The anti-adhesion activity of Aspergillus oryzae cell free supernatant prevents the formation of Klebsiella pneumoniae biofilms on abiotic surfaces

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Abstract

For more than a century, humans have used medical equipment. While very helpful in treating patients, they can cause serious health problems. The spread of bacteria through indwelling medical devices is the beginning of the problem. Adhesion to the medical device's surface then proceeds with forming a biofilm. This structure then becomes a reservoir of resistant bacteria. The purpose of this study was to investigate the potential of the Aspergillus oryzae in inhibiting biofilm and bacterial adhesion to abiotic polystyrene surfaces. We used A. oryzae cell-free supernatant (CFSAO) to challenge Klebsiella pneumoniae (ATCC1705). We studied the potential of CFSAO in inhibiting growth, adhesion, and production of Klebsiella pneumoniae biofilm. The cell-free supernatant of A. oryzae was proven to have bactericidal activity against K. pneumoniae. Cell-free supernatant A. oryzae was able to inhibit the adhesion of K. pneumoniae to abiotic surfaces. In addition, CFSAO firmly controls Klebsiella pneumoniae biofilm formation. In addition, CFSAO was able to destroy K. pneumoniae biofilms that had formed. Together, these results explain that A. oryzae can be used as natural antiadhesive coatings to prevent the colonization of medical devices.

Key Words: anti-adhesion, Aspergillus oryzae cell, supernatant prevents, Klebsiella pneumoniae

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Background to the Study

The use of medical devices has become an essential part of healthcare worldwide (Sabet Sarvestani & Sienko, 2018). Medical devices have helped much medical personnel serve patients in clinics and hospitals setting (Shah & Robinson, 2007). However, the spread of infection due to medical devices has become a severe problem lately (van Epps & Younger, 2016). Bacterial infections caused by medical devices are generally hazardous because bacteria can form biofilms (Maiti et al., 2014; van Epps & Younger, 2016) One of them is Klebsiella pneumoniae. This group of bacteria is more resistant to antibiotics (Naparstek et al., 2014). The biofilm matrix will provide mechanical protection for K. pneumoniae (Yin et al., 2019), making it difficult for antibiotics to reach. Biofilm-forming bacteria are 1000 times stronger than non-biofilm-producing bacteria (Potera, 2010).

The process of K. pneumoniae biofilm formation consists of attachment (adherence), maturation (maturation), and planktonic bacterial reactions (dispersion) (Oleksy-Wawrzyniak et al., 2022). Initially, planktonic bacteria can find appropriate abiotic surfaces, such as indwelling devices. The bacteria then adhere to the abiotic surface (Khatoon et al., 2018). When the number of bacteria increase, they will produce quorum sensing to communicate with each other (Xie et al., 2019). One such communication is the formation of microcolonies and biofilm maturation. Some

bacterial colonies are released in some conditions and will create new colonies in a new place (image taken from Chrzanowski et al., 2011).

Inhibition of K. pneumoniae biofilm can be done by interfering with the initial attachment of bacteria (Segev-Zarko & Shai, 2017). Materials capable of inhibiting this step are called biosurfactants. Biosurfactants are surface-active compounds synthesized by microbes that can reduce the liquid surface and different interfacial tensions and increase the emulsion (Rivardo et al., 2009). Biosurfactants are less toxic and non-allergenic. Biosurfactants also have high biodegradability and high activity at extreme temperatures, pH, and salinity. In addition, biosurfactants can be synthesized from renewable raw materials (Sáenz-Marta et al., 2015). Biosurfactants can be applied as health care products as surface coatings (Znaleziona et al., 2008).

Many studies have proven that Ascomycota is isolated from various environments to produce biosurfactants (Asgher et al., 2020). Aspergillus niger and A. fumigatus are known to be capable of producing glycolipids and lipopeptides. These two compounds act as biosurfactants (Shu et al., 2021). Until now, no research has discussed the potential of A. oryzae biosurfactants against bacteria. At the same time, A. oryzae products is known to be safe and easy to develop (Nacef et al., 2020; Park et al., 2008) . Aspergillus oryzae is known to produce several compounds that act as antibiofilm. Fatty acids can act as antibiofilm by inhibiting the process of attachment, adhesion, EPS, fimbriae, biofilm formation, and biofilm maturation (Kumar et al., 2020). Aspergillus oryzae also contains extracellular enzymes such as -glucosidase, which can synthesize phenolic compounds (Sáenz-Marta et al., 2015). This study aimed to determine the potential of A. oryzae biosurfactant against Klebsiella pneumoniae bacteria.

Methods

Strains and growing conditions

Klebsiella pneumoniae which forms biofilms was obtained from the Surabaya Health Laboratory Center (BAA, 1705). Bacteria were cultured in Mueller Hinton (MH) media and incubated at 37°C for 24 hours. At least 3-5 bacterial colonies were transferred from Mueller Hinton (MH) media to 5 ml BHI. Then shake the incubator for 2-6 hours, or until the turbidity exceeds the Mc Farland standard (108 CFU/mL). After being equal with the Mc. Farland standard sterile saline was diluted 100 times, yielding a concentration of 106 CFU/mL. This bacterial suspension is now ready for testing. The fungi strain A. oryzae was provided by the Indonesian Culture Collection (Ina-CC).

Preparation of Cell-Free Supernatant from Aspergillus oryzae

Precultures were grown in 500 mL Erlenmeyer flasks, each containing a 100 mL preculture medium. It was inoculated with an appropriate number of spores to reach a density of approximately 106–107 spores per mL. The cultures were incubated at 27◦ C and 350 rpm for 72 hours. Following the incubation time, the CFS was filtered through a 0.45 m pore size syringe filter (Advantec, Tokyo, Japan) and stored at 80°C until further use.

Minimum Inhibitory Concentration

The MIC determination test was carried out using the microdilution method. The bacteria were suspended in Luria Bertani (LB) broth, and McFarland standard (6x108 CFU/ml) was used. Serial dilutions were carried out for CFSAO levels of 6.25%, 12.5%, 25%, 50%, and 100% with Phosphate buffered saline (PBS) solution as a solvent. The solvent control was used PBS, the negative control was K. pneumoniae, and the positive control was used the antibiotic ciprofloxacin. The test solution was placed on a 96-well flat-bottom microplate, with a total volume of 100 µL per well using a micropipette. Then, 100 µL of bacterial suspension was added to each well, while the control medium was added with PBS. Plates were incubated for 18-24 hours at an incubator temperature of 36.6°C. Following time, the test solution in the microplate was discarded and washed with running water three times. The plate was then dried and added with 1% 125 µL crystal violet dye and allowed to stand for 15 minutes. After that, it was rewashed with running water three times and allowed to stand for 15 minutes. Then 200 µL of 30% acetic acid was added and allowed to stand for 15 minutes. The test results in the form of optical density (OD) were read using the Bio-rad microplate reader Benchmark at a wavelength of 595 nm. Following time, to determine the MBC, the dilution representing the MIC and at least two of the more concentrated test product dilutions are plated and enumerated to determine viable CFU/ml.

Bacterial biofilm production capability test

A bacterial suspension of as much as 200 µL was

inserted into the microplate. The microplates were closed and incubated at 37o C for 24, 48, 72, and 96 hours. After incubation, the contents of the microplate were removed and washed with distilled water. The microplate was then given 200 µL of 1% crystal violet and incubated for 15 minutes at room temperature. After that, the microplate was washed with distilled water and allowed to dry at room temperature. 200 µL of 30% acetic acid was put into the microplate and incubated for 15 minutes at room temperature. The microplate was then measured using a microplate reader at an optical density of 595nm .

Bacterial Initial attachment

A 200 µL series concentration of CFS was introduced into the microplate. The microplate was then closed and incubated at 27ºC for 1 hour. After incubation, the entire contents of the microplate were removed. The bacterial suspension in the microplate was then closed and incubated at 37o C for 24, 48, and 72 hours (Kragh et al., 2019). The incubated microplate was removed and washed with distilled water. A total of 200 µL of 1% crystal violet solution was added to each well. The microplate was then incubated for 15 minutes at room temperature. The microplate was washed with distilled water and allowed to dry at room temperature. A 200 µL of 30% acetic acid, then put into the microplate and incubated for 15 minutes at room temperature. Afterward, the microplate was measured at an optical density of 595nm using a microplate reader. The tests were carried out in triples, The percentage of biofilm inhibition was calculated using the following formula: [(OD growth control – OD sample) / OD growth control] × 100.

Biofilm Inhibition Assay

The bacterial suspension was co-cultured with CFA. The concentration of the bacterial suspension-CFS was made into 3.75%, 6.25%, 12.5%, 25%, and 50%. A total of 200 µL of the test suspension and bacterial suspension were put into the microplate. The microplate was closed and incubated at 37o C for 24 hours. After incubation, the contents of the microplate were removed and washed with water. The microplate was dyed by adding 200 µL of 1% crystal violet solution and incubating for 15 minutes at room temperature. The dye was then washed with distilled water and allowed to dry at room temperature. After the microplate was dry, 200 µL of 30% acetic acid was put into the microplate. Then incubated for 15 minutes at room temperature. Microplates were measured using a microplate reader at an optical density of 595nm (Bjarnsholt et al., 2011). The test was carried out in triples, The percentage of biofilm inhibition was calculated using the following formula: [(OD growth control – OD sample) / OD growth control] × 100.

Biofilm Degradation assay

200 µL of bacterial suspension was put into the microplate, closed the microplate, and incubated at 37o C for 72 hours. The contents of the microplate that had been incubated were removed. Then 200 µL of series concentration of CFS was inserted into the microplate. The negative control was filled with PBS %, and the positive control was filled with ciprofloxacin. The microplate was closed and incubated at 37o C for 6 hours. After incubation, the contents of the microplate were removed and washed with distilled water. A 200 µL 1% crystal violet solution was added and incubated for 15 minutes at room temperature. The dye was washed with distilled water and allowed to dry at room temperature. Then 200 µL of 30% acetic acid was added to the microplate and incubated for 15 minutes at room temperature. Afterward, the microplate was measured at an optical density of 595nm using a microplate reader. The tests were carried out in triples, The percentage of biofilm inhibition was calculated using the following formula: [(OD growth control – OD sample) / OD growth control] × 100.

Results

Minimum Inhibitory Concentration

The MIC test was carried out to determine the minimum concentration in the attachment resistance and biofilm tests. The concentrations used were 6.25%, 12.5%, 25%, 50%, and 100%. The results of the MIC test can be seen in Figure 1.

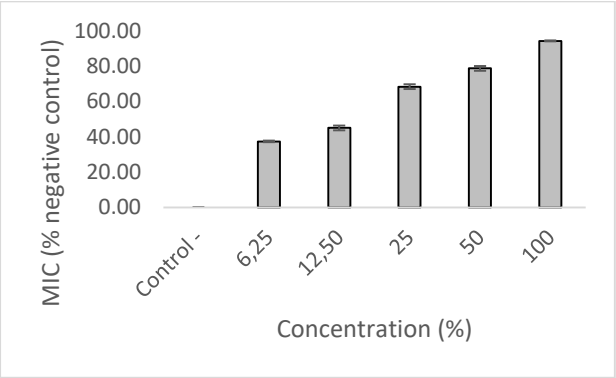

Figure 1. Minimal Inhibitory concentration (MIC) of CFSAO against *Klebsiella pneumoniae* 1705.

MIC Level that can kill 90% of the bacterial population compared to the negative control. MIC at 100% concentration was 94.2% (bold). While the negative control of KP-ESBL without CFSAO exposure was 0%. CFO was able to inhibit the growth of KP. Bars indicate the standard error, and the sign (*) above the bars indicates a significant difference ($p < 0.05$).

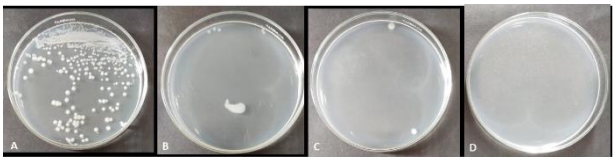

Figure 2. Minimum Bactericidal concentration (MBC) of CFSAO against *Klebsiella pneumoniae* 1705

Concentration from left to right A. 12.5%, B. 25% C. 50%, D. 100%, Minimum bactericidal concentration is a concentration that can kill > 99% isolates at 100% concentration, there was no growth of KP1705.

Figure 1 presents CFSAO's MIC and MBC values in various concentrations. The concentration of CFSAO, which could inhibit > 90% of the bacterial population (MIC), was at a concentration of 100%. At the same time, the concentration capable of killing 99% was obtained through inoculation into the agar medium. The minimum bactericidal concentration of CFSAO against KP1705 is a concentration of 100% (see Figure 2). Results. The largest concentration used for the CFSAO inhibition test on KP biofilm was 50% CFSAO MIC for KP. From the linear regression test results, the R-value is close to 1. The analysis results indicate that CFSAO can inhibit the growth of *Klebsiella pneumoniae* in a dose-dependent manner. Henceforth, the measurement of the inhibitory ability of biofilms and virulence factors using sub_MIC concentrations, namely, 1/8.1/16 and 1/32 MIC, namely 3.75%, 6.25%, 12.5%, 25%, and 50%

Bacterial biofilm production capability test

Prior to the anti-adhesion and antibiofilm tests, the ability of KP 1705 to produce biofilms was tested first.

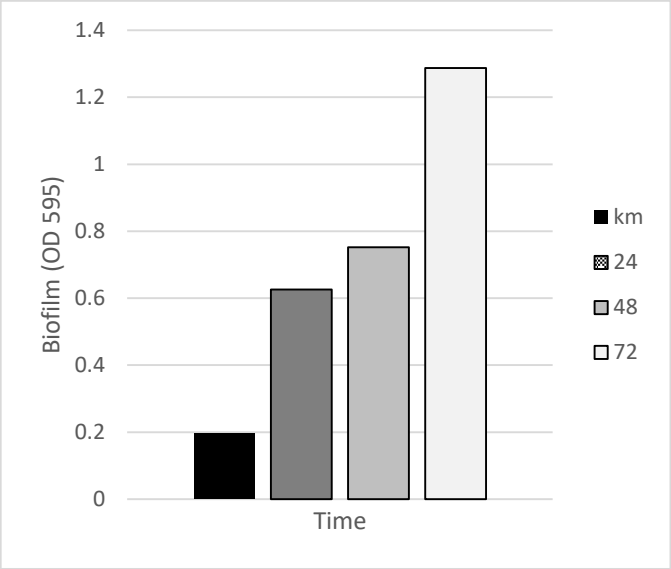

Figure 3. Klebsiella pneumoniae biofilm production.

It appears that KP 1705 is capable of producing solid biofilms (>0.331 as OD cut). The highest and most stable biofilm formation was at 72 hours.

Determine the strength of the biofilm. It can be measured by comparing the ODcut value with the suspension OD value. It is known that the suspension OD value is 1,287, and to find the ODcut value, it can be found by entering the OD value in the ODcut formula = negative control OD + 3 (standard deviation of negative control OD). Moreover, I obtained an ODcut of 0.331. After that, it can be concluded that the biofilm growth test meets the equation 4x ODcut ODsuspense. This means that Klebsiella pneumoniae is a bacterium capable of forming solid biofilms (figure 2). The 24-hour culture was used to test the inhibition of attachment and biofilm production. Meanwhile, the biofilm degradation test used culture for 72 hours. Seventy-two hours is the time needed to form a strong biofilm that is not easily broken down.

Bacterial Initial attachment (adhesion)

The activity of preventing biofilm attachment was carried out by the microdilution method with 1% crystal violet dye. The measurement of biofilm inhibition is indicated by the OD value measured using a microplate reader at a wavelength of 595 nm according to the wavelength that can be absorbed by crystal violet (Sun et al., 2007). In determining OD, if the turbidity level of the test media decreases, the resulting OD value will be smaller. The turbidity level in the test medium indicates how much biofilm is present. The activity of preventing the attachment of CFSAO biofilm to KP can be seen in Figure 4.

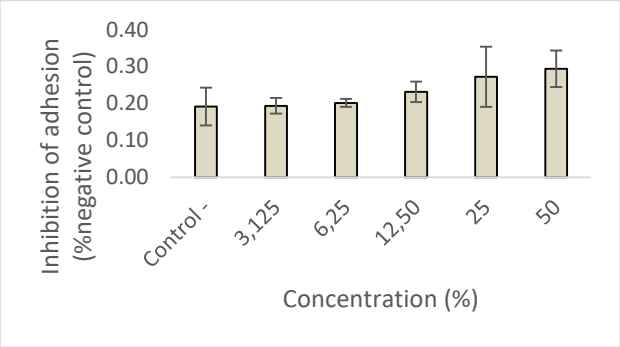

Figure 4. Prevention of Adhesion of CFSAO Biofilm to KP1705.

Preventive effect of CFSAO attachment on biofilms was tested. Microplate incubated for 24 hours with different concentrations of CFSAO. The concentration of CFSAO was given relative to the minimum inhibitory concentration (MIC) of KP1705. CFSAO attachment inhibition concentrations of 3.75%, 6.25%, 12.5%, 25%, and 50% were 19%, 20%, 23%, 27%, and 29%, respectively. Prevention of biofilm attachment was highest at 50% CFSAO concentration. In comparison, the negative control of KP1705 without exposure to CFSAO was 19%. The value of biofilm inhibition by CFSAO was better than that of kanamycin (72.18% < 33.74). Bars indicate the standard error.

Cell-free supernatant A. oryzae (CFSAO) was then administered with a concentration relative to the minimum inhibitory concentration (MIC). The value of the biofilm attachment inhibition [100-(sample abs/control abs x 100)] at a wavelength of 595 nm for each concentration of CFSAO can be seen in Figure 3. In the crystal violet staining test, attachment of the KP1705 biofilm was significantly inhibited at a concentration of 1/2 × MIC. The negative control (KP1705 bacteria without CFSAO exposure) showed the lowest biofilm inhibition value (19%). In administering all four concentrations of CFSAO, the resulting OD value decreased significantly with increasing dose when compared with the OD of the negative control. This indicates the presence of CFSAO inhibition on the attachment of the KP1705 biofilm.

Biofilm Inhibition Assay

The inhibitory effect of CFSAO on biofilms was measured after being incubated with KP1705 for 24 hours with different concentrations of CFSAO. The CFO concentration was given relative to the minimum inhibitory concentration (MIC) KP1705

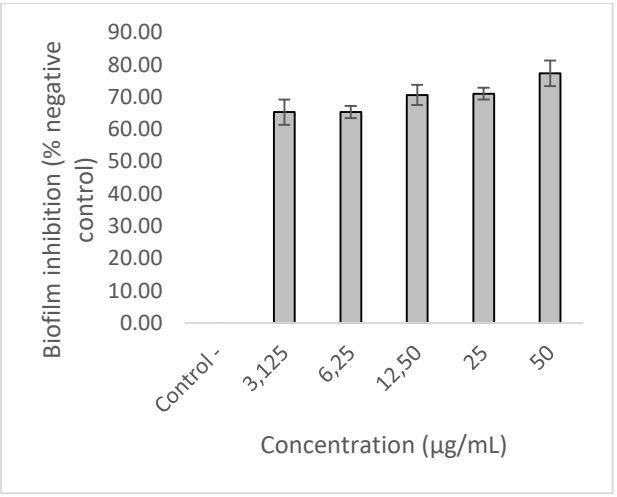

Figure 5. Inhibition of CFSAO against KP1705 biofilm.

Inhibitory effect of CFSAO on biofilm production when co-incubated for 24 h with different concentrations of CFSAO. The concentration of CFSAO was given relative to the minimum inhibitory concentration (MIC) of KP1705. Inhibition of CFSAO biofilm concentrations of 3.75%, 6.25%, 12.5%, 25%, and 50% were 65.32%, 65.37%, 70.65%, 71.05%, and 77, 5% consecutively. The highest biofilm resistance was at 50% CFSAO concentration. At the same time, the negative control of KP1705 without exposure to CFSAO was 0%. Bars indicate the standard error.

The ability of CFSAO to inhibit the production of KP1705 biofilm can be seen in Figure 4. In the crystal violet staining test, KP1705 biofilm was significantly inhibited in all concentrations ($p < 0.05$). The inhibition of biofilm formation was found at concentrations below the MIC. The ability of CFSAO to inhibit biofilm formation on KP1705 was relatively high. The biggest obstacle is the concentration of 50% (77.5%). The negative control (KP1705 bacteria without CFSAO exposure) showed the lowest biofilm inhibition value (0), which indicated that biofilm production was not inhibited at all. In the administration of the four concentrations of CFSAO, the resulting OD value decreased significantly along with increasing the dose compared to the OD of the negative control. This indicates the presence of CFSAO inhibition of the KP1705 biofilm.

Biofilm Degradation assay

Biofilm Degradation assay is carried out to find out whether CFS contains compounds that can destroy biofilms. This test was carried out after the biofilm formed very strongly, after 72 hours. The test results can be seen in Figure 6.

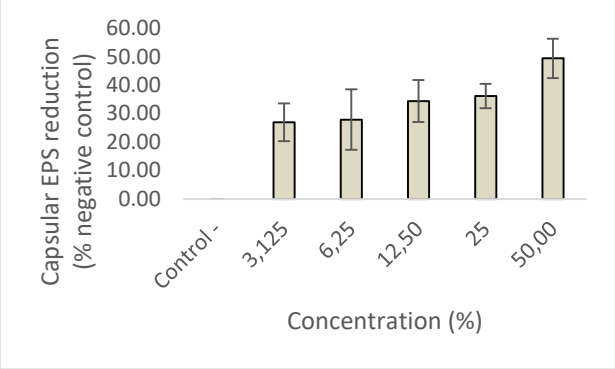

Figure 6. Biofilm degradation test of KP1705.

The destructive effect of CFSAO on the kp1705 biofilm after incubation for 72 hours with different concentrations of CFSAO. The concentration of CFSAO was given relative to the minimum inhibitory concentration (MIC) of KP1705. CFSAO biofilm destruction concentrations of 3.75%, 6.25%, 12.5%, 25%, and 50% were 26.95%, 27.91%, 34.4%, 36.22%, and 49, respectively. 45% consecutively. The highest biofilm destruction power was at a concentration of 50% CFSAO, which was 49.45%. In contrast, the negative control of KP1705 without exposure to CFSAO was 0%. Bars indicate the standard error, and the sign (*) above the bars indicates a significant difference ($p < 0.05$).

The ability of CFSAO to destroy the KP1705 biofilm can be seen in Figure 5. In the crystal violet staining test, the KP1705 biofilm was significantly destroyed at all concentrations ($p < 0.05$). The KP1705 biofilm destruction power was obtained at concentrations below the MIC. The ability of CFSAO to destroy the biofilm formed on KP1705 is relatively high. The biggest obstacle was the concentration of 50% (49.45%). The negative control (KP1705 bacteria without CFSAO exposure) showed the lowest biofilm inhibition value (0), indicating that biofilm production was not destroyed. In the administration of the four concentrations of CFSAO, the resulting OD value decreased significantly along with increasing the dose compared to the OD of the negative control. This indicates the destruction of the KP1705 biofilm by CFSAO.

Discussion

Aspergillus oryzae is known to have the ability to biosynthesize a large number of secondary (Beharka & Nagaraja, 1998; Leonard et al., 2013; Park et al., 2008). Aspergillus oryzae is found abundantly in soil or decaying wood. A. oryzae is commonly used to ferment foodstuffs (Bampidis et al., 2022). However, until now, research on the potential of A. oryzae biosurfactant against biofilm-producing bacteria has not been widely studied. Some studies show that Aspergillus niger and fumigatus are potent biosurfactant producers (Asgher et al., 2020). Because many genes in the Aspergillus family are conserved, it is likely that A. oryzae also has the same ability (Khosravi et al., 2015). In this study, we demonstrated that CFSAO could inhibit the adhesion of K. pneumoniae to the abiotic layer. However, the rate of inhibition of biofilm attachment is not too high, around 29%. This is possible because the material we use is cell-free supernatant. The active ingredients in CFS can be minimal. It is necessary to concentrate using ammonium sulfate or precipitation using acetone (Wingfield, 2016). This may underlie the low inhibition of CFSAO attachment to KP1705. Interestingly, A. oryzae was able to inhibit the biofilm KP1705 vary significantly (77.5%). The ability to inhibit biofilm production is generally due to the activity of the quorum quenching enzyme (Rasouli et al., 2020). Until now, only A. niger is known to produce cellobiose dehydrogenase (Rasouli et al., 2020). Cellobiose dehydrogenase can inhibit the biofilm of Gram-negative bacteria. We also showed that CFSAO was able to leave the KP1705 biofilm as much as 49.45%. This ability indicates that A. oryzae may produce enzymes capable of degrading polysaccharides which are the building blocks of biofilms (Chen et al., 2011). The ability of this biosurfactant derived from A. oryzae is accompanied by antibacterial ability, which is proven by the MBC test. From all the data above, it can be concluded that Aspergillus oryzae is a good candidate for biosurfactant to be further developed in the future. These results may corroborate the development of new natural strategies in the fight against medical device-associated infections. It is necessary to characterize the biosurfactant A. oryzae using chromatography principles chemically.

Conclusion

In this study, we demonstrated that CFSAO can inhibit K adhesion. The ability to inhibit biofilm production is generally due to the activity of quorum quenching enzymes. Cellobiose dehydrogenase can inhibit the biofilm of Gram-negative bacteria. We also showed that CFSAO was able to leave the KP1705 biofilm as much as 49.45%.

This ability indicates that A. oryzae can produce

enzymes capable of degrading polysaccharides which are the constituents of biofilms. The biosurfactant ability of this A. oryzae derivative is accompanied by antibacterial ability as evidenced by the MBC test. From all the data above, it can be concluded that Aspergillus oryzae is a good candidate for biosurfactant to be further developed in the future.

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