**ASIAN J TROP BIOTECHNOL**
Volume 21, Number 2, December 2024
Pages: 89-95

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c210204

# Effectiveness of antibiofilm *Aspergillus niger* cell-free supernatant against *Pseudomonas aeruginosa* biofilm

**TATASA DIVA CAHYAKIRANA, ARI SUSILOWATI♥, ARTINI PANGASTUTI**
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-819-46227085, ♥email: arisusilowati@staff.uns.ac.id

Manuscript received: 19 May 2024. Revision accepted: 5 December 2024.

**Abstract.** *Cahyakirana TD, Susilowati A, Pangastuti A. 2024. Effectiveness of antibiofilm* Aspergillus niger *cell-free supernatant against* Pseudomonas aeruginosa *biofilm. Asian J Trop Biotechnol 21: 89-95. Pseudomonas aeruginosa* is an opportunistic gram-negative pathogenic-bacteria that can cause nosocomial infections. *Pseudomonas aeruginosa* has several virulence factors that can cause infection, including its ability to form biofilms. Biofilm-forming bacteria can limit the penetration of antibiotics toward the microbes. These limitations make it challenging to treat biofilm-producing bacterial infections. Enzymes produced by *Aspergillus niger* with anti-biofilm activity include lactonase, β-glucosidase, acylase, and oxidoreductase. This study aimed to test the effectiveness of cell-free supernatant (CFS) of *A. niger* against *P. aeruginosa* biofilms. The antibiofilm activity test consisted of a biofilm growth inhibition test and *P. aeruginosa* biofilm destruction test. The research method used was the microtiter plate biofilm assay. The results showed that CFS of *A. niger* had the ability to inhibit the growth and destruction of *P. aeruginosa* biofilms. In the biofilm growth inhibition test and biofilm destruction test, the minimum biofilm inhibitory concentration ($MBIC_{50}$) and minimum biofilm eradication concentration (MBEC50) were 56 and 55.7% at 50% concentration, respectively. It was concluded that *A. niger* CFS had antibiofilm activity against *P. aeruginosa* biofilms.

**Keywords:** Antibiofilm, *Aspergillus niger*, cell-free supernatant, microtiter plate biofilm, *Pseudomonas aeruginosa*

## INTRODUCTION

*Pseudomonas aeruginosa* is a Gram-negative bacterium ubiquitous in diverse environments, including air, water, soil, and plant and animal tissues. It is a well-known opportunistic human pathogen. However, this seemingly harmless bacterium can become a formidable foe, causing mild infections in healthy individuals and escalating to severe, life-threatening conditions in those with weakened immune systems (Li et al. 2023; Pachori et al. 2019). *Pseudomonas aeruginosa* frequently targets individuals with weakened immune systems. The infection typically begins when the body's natural protective barriers, such as the skin or mucosal surfaces, are disrupted by factors like trauma, severe burns, surgical procedures, or the presence of medical devices. The progression of infection occurs in three distinct stages: bacterial attachment and colonization at the affected site, localized tissue invasion, and systemic dissemination, which may result in severe or widespread disease (Strateva and Mitov 2011).

*Pseudomonas aeruginosa* is often found in clinical settings and is associated with healthcare-associated infections (HAIs) or nosocomial infections (dos Santos Araújo et al. 2022). Nosocomial infections also referred to as infections acquired during the process of receiving health care that are not present during the time of admission (Isigi et al. 2023). Nosocomial infections cause 1.5 million deaths every day worldwide. In developing countries, around 40% of hospital patients are estimated to suffer from nosocomial infections. Furthermore, as many as 8.7% of hospital patients suffer from nosocomial infections while undergoing treatment at the hospital (Sazkiah and Ismah 2022). The nosocomial infection rate in Indonesia reached 15.74%, much higher than in developed countries, ranging from 4.8 to 15.5% (Mabrurah and Hermawati 2023).

*Pseudomonas aeruginosa* is armed with a range of virulence factors that enable it to cause infection by destroying tissue and spreading within the host. One of the most formidable factors is its ability to form biofilms (Turkina and Vikström 2019). Biofilms, aggregates of microorganisms covered by an extracellular polymer matrix, provide a protective shield for bacteria, rendering them resistant to desiccation, antimicrobials, and other cleaning agents. Moreover, biofilm-forming bacteria can evade the body's immune responses, including phagocytosis and different innate and adaptive immune system mechanisms (Roy et al. 2018). Biofilm-producing bacteria are predicted to have 10,000 times higher antibiotic resistance than bacteria that do not form biofilms. This is because biofilms can limit the penetration of antibiotics to microbes (Sharma et al. 2019). The presence of a barrier to antibiotic penetration makes it challenging to treat biofilm-producing bacterial infections. Therefore, further development of anti-biofilm agents is needed to overcome this problem.

A lot of research has been done out recently focused on finding solutions to inhibit the growth of biofilms in order to reduce the infection rates caused by biofilm-forming bacteria. Among the myriad of microorganisms studied for

their potential antibiofilm properties, *Aspergillus niger* has emerged as a particularly intriguing candidate. *Aspergillus niger* is a filamentous fungus widely known for its ability to produce an array of organic acids, extracellular enzymes, secondary metabolites, and bioactive compounds (Cairns et al. 2021). Cell-free supernatant (CFS) is a promising agent for combating biofilms. Another antibiofilm mechanism of CFS involves reducing biofilm production by lowering cell density, reducing aggregation, and degrading the extracellular polysaccharide matrix (EPS). Several compounds have been investigated for their antibiofilm activity, such as the use of α-amylase and β-glucosidase by *A. niger* (Aliyah et al. 2017). Additionally, enzymes including cellulases, xylanase, mannanase, pectinase, amylases, acylase, oxidoreductase, and alginate lyase have been studied (Kaur et al. 2020), with isolates originating from soil and designated as *A. niger* APS also showing biofilm destruction capabilities. Other research (Nisa' et al. 2013) indicates that cellulase and α-amylase enzymes can be produced by *A. niger* FNCC 6018. The aim of this research was to test the effectiveness of cell-free supernatant (CFS) of *A. niger* against *P. aeruginosa* biofilms.

## MATERIALS AND METHODS

### Materials

The materials used in this research were *Aspergillus niger* FNCC 6018, (obtained from the Laboratory of the Center for Food and Nutrition Studies, Food and Nutrition Culture Collection (FNCC), Gadjah Mada University, Yogyakarta, Indonesia), *Pseudomonas aeruginosa* bacteria forming biofilm ATCC 27853 (purchased from the Laboratory of the Agitama Sinergi Inovasi (AGAVI), agar, distilled water, alcohol, aluminum foil, 30% acetic acid, blue disposal tip, ice cubes/ice pack, falcon, glucose, cotton, gauze, filter paper, crystal violet, Lugol, potato dextrose broth (PDB) media, trypticase soy broth (TSB) media, plastic wrap.

### Research design

This research was conducted in a true experimental design The microtiter plate biofilm assay method was used to evaluate antibiofilm activity by measuring the optical density (OD) value with a microplate reader. The most important study population was *P. aeruginosa*, a biofilm-forming organism of significant scientific interest. The number of repetitions was calculated based on Federer's formula, as below, to ensure a robust and reliable study:

$$(t-1)\,(n-1) \geq 15$$

Where:
t: treatment group
n: number of repetitions for each treatment group

There were 5 treatment groups, namely 1 negative control group and 4 test groups. The control group used in this study was media with *P. aeruginosa* suspension without adding cell-free supernatant (CFS). The test group used was CFS *A. niger* of 100 µL with concentrations of 100, 50, 25, and 12.5%. The research was conducted at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Sebelas Maret University, Surakarta, Indonesia.

### Procedures

*Preparation of cell-free supernatant* A. niger

The culture of *A. niger* which was rejuvenated on Potato Dextrose Broth (PDB), was taken by scratching a needle across the surface of media 8 mm long. Then, the culture was inoculated in 100 mL of sterile PDB, and 1% glucose was added. For one week, the culture was placed in a shaker incubator at a temperature of 28°C at a speed of 120 × g. After one week, 5 mL of culture was taken and inoculated into 45 mL sterile PDB. Then, the culture was placed in an incubator shaker for 4 days to produce cell-free supernatant (CFS).

After incubation for 4 days, the fungal culture was filtered using sterile filter paper number 42 and supernatant was collected in a 15 mL tube. The tube was then stored at 0°C to maintain the fungal enzyme activity. The filtrate tube was placed in the freezer for approximately 15 minutes. Then, the filtrate was centrifuged for 15 minutes at a speed of 10,000 × g. The supernatant formed was filtered again using sterile 0.22 µm membrane filter and collected in a sterile beaker surrounded by ice cubes at 0°C.

Serial dilutions of CFS *A. niger* were prepared using a multilevel dilution technique with a ratio of 1:2. A 15 mL TSB was made and mixed with 1% glucose. 3 mL of media was added to the test tube of each concentration, namely 100, 50, 25, and 12.5% (Prateeksha et al. 2020).

*Preparation of growth media and bacterial suspension for* Pseudomonas aeruginosa

The growth media was prepared using Trypticase Soy Broth (TSB) supplemented with 5% glucose. A total of 10 mL of TSB was sterilized by autoclaving for 15 minutes at 121°C. Additionally, 0.5 mL of TSB with 1% glucose was also sterilized, as the inclusion of 1% glucose strive to enhance the formation of *P. aeruginosa* biofilm. One cycle of *P. aeruginosa* culture was inoculated into the TSB containing 1% glucose. The culture was then homogenized and incubated for 24 hours at 37°C.

*Biofilm formation test of* P. aeruginosa *isolates*

The detection test for *P. aeruginosa* biofilm formation was carried out using the microtiter-plate biofilm assay method. The control group was a mixture of TSB media and 1% glucose. Next, 200 µL of *P. aeruginosa* bacterial suspension was transferred into microplate test well. The microplate was closed and incubated at 37°C for 48 hours without stirring. After incubation, the contents of microplate were removed and washed with sterile phosphate buffer saline (PBS) to remove planktonic cells that were not attached to the microplate and dried them. Next, 200 µL of 0.1% crystal violet dye was added to each well and then incubated at room temperature for 20 minutes. After incubation, the contents of the microplate were discarded, and then the microplate was washed using sterile PBS three times to remove cells that were stained

but not attached to the microplate. Then, the microplates were allowed to dry at room temperature. After drying, 200 µL of 96% ethanol was put into each well and incubated for 20 minutes at room temperature. The microplates were measured using a microplate reader with a wavelength of 595 nm to obtain the optical density (OD) value of the test and control plates.

To determine the strength of test bacterial biofilm, the ODisolate value was compared with the Optical Density cut-off (ODcut) value. The OD value was obtained from the average ODisolate value minus the ODcut value (OD = average ODisolate value – OD cut value). ODcut was three times the standard deviation above the mean OD of the control (culture medium) (Ruchi et al. 2015). The formula for calculating ODcut was as follows:

ODcut = ODC + (3 × SD ODC)

OD value obtained on average ODisolate - ODcut

(OD = average value ODisolate - ODcut)

Where:

ODcut: Optical density cut-off

ODC: Optical density control

SD ODC: Standard deviation optical density control

The criteria of the strength of bacterial biofilm test is presented in Table 1.

Pseudomonas aeruginosa *biofilm growth inhibition test*

*Pseudomonas aeruginosa* biofilm growth inhibition test was carried out using CFS *A. niger* with percentages of 100, 50, 25, and 12.5%. As a test group, 100 µL of CFS with varying percentages and 100 µL of *P. aeruginosa* bacterial suspension were placed in the microplate wells. 100 µL of a mixture of TSB media with 1% glucose and 100 µL of *P. aeruginosa* bacterial suspension were placed in the microplate wells as negative control. Then, the microplates were incubated for 72 hours at 37°C in an incubator (Tobi et al. 2022).

After incubation for 72 hours, the solution of microplate was removed and washed thrice a times with sterile PBS, then dried. After drying, 200 µL of 0.1% crystal violet dye was added in each well and incubated for 20 minutes at room temperature. After 20 minutes, the dye was discarded and washed with sterile PBS, and then microplates were allowed to dry at room temperature. 200 µL of 96% ethanol was added to the wells and incubated for 20 minutes at room temperature. Then, the microplates were observed using a microplate reader with a wavelength of 595 nm.

Inhibition of bacterial biofilm growth was calculated using the following formula (Nikolic et al. 2014):

Inhibition of biofilm growth (%) = (ODkn-ODuji)/ODkn × 100%

Where:

ODkn: Optical Density negative control (K-)

ODuji: Optical Density test group

Pseudomonas aeruginosa *biofilm destruction test*

This test was carried out according to the method of Tobi et al. (2022) with slight modifications. *Pseudomonas aeruginosa* biofilm destruction test used CFS of *A. niger* with 100, 50, 25, and 12.5% concentrations. As much as 200 µL of *P. aeruginosa* bacterial suspension was added to the test well, and the negative control was placed on the microplate. The microplate was closed and incubated at 37°C for 72 hours in an incubator. After incubation for 72 hours, the contents of the microplate were removed, washed with sterile PBS thrice a times, and dried. Next, 200 µL of *A. niger* CFS with various percentages was added to the test well, and 200 µL of a mixture of TSB media and 1% glucose in the negative control well. The microplate was closed again and incubated at 37°C for 60 minutes. After incubation, the contents of the microplate were removed, washed using sterile PBS three times, and dried. After the microplate was dry, 200 µL of 0.1% crystal violet dye was added into all wells and then incubated at room temperature for 20 minutes. Next, the contents of the microplate were washed with sterile PBS. Then, the microplate was allowed to dry at room temperature. Add 200 µL of 96% ethanol into all wells and incubate at room temperature for 20 minutes. Then, the 96-well microplate was observed using a microplate reader at a wavelength of 595 nm. Biofilm destruction was calculated using the following formula:

% Biofilm destruction = (ODkn-ODuji)/ODkn × 100%

Where:

ODkn: Optical Density negative control (K-)

ODuji: Optical Density test group

**Data analysis**

Quantitative data in the form of OD values for each treatment was used to determine the effectiveness of antibiofilm against *P. aeruginosa* biofilms after being treated with the addition of cell-free Supernatant *A. niger*. Quantitative data, including test OD, control OD, cut OD, and SD, were analyzed using Statistical Product and Service Solutions (SPSS). A One-way Analysis of Variance (ANOVA) test with a confidence level of 95% was used to determine the significance of the effect of CFS on *P. aeruginosa* biofilms, followed by a post-hoc test. The Post-Hoc Test was a further use to determine significant differences between each data group. The final analysis used the Pearson correlation test to determine the relationship or correlation between CFS concentration and the OD test value.

**Table 1.** Interpretation of the strength of biofilm formation (Nikolic et al. 2014)

| Average OD value | Biofilm formation strength |
|---|---|
| ODisolate≤ODcut | Not a biofilm former |
| ODcut<ODisolate≤2xODcut | Weak biofilm forming |
| 2xODcut<ODisolate≤4xODcut | Moderate biofilm former |
| 4xODcut<ODisolate | Strong biofilm former |

Note: ODcut: Optical Density cut, ODisolate: Optical Density isolate

## RESULTS AND DISCUSSION

### Cell-free supernatant of *A. niger*

Cell-free supernatant (CFS) of *A. niger* used as antibiofilm test material was obtained from *A. niger* inoculation screening. Filtering was carried out twice and carried out at cold temperatures. The freezing temperature filtration method was selected to eliminate all cells, thereby obtaining a free-cell supernatant while preserving the enzyme content of *A. niger*. The filtration size was selected to ensure only enzymes remained in the medium. As a result of filtering, clear yellow CFS was obtained. Mani-López et al. (2022) stated that CFS refers to a transparent liquid medium produced after the growth of certain microorganisms through a centrifugation and membrane filtration process with a pore size capable of holding all bacteria, generally 0.22 or 0.45 µm.

### Detection of biofilm growth

The OD measurement evaluated the strength of biofilm growth by *P. aeruginosa* bacteria (Table 2). Mira et al. (2022) explains that OD is a method used to measure microbial growth based on its optical density. The OD value indicates the number of microbes in the suspension, which is positively correlated with the turbidity of the medium. An increase in the OD value indicates an increase in microbial cells growing in the medium. In measuring biofilm growth, an increase in the OD value indicates an increase in the amount of biofilm formed on the surface.

Table 2 showed the biofilm growth of *P. aeruginosa* can be assessed by comparing the optical density (OD) of the isolate with the threshold value (ODcut). The results indicate that bacteria formed strong biofilms if the condition 4×ODcut<ODisolate was met, specifically 2.972<3.033. The result suggests that *P. aeruginosa* belongs to the strong biofilm producer category. This finding is supported by research conducted by Nikolic et al. (2014) and Rehman et al. (2018), who reported that *P. aeruginosa* ATCC 27853 is indeed a strong biofilm producer. Strong biofilm-producing bacteria can develop a biofilm matrix more rapidly and thickly compared to those that produce weak biofilms. Consequently, bacteria that generate robust biofilms are often more resistant to antibiotics.

### Inhibition of *P. aeruginosa* biofilm growth

The biofilm growth inhibition test was conducted to detect the ability of *A. niger* CFS concentration to inhibit the growth of *P. aeruginosa* bacterial biofilm. The results showed (Figure 1) that the highest optical density (OD) value of 1.8324 was observed in the negative control group. The OD values for all test group concentrations were lower than that of the negative control. The highest OD value in the test group was 1.2742, recorded at a concentration of 12.5% for the cell-free supernatant (CFS). This may be due to the minimal variation in CFS concentration, which had a limited effect on inhibiting biofilm formation, resulting in a cloudy liquid and a high OD value. Conversely, the lowest OD value of 0.7366 was observed for the test group at a concentration of 100% CFS. This lower value is likely because the highest concentration of CFS was more effective in inhibiting biofilm formation, leading to less turbidity in the liquid and, consequently, the lowest OD value among the test groups. The decrease in OD value relative to the negative control indicates the thickness of biofilm matrix was reduced when treated with *A. niger* CFS. Therefore, it is evident that CFS from *A. niger* possesses the ability to inhibit bacterial biofilm formation. The obtained OD values are then used to calculate the percentage of biofilm inhibition through the formula for inhibition of biofilm growth (%).

The percentage of biofilm growth inhibition activity is illustrated in Figure 2, while the microtiter plate biofilm assay is shown in Figure 3. The highest biofilm growth inhibition activity, at 60%, was observed in the cell-free supernatant (CFS) at a concentration of 100%. In contrast, the lowest inhibition, at 30%, was recorded in the CFS at a concentration of 12.5%. These results indicate that the administration of *A. niger* CFS at a concentration of 12.5% was able to inhibit *P. aeruginosa* bacteria, though this effect falls into the low category. According to Famuyide et al. (2019), anti-biofilm activity is classified as good and effective when the percentage of inhibition is 50% or greater. Conversely, an inhibition rate of 0-49% is considered low. The minimum biofilm inhibitory concentration (MBIC50) at 50% CFS concentration was found to be 56%. When CFS was administered at a concentration of 50%, the percentage inhibition met the MBIC50 requirement, indicating that this concentration can inhibit biofilm growth by at least 50%, thus qualifying as excellent and effective (Sahal et al. 2020).

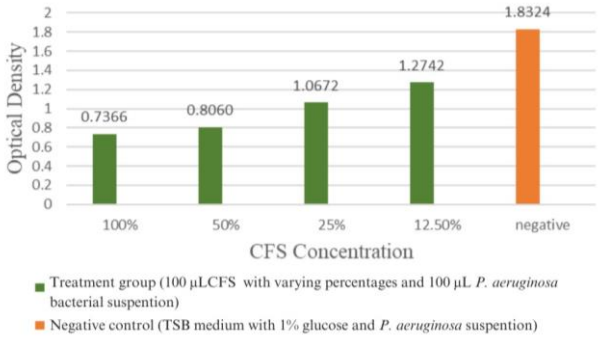


**Figure 1.** Inhibition of *P. aeruginosa* biofilm growth observed at various concentrations of cell-free supernatant treatment, measured by optical density (OD)

**Table 2**. Detection of *P. aeruginosa* biofilm growth by optical density (OD) parameter

| Treatment group | Repetitions | | | | | Average standard deviation | OD cut |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| Test group | 3.834 | 3.241 | 3.066 | 2.522 | 2.502 | 3.033±0.552 | - |
| Control group | 0.480 | 0.525 | 0.995 | 0.705 | 0.338 | 0.609±0.045 | 0.743 |

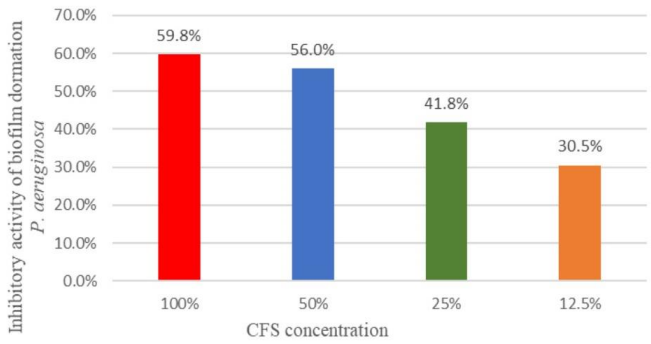


**Figure 2.** Percentage inhibitory activity of *P. aeruginosa* biofilm growth at different cell-free supernatant (CFS) treatment concentrations

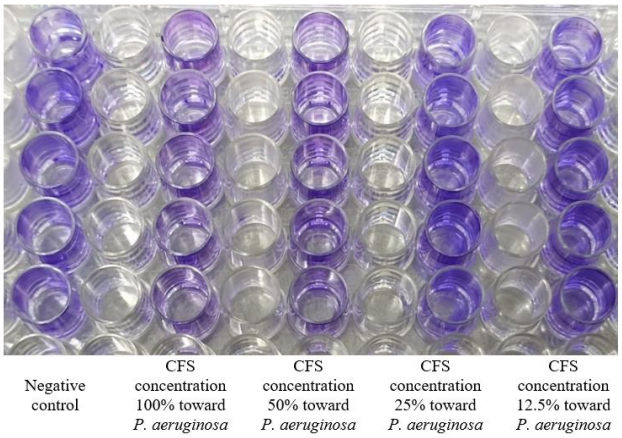


**Figure 3.** Biofilm inhibition after 72 hours' incubation in microtiter plate biofilm assay stained with 0.1% crystal violet

**Table 3.** Post-Hoc Tukey inhibition of biofilm growth

| Concentrations | Probability Value | | | | |
|---|---|---|---|---|---|
| | K- | 100% | 50% | 25% | 12.5% |
| K- | | 0.000* | 0.000* | 0.002* | 0.033* |
| 100% | 0.000* | | 0.996 | 0.367 | 0.045* |
| 50% | 0.000* | 0.996 | | 0.579 | 0.094 |
| 25% | 0.002* | 0.367 | 0.579 | | 0.761 |
| 12.5% | 0.033* | 0.045* | 0.094 | 0.761 | |

Note: K-: Negative control, * indicates significant difference of treatment group

In One-way ANOVA test, the p-value of 0.000 was obtained ($p<0.05$), indicating a significant difference. Table 3 showed significant differences between the negative control group and all treatments with varying concentrations of CFS (cell-free supernatant). To investigate the relationship between the administration of *A. niger* CFS concentrations and the inhibition of biofilm formation in *P. aeruginosa*, Pearson correlation analysis was conducted. The Pearson correlation test yielded a value of 0.000 ($p<0.05$), demonstrating a strong correlation with a coefficient of 0.807. This positive correlation suggests a strong relationship between the concentration of CFS used and the level of biofilm growth inhibition in the tested bacteria, encouraging further research into biofilm inhibition strategies. In the biofilm formation process, bacteria irreversibly adhere to surfaces, forming micro-colonies and producing an extracellular polysaccharide (EPS) matrix. The components in *A. niger*, particularly the α-amylase enzyme, can degrade the polysaccharides that constitute the biofilm structures in bacterial cells. Additionally, the β-glucosidase enzyme can break down glucose bonds, inhibiting bacterial movement, reducing EPS production, and disrupting cellular communication (quorum sensing) (Della Sala et al. 2019). Consequently, β-glucosidase can prevent bacterial attachment and inhibit growth (Gopinath et al. 2024).

**Destruction of *P. aeruginosa* biofilms**

The results indicate that the highest optical density (OD) value recorded was 2.8212 in the negative control group. All test group concentrations showed lower OD values compared to the negative control. Among the test groups, the highest OD value of 1.909 was observed at a 12.5% CFS concentration (Figure 4). This can be attributed to the lower concentration of CFS, which has a minimal effect on inhibiting biofilm destruction, resulting in cloudier liquid and a higher OD reading. In contrast, the lowest OD value of 1.0486 was noted at a concentration of 100%. Here, the higher concentration of CFS significantly impacts biofilm destruction, leading to less turbidity in the liquid and, consequently, the lowest OD value among all test groups. The decrease in OD values relative to the negative control indicates that the thickness of the biofilm was reduced following treatment with CFS. This suggests that *A. niger* CFS can effectively destroy *P. aeruginosa* bacterial biofilm. Thus, higher CFS concentrations correlate with lower OD values, indicating more effective biofilm destruction.

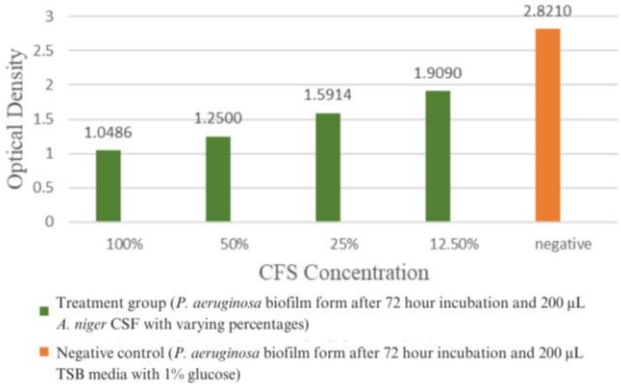


**Figure 4.** Destruction of *P. aeruginosa* biofilm as measured by the optical density (OD) parameter

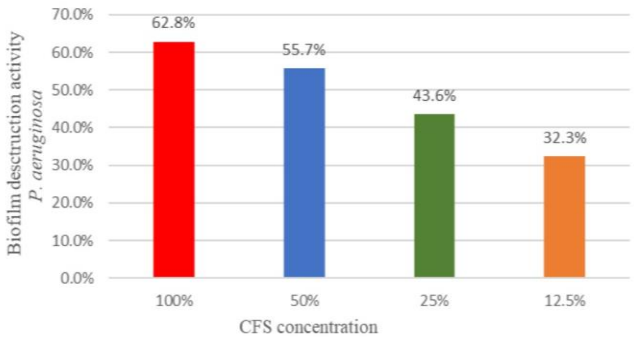


**Figure 5.** Destruction percentage of *P. aeruginosa* biofilm due to varying concentrations of cell-free supernatant (CFS) treatment

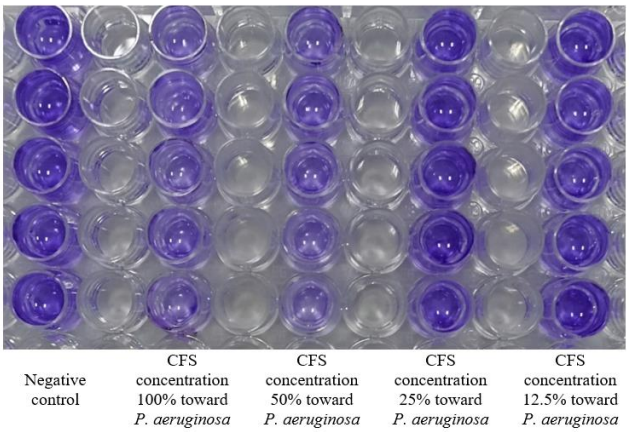


**Figure 6.** Biofilm destruction after administration of CFS and inhibition after 72 hours of incubation in a microtiter plate biofilm assay stained with 0.1% crystal violet

**Table 4.** Post-Hoc Tukey of biofilm destruction

| Concentrations | Probability Value | | | | |
|---|---|---|---|---|---|
| | **K-** | **100%** | **50%** | **25%** | **12.5%** |
| K- | | 0.000* | 0.000* | 0.000* | 0.000* |
| 100% | 0.000* | | 0.665 | 0.003* | 0.014* |
| 50% | 0.000* | 0.665 | | 0.058 | 0.230 |
| 25% | 0.000* | 0.003* | 0.058 | | 0.960 |
| 12.5% | 0.000* | 0.014* | 0.203 | 0.960 | |

Note: K-: Negative control, * indicates significant difference of treatment group

The percentage of biofilm destruction activity is illustrated in Figure 5, while the microtiter plate biofilm assay results are shown in Figure 6. The highest biofilm destruction activity, at 63%, was observed in the cell-free supernatant (CFS) at a concentration of 100%. In contrast, the lowest activity was found in CFS at concentrations of 12.5 and 32%.

The Post Hoc Tukey analysis reveals significant differences between the negative control and all treatment groups (Table 4). A Pearson correlation analysis demonstrates a relationship between the administration of CFS (Cell-Free Supernatant) concentrations of *A. niger* and the degradation of *P. aeruginosa* biofilms. The analysis yielded a p-value of 0.000 ($p<0.05$), indicating a strong correlation with a correlation coefficient of 0.846. This suggests a positive relationship, indicating that higher CFS concentrations correlate with increased destruction of the biofilm growth of the test bacteria. The effectiveness of a crude fungal supernatant (CFS) from *A. niger* in destroying biofilms relies on the ability of its active components to penetrate and break down the extracellular polymeric substance (EPS) matrix.

Research indicates that the enzyme α-amylase found in *A. niger* can degrade EPS by disrupting its main structural components, which include proteins and carbohydrates. Supporting this, a study by Lahiri et al. (2021) conducted in silico revealed that the interaction between α-amylase and *Pseudomonas polysaccharides* is spontaneous. This is demonstrated by a significant correlation between the energy released during the action of α-amylase and the biofilm EPS, highlighting the role of α-amylase in lysing the EPS layer that surrounds the bacteria, thus weakening the biofilm. Alginate lyase is another hydrolytic enzyme that catalyzes the cleavage of β-elimination bonds in the alginate polymer chain, resulting in the production of short-chain oligosaccharides. This enzymatic process disrupts the three-dimensional structure of the biofilm, allowing alginate lyase to compromise the biofilm's ability to protect bacterial colonies from external disturbances (Daboor et al. 2021; Zhang et al. 2021).

In conclusion, based on the results, it can be concluded that CFS of *A. niger* had the ability to inhibit the growth and destruction of *P. aeruginosa* biofilms. It was also observed that the minimum biofilm inhibitory concentration ($MBIC_{50}$) and minimum biofilm eradication concentration ($MBEC_{50}$) of *A. niger* against *P. aeruginosa* were obtained at a concentration of 50% with a percentage of 56 and 55.7%, respectively. Further research is needed regarding the specific active compounds contained in *A. niger* CFS and the mechanism of action of these compounds as anti-biofilm agents.

## ACKNOWLEDGEMENTS

This research was funded by Universitas Sebelas Maret (UNS), Surakarta, Indonesia non-APBN funds in 2024 through the Research Group Grant, under contract number 194.2/UN27.22/PT.01.03/2024.

## REFERENCES


Aliyah A, Alamsyah G, Ramadhani R, Hermansyah H. 2017. Production of α-Amylase and β-Glucosidase from *Aspergillus niger* by solid state fermentation method on biomass waste substrates from rice husk, bagasse and corn cob. Energy Procedia 136: 418-423. DOI: 10.1016/j.egypro.2017.10.269.

Cairns TC, Barthel L, Meyer V. 2021. Something old, something new: challenges and developments in *Aspergillus niger* biotechnology. Essays Biochem 65 (2): 213-224. DOI: 10.1042/EBC20200139.

Daboor SM, Rohde JR, Cheng Z. 2021. Disruption of the extracellular polymeric network of *Pseudomonas aeruginosa* biofilms by alginate lyase enhances pathogen eradication by antibiotics. J Cyst Fibros 20 (2): 264-270. DOI: 10.1016/j.jcf.2020.04.006.

Della Sala G, Teta R, Esposito G, Costantino V. 2019. The chemical language of gram-negative bacteria. In: Tommonaro G (eds.). Quorum Sensing: Molecular Mechanism and Biotechnological Application. Academic Press, London. DOI: 10.1016/B978-0-12-814905-8.00001-0.

dos Santos Araújo, MA, Rodrigues JS, de Lisieux Guedes Ferreira Lobo, T de Albuquerque Maranhão FC. 2022. Healthcare-associated infections by *Pseudomonas aeruginosa* and antimicrobial resistance in a public hospital from Alagoas (Brazil). J Bras Patol Med Lab 58 (4): 14-19. DOI: 10.1900/JBPML.2022.58.447.

Famuyide IM, Aro AO, Fasina FO, Eloff JN, McGaw LJ. 2019. Antibacterial and antibiofilm activity of acetone leaf extracts of nine under-investigated south African *Eugenia* and *Syzygium* (Myrtaceae) species and their selectivity indices. BMC Complement Altern Med 19: 141. DOI: 10.1186/s12906-019-2547-z.

Gopinath V, Mitra K, Chadha A, Doble M. 2024. Disrupting *Mycobacterium smegmatis* biofilm using enzyme-immobilized rifampicin loaded silk fibroin nanoparticles for dual anti-bacterial and anti-biofilm action. Microb Pathog 196: 106999. DOI: 10.1016/j.micpath.2024.106999.

Isigi S, Parsa A Alasqah, I Mahmud, I Kabir R. 2023. Predisposing factors of nosocomial infections in hospitalized patients in the United Kingdom: Systematic Review. JMIR Public Health Surveill 9: e43743. DOI: 10.2196/43743.

Kaur A, Rishi V, Soni SK, Rishi P. 2020. A novel multi-enzyme preparation produced from *Aspergillus niger* using biodegradable waste: A possible option to combat heterogeneous biofilms. AMB Expr 10: 36. DOI: 10.1186/s13568-020-00970-3.

Lahiri D, Nag M, Banerjee R, Mukherjee D, Garai S, Sarkar T, Dey A, Sheikh HI, Pathak SK, Edinur HA, Pati S, Ray RR. 2021. Amylases: Biofilm inducer or biofilm inhibitor? Front Cell Infect Microbiol 11: 660048. DOI: 10.3389/fcimb.2021.660048.

Li X, Gu N, Huang TY, Zhong F, Peng G. 2023. *Pseudomonas aeruginosa*: A typical biofilm forming pathogen and an emerging but underestimated pathogen in food processing. Front Microbiol 13: 1114199. DOI: 10.3389/fmicb.2022.1114199.

Mabrurah F, Hermawati. 2023. The description of nurses knowledge level about healthcare associated infections (HAIs) at Dr. Soehadi regional hospital Prijonegoro Sragen. J Kegawatdaruratan Medis Indonesia 2 (2): 190-202. DOI: 10.58545/jkmi.v2i2.190.

Mani-López E, Arrioja-Bretón D, López-Malo A. 2022. The impacts of antimicrobial and antifungal activity of cell-free supernatants from lactic acid bacteria in vitro and foods. Compr Rev Food Sci Food Saf 21 (1): 604-641. DOI: 10.1111/1541-4337.12872.

Mira P, Yeh P, Hall BG. 2022. Estimating microbial population data from optical density. PLoS One 17 (10): e0276040. DOI: 10.1371/journal.pone.0276040.

Nikolic M, Vasic S, Djurdjevic J, Stefanovic O, Comic L. 2014. Antibacterial and anti-biofilm activity of ginger (*Zingiber officinale (Roscoe)*) ethanolic extract. Kragujevac J Sci 36: 129-136. DOI: 10.5937/KgJSci1436129N.

Nisa' K, Wuryanti, Taslimah. 2013. Isolasi, Karakterisasi dan Amobilisasi α-Amilase dari *Aspergillus niger* FNCC 6018. Chem Inf 1: 141-149.

Pachori P, Gothalwal R, Gandhi P. 2019. Emergence of antibiotic resistance *Pseudomonas aeruginosa* in intensive care unit. A critical review. Genes Dis 6 (2): 109-119. DOI: 10.1016/j.gendis.2019.04.001.

Prateeksha, Bajpai R, Yusuf MA, Upreti DK, Gupta VK, Singh BN. 2020. Endolichenic fungus, *Aspergillus quandricinctus* of *Usnea longissima* inhibits quorum sensing and biofilm formation of *Pseudomonas aeruginosa* PAO1. Microb Pathog 140: 103933. DOI: 10.1016/j.micpath.2019.103933.

Rehman SA, Shailaja VV, Rao A. 2018. Comparison of phenotypic methods for the detection of biofilm production in indwelling medical devices used in NICU & PICU in a tertiary care hospital in Hyderabad, India. Intl J Curr Microbiol Appl Sci 7: 3265-3273. DOI: 10.20546/ijcmas.2018.709.405.

Roy R, Tiwari M, Donelli G, Tiwari V. 2018. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence 9 (1): 522-554. DOI: 10.1080/21505594.2017.1313372.

Ruchi T, Sujata B, Anuradha D. 2015. Comparison of phenotypic methods for the detection of biofilm production in uro-pathogens in a tertiary care hospital in India. Intl J Curr Microbiol Appl Sci 4 (9): 840-849.

Sahal G, Woerdenbag HJ, Hinrichs WLJ, Visser A, Tepper PG, Quax WJ, van der Mei H C, Bilkay IS. 2020. Antifungal and biofilm inhibitory effect of *Cymbopogon citratus* (lemongrass) essential oil on biofilm forming by *Candida tropicalis* isolates; an in vitro study. J Ethnopharmacol 246: 112188. DOI: 10.1016/j.jep.2019.112188.

Sazkiah ER, Ismah Z. 2022. The epidemiological determination of nosocomial infection in inpatients at Adam Malik general hospital, Medan. Placentum: Jurnal Ilmiah Kesehatan dan Aplikasinya 10 (2): 99-111. DOI: 10.20961/placentum.v10i2.54505.

Sharma D, Misba L, Khan AU. 2019. Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrob Resist Infect Control 8: 76. DOI: 10.1186/s13756-019-0533-3.

Strateva T, Mitov I. 2011. Contribution of an arsenal of virulence factors to pathogenesis of *Pseudomonas aeruginosa* infections. Ann Microbiol 61: 717-732. DOI: 10.1007/s13213-011-0273-y.

Tobi CHB, Saptarini O, Rahmawati I. 2022. Aktivitas antibiofilm ekstrak dan fraksi-fraksi biji pinang (*Areca catechu L.*) terhadap *Staphylococcus aureus* ATCC 25923. J Pharm Sci 1: 57. DOI: 10.20961/jpscr.v7i1.43698. [Indonesian]

Turkina M V, Vikström E. 2019. Bacteria-host crosstalk: Sensing of the quorum in the context of *Pseudomonas aeruginosa* infections. J Innate Immun 11: 263-279. DOI: 10.1159/000494069.

Zhang L, Li X, Zhang X, Li Y, Wang L. 2021. Bacterial alginate metabolism: An important pathway for bioconversion of brown algae. Biotechnol Biofuels 14: 158. DOI: 10.1186/s13068-021-02007-8.