13–17 mm and a diameter of 22 mm) in 12-well microtiter plates (Corning® Costar®, Sigma-Aldrich, MO, USA) with EA and chloroform extract (1/4-1/2MIC), and untreated cells for 24 hours at 37°C. After that, the coverslips were washed twice with sterile PBS (0.1 M and pH 7.2). The samples were dehydrated with ethanol series (70% for 10 minutes, 95% for 10 minutes, and 100% for 20 minutes) and air-dried overnight in a desiccator [21,22]. The coverslip was coated twice with platinum vanadium using a sputter ion (Bal-Tec SCD 005), then bonded to carbon double-sided tape for examination by SEM (JEOL JED-2300, Japan).

Fractination, isolation, and identification of the compounds of *Actinomycetes InaCC* A758

The dried *Actinomycetes* extract was then fractinated using semi-preparative high-performance liquid chromatography (HPLC) (waters) with a C18 column. The mobile phase used methanol: water = 50:50 with a running time of 33 minutes. A total of 20 µl of *Actinomycetes* extract filtered with a syringe filter was injected into the HPLC machine. Each peak that appears is collected using a clean bottle and evaporated to be dried and tested for bioactivity. The active peak was then purified again similarly but with a different solvent composition, i.e., methanol: water = 80:20 with a running time of 20 minutes to obtain a single peak. Furthermore, the single peak is collected and dried for identification.

The pure compound was determined [23] using GC-MS-QP2010S (Shimadzu, Japan) [23]. The column type used was Agilent HP-5MS UI (30 m length, 0.25 mm diameter, 0.25 m film thickness), and used helium carrier gas. The compound identification was analyzed based on m/z using chromeleon™ chromatography data system software.

Statistical analysis

We used probit analysis to determine 50% biofilm inhibition concentration (BIC_{50}), 80% biofilm inhibition concentration (BIC_{80}), 50% biofilm reduction concentration (BRC_{50}), and 80% biofilm reduction concentration (BRC_{80}).

RESULTS AND DISCUSSION

Actinomycetes InaCC A758 extraction product

Two liters of fermented liquid from *Actinomysetes InaCC A758* culture produced 290 mg of EA extracts (758 EA) and 187 mg of chloroform extracts (758 Cl).

Antimicrobial activity of *InaCC* A758

The minimum inhibitory (MIC) value of *InaCC* A758 ethyl acetate extract (758 EA) against *S. aureus* was eight times smaller than that of *C. albicans* (Table 1). This MIC indicated

Table 1. MIC values of InaCC A758 extracts against *C. albicans* and *S. aureus* planktonic cells (mono-species and dual-species).

Extractsa	Mono-species MIC values (µg/ml)		Dual-species MIC values (µg/ml)
	C. albicans	*S. aureus*	
758 EA	50	6.25	400
758 Cl	6.25	12.5	400
Flu/Gen	1.56	3.125	50/50

[a]758 EA: ethyl acetate extract of InaCC A758; 758 Cl: Cloroform extract of InaCC A758; Flu: fluconazole; Gen: gentamycin.

that A758 EA had better inhibitory activities against Gram-positive bacteria than fungi. In contrast, the MIC value of *InaCC* A758 chloroform extract (758 Cl) against *C. albicans* was twice as small as the MIC value of *S. aureus*. These results show that A758 Cl was more active in inhibiting fungi than Gram-positive bacteria. Meanwhile, the MIC values of both extracts, i.e., 758 EA and 758 Cl against dual-species *C. albicans* and *S. aureus* planktonic cells, were 8–64 times greater than MIC values in mono-species. Both EA and chloroform extracts have the same activities in inhibiting dual-species planktonic cells (MIC: 400 µg/ml) (Table 1).

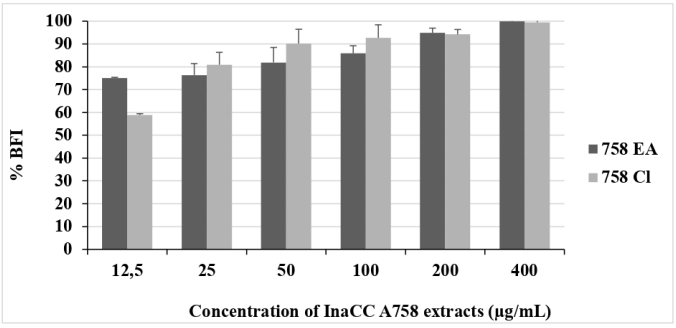

Figure 1. BFI percentation of *InaCC* A758 extracts against dual-species biofilm: *C. albicans* ATCC 10231 and *S. aureus* ATCC 6538. Inhibitory activities were dose-dependent. Statistical analysis by probit analysis determined BIC_{50} and BIC_{80} value of EA extracts were 3.57 ± 1.384 and 30.5 ± 6.05 mg/ml, respectively. The BIC_{50} and BIC_{80} values of chloroform extracts ranged from 6.66 ± 1.59 and 29.25 ± 4.64 mg/ml, respectively. The experiments were carried out three times replication. 758 EA: ethyl acetate extract of *InaCC* A758; 758 Cl: chloroform extract of *InaCC* A758.

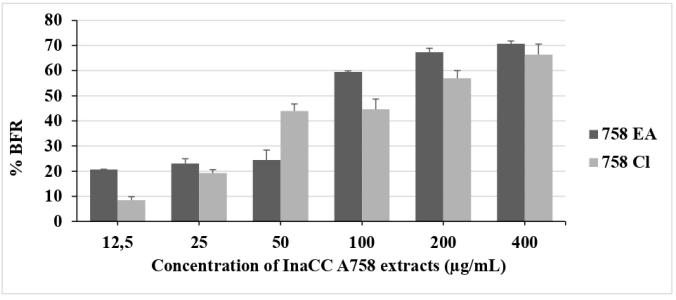

Figure 2. BFR percentation of *InaCC* A758 extracts against dual-species biofilm: *C. albicans* ATCC 10231 and *S. aureus* ATCC 6538. Statistical analysis by probit analysis determined BRC_{50} and BRC_{80} of EA extracts were 100.62 ± 0.14 and 596.4 ± 24.4 mg/ml, respectively. The BIC_{50} and BIC_{80} value of chloroform extracts ranged from 131.85 ± 12.9 and 849.6 ± 29.03 mg/ml, respectively. The experiments were carried out three times replication. 758 EA: ethyl acetate extract of *InaCC* A758; 758 Cl: chloroform extract of *InaCC* A758.

Biofilm inhibition activity of *InaCC* A758 against dual-species biofilms

The biofilm inhibition percentage of *InaCC* A758 extracts is shown in Figure 1. The increase of dual-species biofilm inhibition is linear with adding the extract doses. These results indicated that the activities of *Actinomycetes InaCC* A758 extract were dose dependent. Both EA and chloroform extracts could inhibit 50% formation of dual-species biofilms at concentrations less than 12.5 µg/ml. In inhibiting 80% of dual-species biofilm formation, chloroform extract was better than EA extract (25 and 50 µg/ml, respectively). Both extracts could inhibit 100% dual-species biofilms at 400 µg/ml.

Biofilm reduction activity of *InaCC* A758 against dual-species biofilms

The biofilm reduction activity of *InaCC* A758 extracts is shown in Figure 2. EA extract reduced more than 50% of the biofilm formed at 100 µg/ml concentration, while chloroform extract required a two-fold concentration (200 µg/ml). At the highest concentrations tested (400 µg/ml), EA and chloroform extracts could reduce the formation of dual-species biofilms by more than 60%. However, EA extract has better activities than chloroform extract in reducing the dual-species biofilms.

The probit analysis for determining the value of dual-species BIC_{50} and BIC_{80} and BRC_{50} and BRC_{80} was shown in Table 2. The potency of EA extract to reduce dual-species biofilm was better than chloroform extract.

Biofilm structures observation by SEM

The structures of dual-species biofilm without treatment (control) were pseudohifa of *C. albicans* with smooth surfaces. The structure of *S. aureus* cells was round-shaped, clustered, and attached to the surface of pseudohifa. The surface of *S. aureus* cells was also smooth (Fig. 3a).

The structure of dual-species biofilm cells exposed by 758 EA at a concentration of 1/4 MIC (100 µg/ml) showed a reduced number of *C. albicans* and *S. aureus* cells. Yeast cells with oval shapes were stuck together and had varying sizes and smooth surfaces. *Staphylococcus aureus* cells appeared round-shaped, clustered, and partially attached to the surface of the yeast cells. Some bacterial cells seen have structural damage (Fig. 3b). Cells exposed to 758 EA at a concentration of 1/2 MIC (200 µg/ml) appeared to undergo morphological changes where yeast cells became wrinkled and damaged (Fig. 3c). Cells were exposed to 758 Cl at a concentration of 1/4 MIC (100 µg/ml), and the yeast cells were attached by *S. aureus* cells. The surface of the yeast cells began to wrinkle and become uneven. Bacterial cells appeared round-shaped, and the membrane cells seemed to be in intake, but the cells' numbers were reduced

Table 2. BIC and BRC of InaCC A758 extracts against dual-species *C. albicans* and *S. aureus* biofilm based on probit analysis.

Extracts	BIC50 (µg/ml) (±SD)	BIC80 (µg/ml) (±SD)	BRC50 (µg/ml) (±SD)	BRC80 (µg/ml) (±SD)
758 EA	3.57 ± 1.384	30.50 ± 6.05	100.62 ± 0.14	596.4 ± 24.4
758 Cl	6.66 ± 1.59	29.25 ± 4.64	131.85 ± 12.9	849.6 ± 29.03

758 EA: ethyl acetate extract of InaCC A758; 758 Cl: Chloroform extract of InaCC A758; BIC:biofilm inhibition concentration; BRC: biofilm reduction concentration.

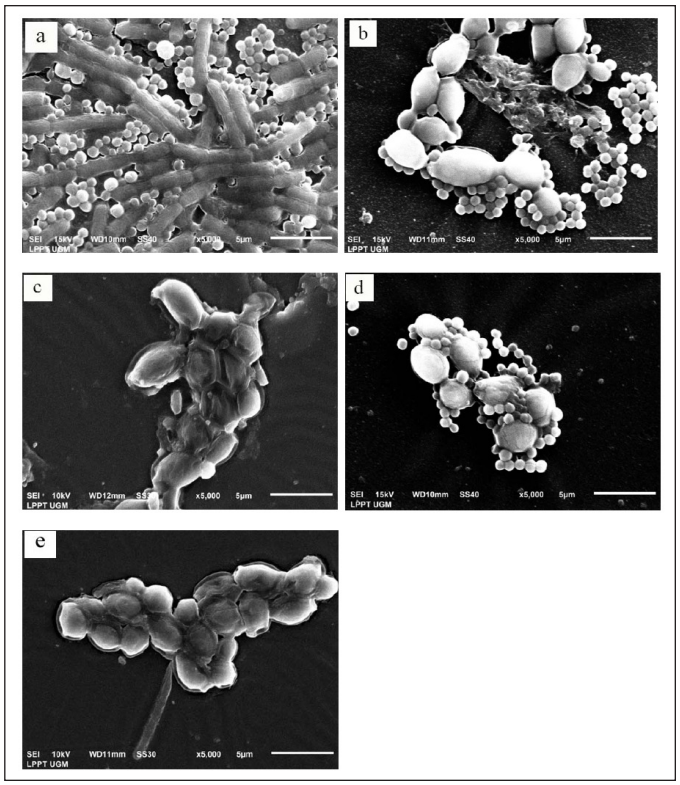

Figure 3. SEM observation of dual-species biofilm: *C. albicans* ATCC 10231 and *S. aureus* ATCC 6538. (a) Cells without pre-treatment (control) seemed pseudo hyphae of *C. albicans* with smooth surfaces and overlapping. *Staphylococcus aureus* cells were round in shape and clique. Bacterial cells are attached to the surface of pseudo hypha. The membrane surface of *S. aureus* cells was smooth; (b) cells were exposed to EA extract of *InaCC* A758 at a concentration of 1/4 MIC (100 mg/ml), which showed a reduced amount of yeast and bacterial cells. Bacterial cells are attached to the surface of the yeast cells partially. There was structural damage of bacterial cells; (c) cells were exposed to EA extract of *InaCC* A758 at concentration 1/2 MIC (200 mg/ml) appeared to undergo morphological changes where yeast cells become wrinkled and damaged; (d) cells were exposed to chloroform extract at concentration 1/4 MIC (100 mg/ml) seemed the surface of the yeast cells was wrinkled and uneven; (e) cells were exposed to chloroform extract at concentration 1/2 MIC (200 mg/ml) appeared only yeast cells with irregular oval-shaped, surface wrinkled, uneven, and damaged.

(Fig. 3d). Yeast cells with irregular oval-shaped surfaces were wrinkled, uneven, and damaged when exposed to 758 Cl at a concentration of 1/2 MIC (200 mg/ml) (Fig. 3e).

Fractination, isolation, and identification of active compounds of *InaCC* A758

Fractionation of *InaCC* A758 EA extract resulted in eight fractions (F1 EA–F8 EA). In comparison, chloroform extract obtained seven fractions (F1 Cl–F7 Cl) with the retention time (Rt) of each peak and its area percentage shown in Table 3. The chromatograms of the fractions results using semipreparative HPLC can be seen in Figure 4. The Identification of the active compound *InaCC* A758 using gas chromatography-mass spectrometry (GC-MS) is shown in Figure 5 and Table 4. Figure 5 shows that the EA fraction has seven peaks with possible compounds that can be seen in Table 4.

Table 3. *Retention time (Rt) and percentage of the area of the InaCC A758 fraction.*

	Fraction	Rt (minute)	Area (%)	The colour of fraction
	EA fraction			
1	F1 EA	4.261	20.62	Clear
2	F2 EA	9.245	3.55	Clear
3	F3 EA	14.896	11.50	Clear
4	F4 EA	16.551	3.55	Clear
5	F5 EA	24.185	1.76	Light yellow
6	F6 EA	25.674	13.65	Yellow
7	F7 EA	26.529	28.31	Tumeric yellow
8	F8 EA	36.334	17.06	Clear
	Cllorofom fraction			
1	F1 Cl	9.263	11.51	Clear
2	F1 Cl	14.971	54.99	Clear
3	F3 Cl	16.626	6.73	Clear
4	F4 Cl	17.847	3.37	Clear
5	F5 Cl	23.917	1.27	Light yellow
6	F6 Cl	25.449	5.27	Yellow
7	F7 Cl	26.288	16.86	Tumeric yellow

This study showed that the MIC values of *Actinomycetes InaCC* A758 extract against dual-species *C. albicans* and *S. aureus* were higher than those of monospecies (Table 1). Interactions between microbes in multi-species communities could increase virulences and drug tolerance [24] and require higher doses for treatment. The co-infection of *C. albicans* and *S. aureus* could increase mortality because there was "lethal synergy" when they were together [24]. Todd *et al.* [25] reported an increase in mortality range at 80%–100% in mice with intraabdominal infection models inoculated with dual-species *C.albicans* and *S. aureus*, while in monospecies infections, there was no mortality in the mouse model. The ability of *C. albicans* to form hyphae is an essential factor in increasing mortality because the hyphal can invade and penetrate human tissue [26]. It was difficult to eradicate the dual-species biofilm because the polysaccharides contained in the biofilm matrix of *C. albicans* could influence the tolerance of *S. aureus* to antimicrobials when they were grown together [27]. Extracellular biofilm matrix could protect microbes against antimicrobial agents. The protection effect of the extracellular matrix against antimicrobial agents in dual-species biofilm communities occurred through two mechanisms: the first is that the extracellular matrix binds drug components; thus, the drugs could not penetrate biofilms. The second is the presence of the matrix material itself, which induces drug tolerance due to the expression of related genes [24]. Interestingly, the *Actinomycetes InaCC* A758 extracts in this study could inhibit the dual-species biofilm up to 80% at concentrations 30.50 ± 6.05 µg/ml (Table 2). This result is an important finding regarding the potential of *Actinomycetes* extract as an anti-biofilm agent.

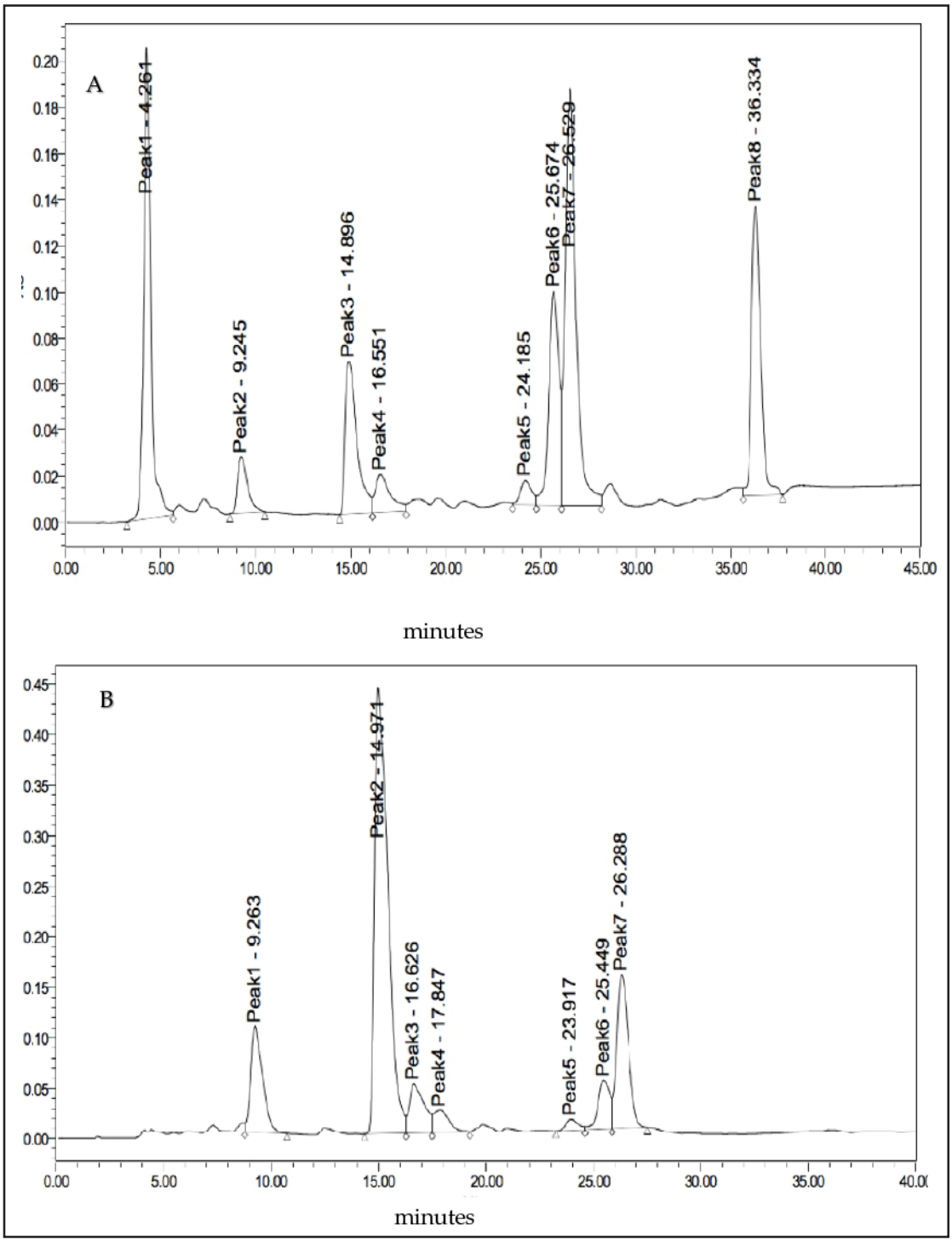

Figure 4. Chromatograms of the *InaCC* A758 isolated fractions. (A) EA extracts. (B) Chloroform extracts.

This study found the unique structure of dual-species *C. albicans* and *S. aureus* biofilm (Fig. 3). This result is related to Peters *et al.* [2], which reported the physical interactions within the dual-species biofilm communities. The dual-species of *S. aureus* and *C. albicans* biofilms formed a unique architecture where *S. aureus* attached to the *C. albicans* hypha. *Staphylococcus aureus* would form microcolonies attached to the surface of the biofilm with *C. albicans* as the basis [5]. The *S. aureus*-hypha binding would influence the formation of yeasts into hyphae in the biofilm communities [24]. The specific interactions between *S. aureus* and *C. albicans* could affect the growth of biofilms [27]. In the dual-species community, *S. aureus* is attached to the hyphal of *C. albicans* specifically [2], and this hypha acts as *an S. aureus* courier to invade human tissue [28]. *Staphylococcus aureus* was bound in the matrix secreted by *C. albicans*, i.e., β-1,3-

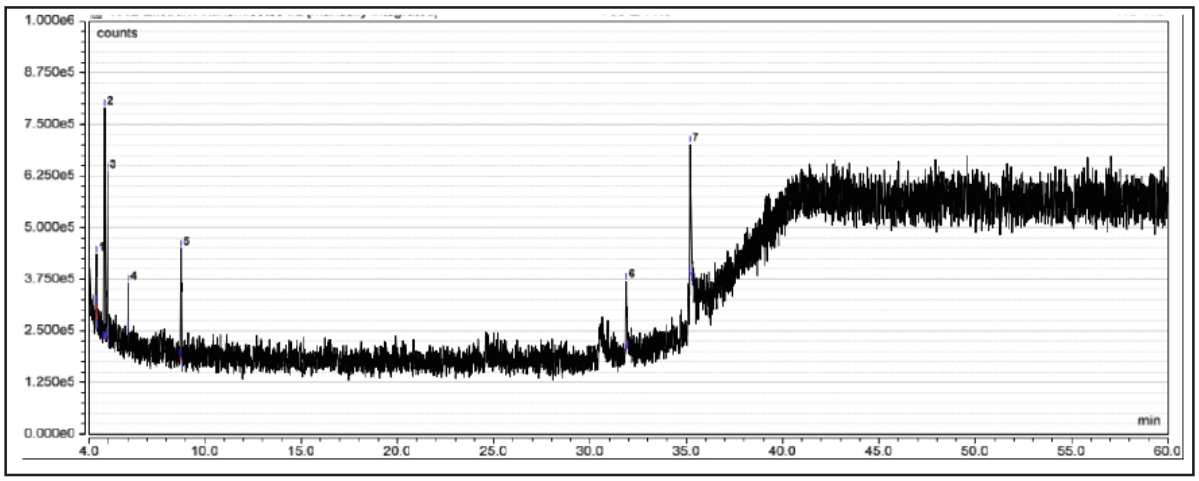

Figure 5. The GC-MS spectra of EA InaCC A758 fraction.

Table 4. Molecular weight and compounds contained of the EA InaCC A758 fraction.

Rt (minute)	Compounds	Chemical formula	Molecular weight	Area (%)
4.38	Dodecanoic acid 3-hydroxy	$C_{12}H_{24}O_3$	216	3,033.534
4.80	1,3,5-Pentanetriol,3-methyl	$C_6H_{14}O_3$	134	22,200.553
4.95	1,3-Dioxolane-4-methanol,2-ethyl	$C_6H_{12}O_3$	132	16,313.901
6.02	2-Cyclopropylcarbonyloxytridecane	$C_{17}H_{32}O_2$	268	2,130.146
8.76	2-Hepten-1-ol,(E)	$C_7H_{14}O$	114	9,300.922
31.89	Methyl 6-methyl heptanoate	$C_9H_{18}O_2$	158	7,882.348
35.21	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	16,855.053

glucan [27], affecting its resistance to vancomycin [5]. Protein adhesin plays an essential role in bacterial-fungal interaction in biofilm communities. The Als3 protein was a specific receptor secreted by *C. albicans* hypha, which could bind *S. aureus* [28]. Kean *et al.* [29] reported another component in the biofilm matrix, i.e., extracellular DNA, which supported the attachment of *S. aureus* to fungal cells and affected the stability of the biofilm matrix.

The structures of dual-species biofilm cells exposed by *Actinomycetes InaCC* A758 extracts displayed morphological damage to the cells (Fig. 3). Alkylphenol compounds found in *Actinomycetes* could inhibit the germination of spores [30]. Polyketide compounds such as Forazaline A can damage the integrity of the cell membrane by deregulating phospholipids. Phenolic compounds worked as antifungals by influencing the morphological changes of fungal cells. This study also showed that untreated cells appeared in the pseudohyphae while treated cells appeared only in yeast cells (Fig. 2). This result was possible because *Actinomycetes* extracts act as antimicrobial and antibiofilm in dual-species by damaging the membrane cells and inhibiting the yeast-hyphal transition. Phenolic compounds found in *Actinomycetes* effectively inhibited and damaged *C. albicans* biofilms because they inhibited hyphal development. Hyphae were very involved in the initial adhesion stage of biofilms [31]. Glycolipid compounds produced from actinobacterium *Brevibacterium casei* MS419 isolated from the Dendrilla nigra sponge inhibited the initial attachment phase of *E. coli* and *Vibrio alginolyticus* biofilm cells [32].

The GC-MS results showed that the EA of *InaCC* A758 fraction contained seven compounds, i.e., 3-hydroxy dodecanoic acid; 1,3,5-pentanetriol,3-methyl; 1,3-dioxolane-4-methanol,2-ethyl; 2-cyclopropylcarbonyloxytridecane; 2-hepten-1-ol,(E); methyl 6-methyl heptanoate; and 11-octadecenoic acid, a methyl ester. Dodecanoic acid 3-hydroxy is a medium-chain saturated fatty acid compound. Fatty acids are compounds that are found in many living organisms. Fatty acids are essential in forming cell structures and play an important role in energy for living things.

Several studies have reported that fatty acid compounds are potential antimicrobials [33] and antibiofilms [34]. Several fatty acids are reported to be able to damage and inhibit biofilm formation from various pathogens such as *S. aureus* [35], *P. aeruginosa* [36], and *C. albicans* [37]. Lee *et al.* [38] reported the activity of dodecanoic acid, which could inhibit *C. albicans* biofilms up to more than 75% with MIC values ranging from 100 to 200 µg/ml. This compound inhibited the growth of hyphae and cell aggregation and reduced the production of farnesol and sterols. Farnesol plays an essential role in quorum sensing activity, which affects the formation of biofilms, and sterols play an important role in the structure of cell membranes.

The 1,3,5-pentane-triol,3-methyl compound is an alkane compound class of aliphatic compounds. The 1,3,5-pentanetriol,3-methyl compounds are contained in several extracts from the stems and stalks of the *Buchanania cocinchinensis* plant and are reported to have antioxidant and antimicrobial activity [39]. The 1,3-dioxolane is a compound

that is widely used in the synthesis of compounds that have biological activity. In synthesizing compounds of natural origin, these compounds are often used as protective groups for ketones and aldehydes. Several compounds containing ring 1,3 dioxolane were reported to have antifungal, antibacterial, antiviral, and antineoplastic activity [40] succeeded in synthesizing compounds derived from 1,3-dioxolane and reported that these compounds have activity against Gram-positive bacteria (*S. aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, and *Enterococcus faecalis*) and Gram-negative bacteria (*P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, and *Proteus mirabilis* ATCC 14153). The compound also has antifungal activity against *C. albicans* 10231. The compound 11-octadecenoic acid, methyl ester, has anti-inflammatory, anticancer, and hypocholesterolemic activity.

However, research on secondary metabolites of *Actinomycetes* as dual-species antibiofilm is very rare. In fact, in the human body, biofilms are usually formed in multi-species biofilms. This result can lead to further studies to develop the *Actinomycetes InaCC* A758 as an antibiofilm, especially in dual-species.

CONCLUSION

Actinomycetes InaCC A758 have activities in inhibiting and reducing dual-species biofilms. The activities of EA extract and chloroform extract as antibiofilm were almost similar. The *InaCC* A758 extracts affect the morphological shape of bacteria and fungal cells. However, this research was still fundamental, so further study of the fractionation and isolation of bioactive compounds is still needed.

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AUTHOR CONTRIBUTIONS

Concept and design: Mustofa Mustofa and Puspita Lisdiyanti.; data acquisition: Titik Nuryastuti and Setiawati. Setiawati; data analysis, Setiawati Setiawati, Eti Nurwening Sholikhah, and Jumina Jumina; drafting manuscript: Setiawati Setiawati, Titik Nuryastuti, Eti Nurwening Sholikhah.; critical revision of manuscript: Mustofa Mustofa, Jumina Jumina, and Puspita Lisdiyanti.; Statistical analysis: Setiawati Setiawati, Eti Nurwening Sholikhah; funding acquisition: Eti Nurwening Sholikhah. Project administration, Setiawati Setiawati; supervision: Mustofa Mustofa; Titik Nuryastuti; final approval: Eti Nurwening Sholikhah.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest. The funders had no role in the study's design, in the collection, analysis, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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