e-ISSN 2716-697X

Antibiofilm Activity of *Pseudomonas Sp.* (Mb273n) Cell-Free Supernatant Against Pathogenic Bacteria

Izzah Mardhiyyah Nurrohmah[1], Anto Budiharjo[2*], Zarina Amin[3*]

[1]Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Jl. Prof. Soedarto, SH, Tembalang, 50275 Semarang, Central Java, Indonesia.
[2]Biotechnology Study Program, Faculty of Science and Mathematics, Diponegoro University, Jl. Prof. Soedarto, SH, Tembalang, 50275 Semarang, Central Java, Indonesia.
[3]Biotechnology Research Institute, University Malaysia Sabah, Jalan UMS, 88450, Kota Kinabalu, Sabah, Malaysia.
*Corresponding author's e-mail: zamin@ums.edu.my
Received: 15 October 2025 | Accepted: 20 December 2025 | Published: 31 December 2025

ABSTRACT

Bacterial biofilms pose a significant global health challenge due to their resistance to antibiotics and the host immune system, leading to persistent infections. Developing effective strategies to control biofilm formation is therefore crucial for preventing persistent infections. This study investigated the antibiofilm potential of the cell-free supernatant (CFS) from *Pseudomonas* sp. (MB273N) against biofilms of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Chromobacterium violaceum*. Additionally, the study examined biofilm structural changes following CFS treatment. The CFS was obtained by centrifuging bacterial cultures three times at 11,000 rpm for 15 minutes. A biofilm inhibition assay was conducted to assess the antibiofilm activity of CFS at a sub-lethal concentration, and biofilm morphology was examined using scanning electron microscopy (SEM). Statistical analysis was performed using SPSS version 26. The CFS significantly ($p<0.05$) inhibited biofilm formation, with inhibition rates of 14.15% for *E. coli*, 32.46% for *P. aeruginosa*, 30.83% for *S. aureus*, and 37.5% for *C. violaceum*. SEM analysis revealed that untreated biofilms formed dense, multilayered structures, while CFS-treated biofilms appeared less dense, more dispersed, and exhibited altered cell morphology. The CFS of *Pseudomonas* sp. (MB273N) demonstrates potential as an antibiofilm agent against *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. violaceum*, highlighting its possible application in biofilm control strategies.

e-ISSN 2716-697X

INTRODUCTION

Biofilms have become a major concern in public health because biofilm-associated microorganisms are less susceptible to antimicrobial treatments (Arampatzi *et al.*, 2011). Biofilms are responsible for various infections. It has been reported that around 50% of hospital-acquired infections in immunodeficient patients are linked to biofilms, and as much as 80% of chronic infections globally are also associated with biofilms (Asma *et al.*, 2022; Meroni *et al.*, 2021). Therefore, effective control of biofilm is necessary to overcome various health problems.

This study investigated four model organisms, namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Chromobacterium violaceum* to investigate biofilm inhibition. Urinary tract infections (UTIs) associated with biofilms are often caused by *E. coli*, and Uropathogenic *Escherichia coli* (UPEC) is the primary etiological agent of UTIs (Alshammari *et al.*, 2023; Zagaglia *et al.*, 2024). *P. aeruginosa* is known for its ability to form biofilms, which play a crucial role in establishing persistent lung infection (Davis and Brown, 2016). *S. aureus* biofilm infections are very common in orthopedic infections, and it has been demonstrated *in vitro* that *S. aureus* biofilms may cause bone loss during chronic osteomyelitis (Sanchez *et al.*, 2013; Yu *et al.*, 2020). Moreover, *C. violaceum* can become a pathogen and possesses the ability to form biofilm, which may increase its resistance to antibiotics and promote the initiation of infections in the host (Alisjahbana *et al.*, 2021; Dimitrova *et al.*, 2024). These four model organisms are recognized as the causative agents of many infections.

Cell-free supernatant (CFS), a microbiological culture medium containing metabolites that exclude bacterial cells (Liu *et al.*, 2023), may serve as an alternative to antimicrobial agents against biofilms. Prior studies have found that CFS from certain bacteria contain compounds with antibiofilm properties that inhibit biofilm development of bacterial pathogens (Al-Barhawee and Al-Rubyee, 2024; Ray *et al.*, 2023). Therefore, cell-free supernatant may become an effective solution for controlling biofilms.

A previous study identified *Pseudomonas* sp. (MB273N), a novel bacterial species isolated from the Maliau Basin Conservation Area (data not published). The antibiofilm activity of *Pseudomonas* sp. (MB273N) cell-free supernatant against biofilms formed by *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. violaceum* has not been evaluated. This study aimed to investigate whether there is a potency of *Pseudomonas* sp. (MB273N) cell-free supernatant as an antibiofilm agent against these bacteria, and to observe the characteristics of these four bacterial biofilms both before and after treatment with CFS.

MATERIALS AND METHODS

e-ISSN 2716-697X

Bacterial Cultivation

The bacterial strains used in this study (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Chromobacterium violaceum,* and *Pseudomonas* sp. (MB273N)) were obtained from the frozen stocks of the Biotechnology Research Institute, Universiti Malaysia Sabah. Glycerol stocks of these strains were stored at -80°C. Prior to use, the stocks were thawed at room temperature until liquid cultures were observed. A loopful of each bacterial strain was streaked onto nutrient agar (Oxoid) and incubated at 28°C overnight. Single colonies were then sub-cultured in nutrient broth (Merck KGaA) and incubated at 28°C for 14 hours in a shaking incubator (Innova) at 180 rpm.

Preparation of Cell-Free Supernatant (CFS)

The culture of *Pseudomonas* sp. (MB273N) was transferred into Falcon tubes and centrifuged at 11,000 rpm for 15 minutes at 4°C. The supernatant was separated from the pellet and centrifugation was repeated three times. The final supernatant was filter-sterilized using a 0.22 µm syringe filter. The bacterial pellet was used for colony-forming unit (CFU) enumeration by resuspending in 100 mL of NaCl solution, followed by a 1:10 serial dilution. The cultures were then plated onto nutrient agar using the spread plate method and incubated overnight at 28°C. The bacterial concentration was determined to be 1.1×10^{11} CFU/mL, representing the neat concentration of *Pseudomonas* sp. (MB273N) CFS.

Preparation of 2,3,5-Triphenyltetrazolium Chloride (TTC) Solution

TTC solution was prepared following Mukti (2019) with modifications. Briefly, 75 mg of TTC was dissolved in 15 mL of sterile distilled water at room temperature, filtered through a 0.22 µm syringe filter, and stored at 4°C until use.

Minimum Inhibitory Concentration (MIC) Assay

The MIC assay was adapted from Mukti (2019) with modifications. A 96-well microtiter plate was prepared with 100 µL of nutrient broth (NB) per well. CFS was serially diluted two-fold in NB, and 50 µL of bacterial suspension (OD600 = 0.5, adjusted using a Bio-Rad SmartSpec Plus spectrophotometer) was added to each well.

Controls included:

a) Positive control: 100 µL NB + 50 µL bacterial suspension
b) Negative control: 100 µL NB + 100 µL CFS

e-ISSN 2716-697X

The plate was incubated at 37°C overnight. After incubation, 40 µL of TTC was added to each well and further incubated for 3 hours at room temperature. Bacterial growth was indicated by red spot formation at the well bottom. The MIC was determined as the lowest CFS concentration that inhibited visible bacterial growth (absence of red spot formation). The assay was performed in technical triplicates. The sub-lethal concentrations of CFS were then determined and used for the biofilm inhibition assay.

Biofilm Inhibition Assay

The biofilm inhibition assay followed Mukti (2019) with modifications. A 96-well microtiter plate was prepared by adding 100 µL of NB per well. CFS was added and serially diluted two-fold. Then, 50 µL of bacterial suspension (OD600 = 0.5) of *P. aeruginosa, E. coli, S. aureus,* and *C. violaceum* was introduced into each well.

Controls included:

a) Positive control: 100 µL NB + 50 µL bacterial suspension (untreated sample)

b) Negative control: 100 µL NB + 100 µL CFS

The plate was incubated at 37°C overnight. After incubation, culture media were aspirated, and the wells were rinsed three times with sterile distilled water to remove planktonic bacteria. Wells were then stained with 200 µL of 0.1% (w/v) crystal violet solution for 30 minutes at room temperature. After washing and air-drying, bound crystal violet was solubilized with 95% ethanol. The absorbance (OD570) of solubilized dye was measured using a microplate reader (Tecan Sunrise). The percentage of biofilm inhibition was calculated as follows:

$$\text{Biofilm inhibition} = \left(1 - \frac{\text{Mean OD treated}}{\text{Mean OD untreated}}\right) \times 100$$

Scanning Electron Microscopy (SEM) Analysis

SEM was used to visualize the effects of *Pseudomonas* sp. (MB273N) CFS on biofilms of *E. coli, P. aeruginosa, S. aureus,* and *C. violaceum*, following Mukti (2019) with modifications. Test bacteria were cultured in the presence and absence of CFS and incubated at 37°C for 24 hours without shaking. The optical density of the cultures was adjusted to OD600 = 0.5 before biofilm samples were fixed in fixative solution and incubated at 4°C for a minimum of 4 hours.

The samples were dehydrated through a graded ethanol series (35%, 50%, 75%, 95%, and 100%) and dried overnight using silica gel, following the method of Raab and Bachelet (2017), where silica gel reduces moisture

e-ISSN 2716-697X

by gradually absorbing water vapor. Dried samples were sputter-coated with gold and examined under a Scanning Electron Microscope (Hitachi S-3400N).

Data Analysis

Statistical analyses were conducted using SPSS version 26. Data normality was assessed using the Shapiro-Wilk test. Paired t-tests and Wilcoxon signed-rank tests were used to compare mean values before and after CFS treatment, with statistical significance set at $p < 0.05$.

RESULTS AND DISCUSSION

MIC and Sub-lethal Concentration of CFS for Pathogenic Bacteria

MIC assay results of *Pseudomonas* sp. (MB273N) CFS are shown in figures 1 and 2. The MIC value of CFS against *E. coli* was observed at a concentration of 1.4×10^{10} CFU/mL, whereas the CFS demonstrated the same MIC value against *P. aeruginosa*, *S. aureus*, and *C. violaceum* bacteria, with a concentration of 1.1×10^{11} CFU/mL. The MIC results showed that *Pseudomonas* sp. (MB273N) CFS inhibited bacterial growth. The solution in the well remained clear or turned pale pink after adding TTC and being incubated, indicating growth inhibition (Panphut *et al.*, 2020). In accordance with our study, Chythanya *et al.* (2002) revealed that the chloroform extract of *Pseudomonas* CFS exhibited inhibitory effects on the growth of a bacterial pathogen, as determined by the MIC assay. MIC is the lowest antimicrobial concentration that will inhibit a microorganism's visible growth after overnight incubation (Wahi *et al.*, 2011). Referring to Table 1, the higher MIC value of 1.1×10^{11} CFU/mL for *P. aeruginosa*, *S. aureus*, and *C. violaceum* indicates greater resistance compared to *E. coli*. According to Mombeshora and Mukanganyama (2017), a high MIC value demonstrates that a high concentration of the antimicrobial agent is needed to inhibit microbial growth, hence, the test isolate is resistant to the specific antimicrobial.

e-ISSN 2716-697X

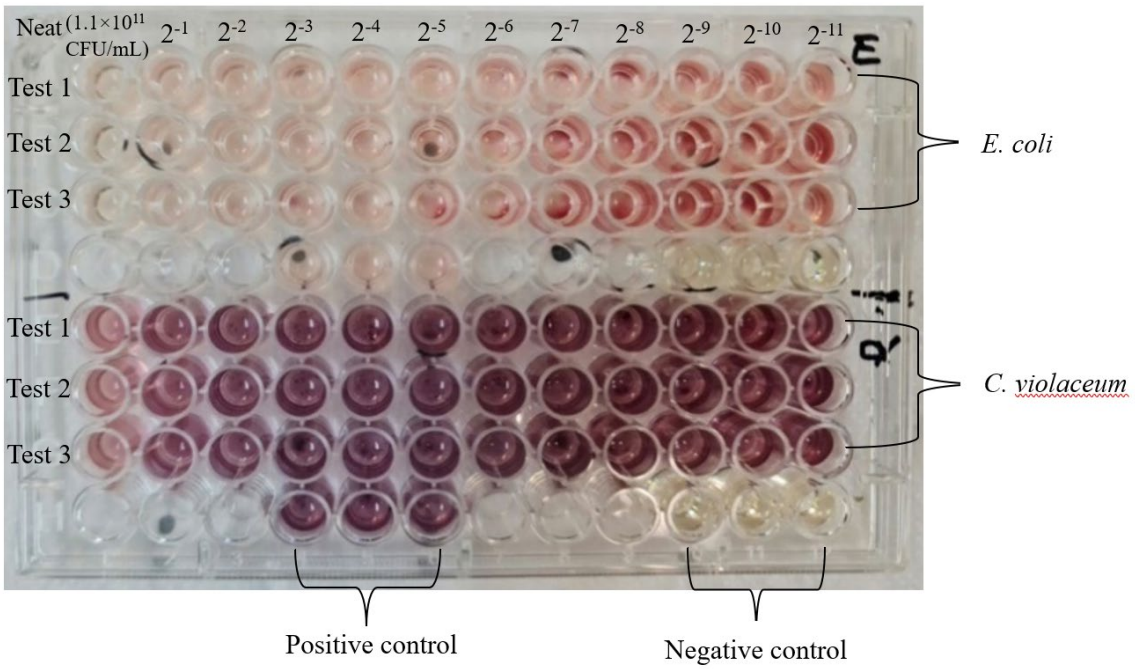


Figure 1 Minimum inhibitory concentration of (MB272N) CFS against *E. coli* and *C. violaceum*.

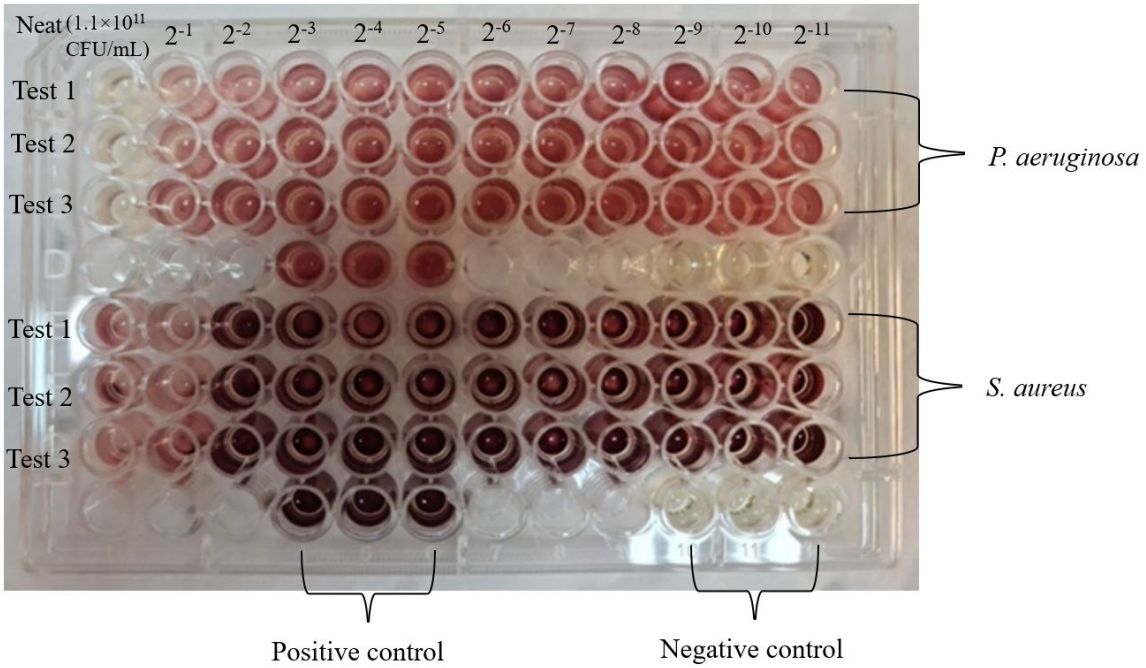


Figure 2 Minimum inhibitory concentration of (MB272N) CFS against *P. aeruginosa* and *S. aureus*.

The sub-lethal concentration of CFS against *E. coli* and *P. aeruginosa* was 0.07×10^{11} and 0.55×10^{11} respectively. CFS exhibited the same sub-lethal concentration value against *S. aureus* and *C. violaceum*, measuring 1.1×10^{11}

e-ISSN 2716-697X

CFU/mL. Sub-lethal concentration is the concentration of antimicrobial agent in the well immediately before the MIC concentration. The concentration of *Pseudomonas* sp. (MB273N) CFS for the biofilm inhibition assay was below the MIC to ensure that the concentration used to inhibit biofilm formation was not lethal to the test bacteria. The selection of sub-lethal concentration for the CFS was based on the premise that alternative anti-infective treatments should not directly cause bacterial death, as antimicrobial resistance to various antibiotics arises from gene mutations in pathogenic bacteria, which serves as a defense mechanism against cell death (Mukti *et al.*, 2019).

Table 1 MIC and sub-lethal concentration of *Pseudomonas* sp. (MB272N) CFS against tested bacteria.

Microorganisms	*Pseudomonas sp.* (MB273N) CFS	
	MIC (CFU/mL)	Sub-lethal (CFU/mL)
Pseudomonas aeruginosa	1.1×10^{11}	0.55×10^{11}
Escherichia coli	0.14×10^{11}	0.07×10^{11}
Staphylococcus aureus	1.1×10^{11}	1.1×10^{11}
Chromobacterium violaceum	1.1×10^{11}	1.1×10^{11}

Biofilm Inhibition of Pseudomonas sp. (MB273N) CFS

A biofilm inhibition assay was conducted to evaluate the ability of *Pseudomonas* sp. (MB273N) CFS in inhibiting bacterial biofilm formation. In figure 3, it can be seen that the OD values of tested microorganisms after exposure to a sub-lethal concentration of CFS were lower than the OD values before CFS treatment, indicated a reduction in biomass. According to Liu *et al.* (2021), a reduction in the biomass may indicates a decrease in the biofilm matrix content. These results suggested that CFS *Pseudomonas* sp. (MB273N) exhibited biofilm inhibitory activity against *P. aeruginosa*, *E. coli*, *S. aureus*, and *C. violaceum*.

e-ISSN 2716-697X

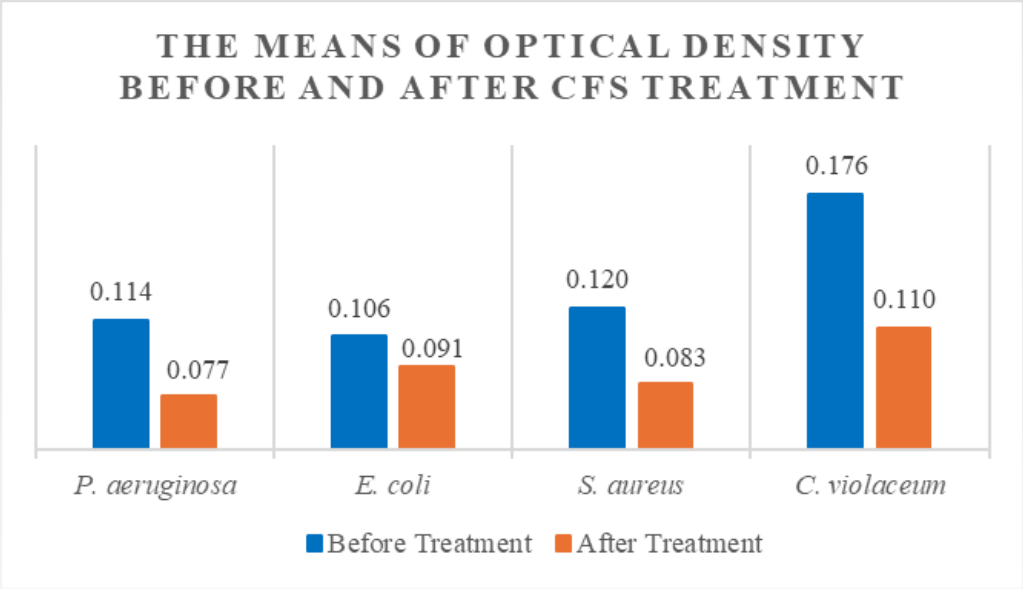


Figure 3 The means of optical density before and after CFS treatment

Biofilm inhibition percentage of tested bacteria is shown in Figure 4. Biofilm formation of *P. aeruginosa*, *S. aureus*, and *C. violaceum* was significantly inhibited by *Pseudomonas* sp. (MB273N) CFS ($p < 0.05$). The biofilm inhibition percentages of CFS-treated *S. aureus*, *P. aeruginosa*, and *C. violaceum* were 30.83%, 32.46%, and 37.5%, respectively. No significant difference was observed between before and after CFS treatment biofilm inhibition of *E. coli* ($p > 0.05$). Biofilm inhibition of 14.15% was noted when *E. coli* was exposed to CFS. According to Famuyide et al. (2019), antibiofilm activity is considered good when the percentage inhibition is above 50%, whereas percentages between 0% and 49% indicate poor antibiofilm activity. Thus, it can be concluded that the CFS from *Pseudomonas* sp. (MB273N) at sub-lethal concentration exhibited low biofilm inhibition against *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. violaceum*, as the inhibition percentages were below 50%. However, the observed low inhibition does not indicate an absence of antibiofilm effect, as the CFS may still contain bioactive compounds that exert partial or concentration-dependent antibiofilm activity, which may become more pronounced under different experimental conditions or at higher concentrations.

e-ISSN 2716-697X

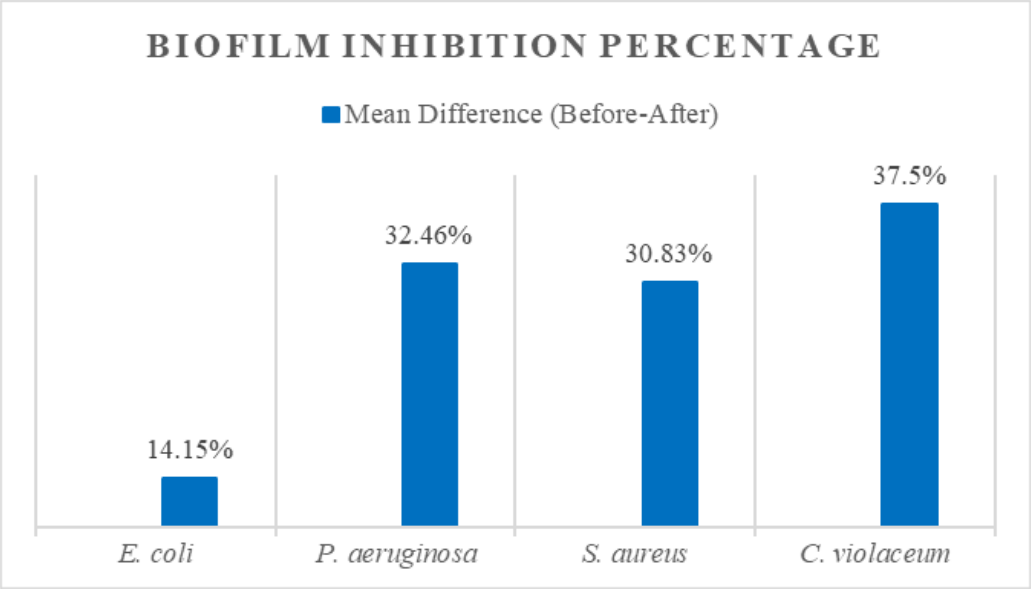


Figure 4 Biofilm inhibition percentage

The inhibition of biofilm formation was assessed using crystal violet solution. Aiyer and Vijaykumar (2019) noted that crystal violet stains living biomass and the extracellular matrix of the biofilm. Additionally, crystal violet is suitable for qualitative and quantitative measurements of biofilms adhered to various surfaces (Haney *et al.*, 2018). Gene expression of biofilm is regulated by quorum sensing, and the activity of biofilm inhibition may occur due to the disruption of quorum sensing (Theodora *et al.*, 2019). Besides, interfering quorum sensing system may hinder the assembly of biofilm matrix and the further establishment of infection in host cells (Venkatramanan *et al.*, 2020). The biofilm inhibition may occur because the bacterial cells produce compounds that can disrupt quorum sensing. These compounds, known as quorum quenching compounds, can work in several ways, by interfering synthesis of autoinducer, degrading the autoinducer, or preventing the binding of a signal molecule to the receptor (Mulya and Waturangi, 2021).

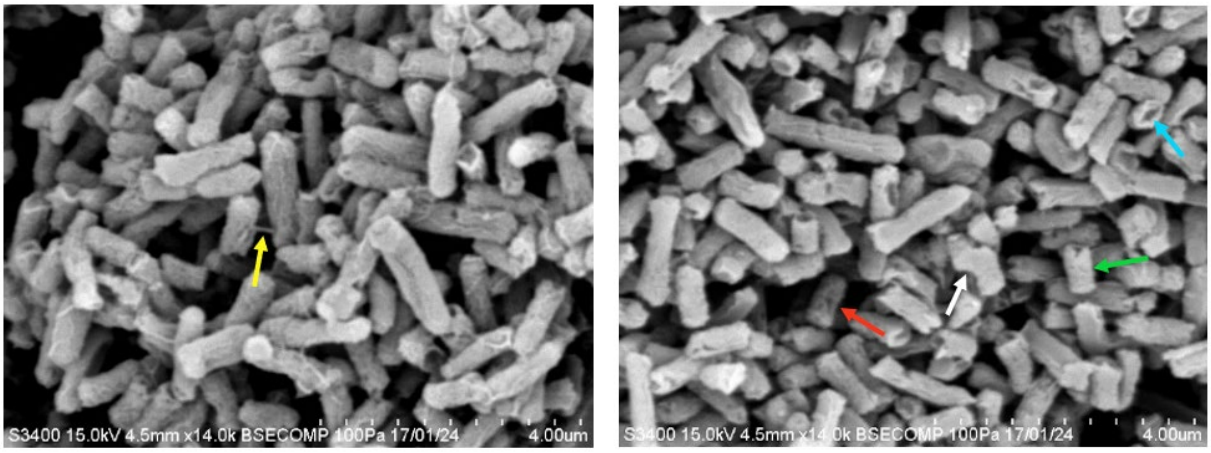
Biofilms With and Without CFS Treatment under Scanning Electron Microscope

To verify that *Pseudomonas* sp. (MB273N) CFS exhibited biofilm inhibitory activity of *P. aeruginosa*, *E. coli*, *S. aureus*, and *C. violaceum*, the bacterial biofilm was observed using Scanning Electron Microscope (SEM). In untreated *E. coli* group, biofilms displayed dense cellular aggregates, typical rod-shaped cells with normal size, and the presence of extracellular polysaccharide (EPS) matrix (Figure 5a). Meanwhile, morphological or ultrastructural changes were observed in the microbial cells treated with CFS. Some cells became shorter, appeared wrinkled, and had altered shape, invaginations and looser cell

e-ISSN 2716-697X

aggregation after CFS treatment. Furthermore, an obvious reduction of the EPS matrix was also detected (Figure 5b).

Our results were similar to those reported in previous studies. Chaichana *et al.* (2023) found that *E. coli* strain exhibited looser cell aggregation and less extracellular matrix production when treated with *Staphylococcus warneri* CFS. Famuyide *et al.* (2020) reported that the exposure of *E. coli* to *Syzygium legatii* acetone leaf extracts caused invagination of some cells, wrinkled surface, and alteration in bacterial cell shape. These characteristics, which are like our findings, demonstrated that *E. coli* biofilm treated with *Pseudomonas* sp. (MB273N) CFS was disrupted compared to the untreated group.



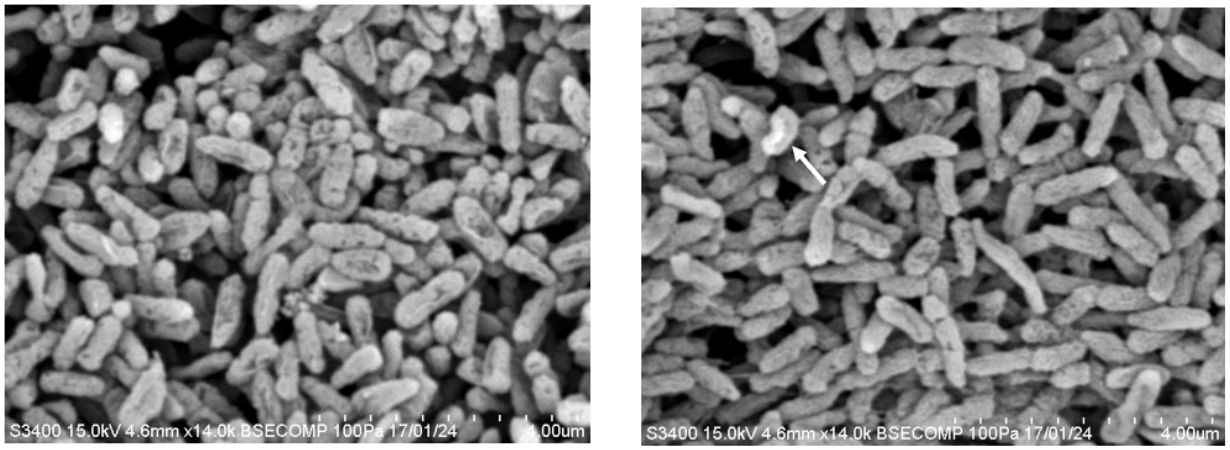
(a) (b)

Figure 5 *E. coli* untreated [(a) (15.0 kV; magnification: 14000 ×)] and treated [(b) (15.0 kV; magnification: 14000 ×)] with *Pseudomonas* sp. (MB273N) CFS. In Figure 5.a, the yellow arrow indicates the EPS matrix, while Figure 5.b shows several changes: shorter cells (green arrow), a wrinkled cell surface (red arrow), cell shape alteration (white arrow), and invagination (blue arrow).

Untreated *P. aeruginosa* cells exhibited tight cellular aggregates (Figure 6a), whereas more dispersed cells and alteration in bacterial cell shape were observed after CFS treatment (Figure 6b). These findings were consistent to those reported by Drumond *et al.* (2023), who revealed that the treatment of *P. aeruginosa* with the cell-free spent medium (CFSM) of lactic acid bacteria caused a reduction in the cell's compaction. Moreover, Liu *et al.* (2020) showed that bacterial cells were bent after treated with linalool. As confirmed by these results, it can be concluded that CFS of *Pseudomonas* sp. (MB273N) was effective in altering the structure of biofilm.

Pseudomonas sp. is known to produce antimicrobials that can inhibit closely related species. A previous study done by Snopková *et al.* (2022) reported that selected *Pseudomonas* sp. strains isolated from Antarctica were able to inhibit one other *Pseudomonas* strain. These strains produced antimicrobials, and no strain was sensitive to its own antimicrobial agent.

e-ISSN 2716-697X



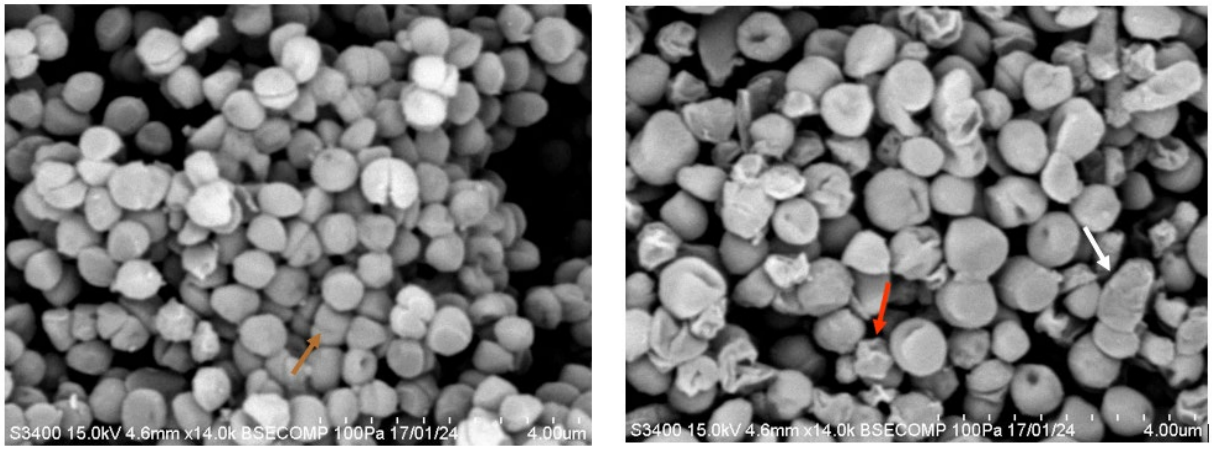
(a) (b)

Figure 6 *P. aeruginosa* untreated [(a) (15.0 kV; magnification: 14000 ×)] and treated [(b) (15.0 kV; magnification: 14000 ×)] with *Pseudomonas* sp. (MB273N) CFS. The white arrow indicates an altered cell shape.

S. aureus groups without CFS treatment portrayed dense and clumped biofilm structures with multiple layers were formed. Many cells showed spherical shape intact cell morphology, and the formation of a septum and cell splitting was also observed (Figure 7a). On the other hand, CFS-treated biofilms exhibited altered morphology, more dispersed cells, and wrinkles on the cell surface. The treated cells appeared elongated, and septum formation was reduced compared with the untreated group (Figure 7b).

Similar findings were also observed in previous studies on *S. aureus* cells treated with various antimicrobial agents. Kim *et al.* (2020) reported that *S. aureus* treated with *S. cerevisiae* CFS exhibited decreased cell attachment and aggregation. Furthermore, *S. aureus* treated with *Perilla frutescens var. acuta* leaf extract presented wrinkled abnormalities on the morphology of the cells (Kim *et al.*, 2011). Moreover, Matijašević *et al.* (2016) revealed that untreated *S. aureus* cells were spherical and exhibited a septum formation, whereas those treated with *Coriolus versicolor* extracts appeared elongated and deformed. Interestingly, the septum formation in the treated sample was not detected. The formation of a septum indicated active proliferation, and the authors assumed that the treatment of *S. aureus* with methanol extract of *C. versicolor* disables cell division because the septum formation in the treated sample was not observed. From the previous study, we could conclude that *Pseudomonas* sp. (MB273N) CFS had an inhibitory effect on cell division, as evidenced by the reduction in the formation of septa, and similar changes obtained from previous works also proved that the CFS of *Pseudomonas* sp. (MB273N) caused damage to the cell shape of *S. aureus*.

e-ISSN 2716-697X



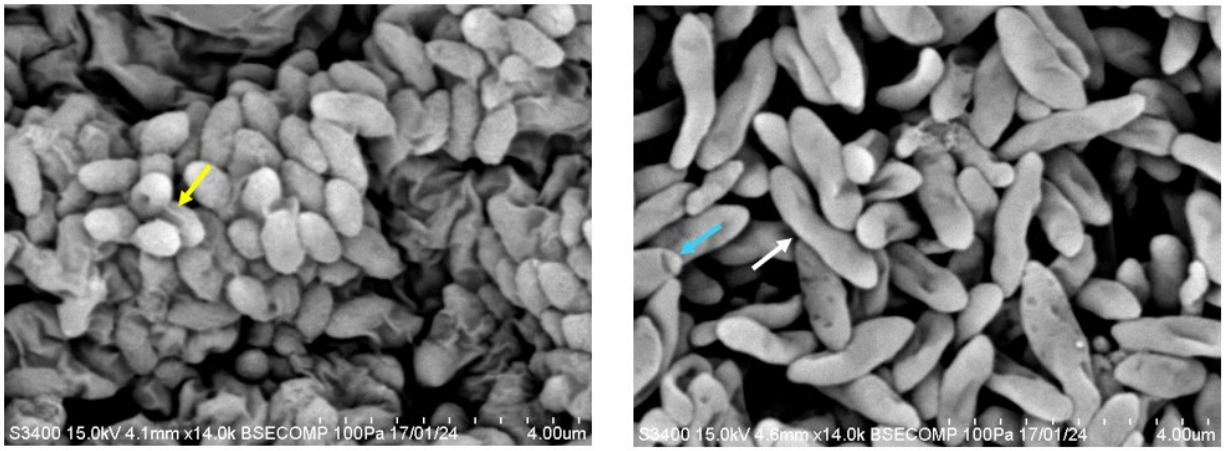
(a) (b)

Figure 7: *S. aureus* untreated [(a) (15.0 kV; magnification: 14000 ×)] and treated [(b) (15.0 kV; magnification: 14000 ×)] with *Pseudomonas* sp. (MB273N) CFS. The brown arrow shows septum formation. The red arrow indicates a wrinkled cell, and the white arrow shows a shape alteration.

C. violaceum biofilm without CFS formed a cohesive aggregate with rod-shaped cells closely adhered to each other. Moreover, the bacterial cells can also be seen enclosed in a polymeric matrix (EPS) forming a clump of cells (Figure 8a). In contrast, CFS-treated samples exhibited reduced EPS matrix, dispersed cells, and surface invagination. Additionally, an elongation of cell size with abnormally shaped bacterial cells was observed, likely resulting from incomplete cell division (Figure 8b). This observation is consistent with findings reported by Dimitrova *et al.* (2024) utilizing lactone-enriched fraction from *I. Britannica*, where the cell elongation was observed in *C. violaceum*. Their study also highlighted incomplete cell division as the likely cause of the abnormal bacterial cell shape.

Similar observations have also been reported by previous studies on *C. violaceum* treated with various antibiofilm agents. The SEM results of *C. violaceum* treated with silver nanoparticles made from *Moringa oleifera* revealed biofilm inhibition with scattered cells compared with untreated cells (Haris and Ahmad, 2024). Furthermore, Dimitrova *et al.* (2024) reported that invagination on the cell surface and alterations in bacterial cell shape were found in *C. violaceum* treated with plant extracts of the genus *Inula*. These findings supported the present study, indicating that *C. violaceum* treated with *Pseudomonas* sp. (MB273N) CFS exhibited morphological deformations.

e-ISSN 2716-697X



(a) (b)

Figure 8 *C. violaceum* untreated [(a) (15.0 kV; magnification: 14000 ×)] and treated [(b) (15.0 kV; magnification: 14000 ×)] with *Pseudomonas* sp. (MB273N) CFS. The yellow arrow indicates EPS matrix. The blue arrow shows invagination of the cell. The white arrow indicates elongated cell.

The present study found that four bacterial biofilms treated with CFS exhibited reduced cell attachment and aggregation. This demonstrates that the treatment with the CFS resulted in a reduction in the ability of cells to adhere to each other, which led to less dense and more dispersed biofilm structures. The EPS matrix plays a crucial role in microbial adhesion to a surface, aggregation in multilayered biofilms, and protection against antimicrobial compounds, as well as antibiotics (Mishra *et al.*, 2020). According to Saptami *et al.* (2022), a reduction in EPS may lower the surface adhesion resulting in the loosening and dispersion of the cells. EPS contains exopolysaccharides, which play a major role in the integrity of the biofilm matrix. Biofilm maturation requires the secretion of EPS to bind cells together and maintains cohesive biofilm architecture. Therefore, a reduction in EPS production will cause the biofilm structure weak and susceptible (Husain *et al.,* 2015).

The CFS treatment also altered bacterial morphology. When bacteria change shape from a rod-like shape to shortened rods, they may decrease their surface area in contact with the surface and adjacent cells, resulting in impaired attachment to the surface and other cells (van Teeseling *et al.*, 2017). According to Baquero *et al.* (2024), environmental stress, either natural or anthropogenic, affects the shape of bacterial cells. Antimicrobial agents alter bacterial functions and cellular structures, causing cellular stress and alterations to bacterial forms.

Several changes observed within the biofilms treated with *Pseudomonas* sp. (MB273N) CFS are assumed to be the results of the activity of compounds present in the CFS, which possess antimicrobial or antibiofilm

properties. A previous study reported that CFS from *Pseudomonas*, isolated from a hot spring, produced a bacteriocin-like substance and exhibited a large antibacterial activity against *E. coli* and *Pseudomonas* sp. (Ghrairi *et al.*, 2014). Bacteriocins can interfere in the early stages of biofilm formation by inhibiting the attachment of bacterial cells to other cells or their surface, or by killing the cells before they can integrate into biofilm structure (Duraisamy *et al.*, 2020). Additionally, pyocyanin from *Pseudomonas* has demonstrated antibiofilm activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) by reducing bacterial viability and EPS matrix (Kamer *et al.*, 2023). Based on these findings, it can be assumed that *Pseudomonas* sp. (MB273N) CFS may contain these compounds. However, further studies are needed to verify whether these compounds contribute to its antibiofilm activity.

The results of our study demonstrated that the cell-free supernatant of *Pseudomonas* sp. (MB273N) affected the structure and morphological characteristics of bacterial biofilms, thereby disrupting biofilm formation process in *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. violaceum*. Based on the data obtained from the Scanning Electron Microscopy (SEM) observation, the interference of biofilm formation was indicated by reduced cell aggregation and morphological alterations in bacterial cells.

CONCLUSION

Cell-free supernatant of *Pseudomonas* sp. (MB273N) exhibited antibiofilm activity against *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. violaceum*, with inhibition percentages below 50%. These findings were further supported by scanning electron microscopy observations, which showed that before CFS treatment, the four bacterial biofilms displayed dense cellular aggregates and multilayered structures, whereas after CFS treatment, the biofilms became less dense, more dispersed, and exhibited alterations in cell morphology.

REFERENCES

Aiyer, K. S. and Vijayakumar, B. S. (2019). An improvised microtiter dish biofilm assay for non-invasive biofilm detection on microbial fuel cell anodes and studying biofilm growth conditions. *Brazilian Journal of Microbiology* 50(3), 769-775.

Al-Barhawee, N. I. K. and Al-Rubyee S. S. (2024). Inhibition of Biofilm Formation in *Agrobacterium tumefaciens* by Cell-Free Supernatants of *Pseudomonas aeruginosa* Analyzed by GC-MS. *Baghdad Science Journal* 21(7), 2222-2236.

Alisjahbana, B., Debora, J., Susandi, E. and Darmawan, G. (2021). *Chromobacterium violaceum*: a review of an unexpected scourge. *International journal of general medicine* 14, 3259-3270.

Alshammari, M., Ahmad, A. AlKhulaifi, M., Al Farraj, D., Alsudir, S., Alarawi, M., Takashi, G. and Alyamani, E. (2023). Reduction of biofilm formation

e-ISSN 2716-697X

of *Escherichia coli* by targeting quorum sensing and adhesion genes using the CRISPR/Cas9-HDR approach, and its clinical application on urinary catheter. *Journal of Infection and Public Health* 16(8), 1174-1183.

Arampatzi, S. I., Giannoglou, G. and Diza, E. (2011). Biofilm formation: A complicated microbiological process. *Aristotle University Medical Journal* 38(2), 21-29.

Asma, S.T., Imre, K., Morar, A., Herman, V., Acaroz, U., Mukhtar, H., Arslan-Acaroz, D., Shah, S. R. A. and Gerlach, R. (2022). An Overview of Biofilm Formation–Combating Strategies and Mechanisms of Action of Antibiofilm Agents. *Life* 2022 12(8), 1110.

Baquero, F., Moreno-Blanco, A. and del-Campo, R. (2024). Environmental Stress, Bacterial Cell Differentiation, and Antimicrobial Resistance.

Chaichana, N., Muneerungsee, N., Sukpondma, Y. and Sermwittayawong, N. (2023). *Escherichia coli* virulence inhibition by cell-free supernatants from mangrove forest bacteria producing quorum sensing inhibitor. *LWT* 185, 115182.

Chythanya, R., Karunasagar I. and Karunasagar. I (2002). Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. *Aquaculture* 208(1-2), 1-10.

Davis, R. and Brown, P. D. (2016). Multiple antibiotic resistance index, fitness and virulence potential in respiratory *Pseudomonas aeruginosa* from Jamaica. *Journal of medical microbiology* 65(4), 261-271.

Dimitrova, P. D., Ivanova, V., Trendafilova, A. and Paunova-Krasteva, T. (2024). Anti-Biofilm and Anti-Quorum-Sensing Activity of Inula Extracts: A Strategy for Modulating Chromobacterium violaceum Virulence Factors. *Pharmaceuticals* 17(5), 573.

Drumond M. M., Tapia-Costa, A. P., Neumann, E., Nunes, Á. C., Barbosa, J. W., Kassuha, D.E. and Mancha-Agresti, P. (2023). Cell-free supernatant of probiotic bacteria exerted antibiofilm and antibacterial activities against *Pseudomonas aeruginosa*: A novel biotic therapy. *Front. Pharmacol* 14, 1152588.

Duraisamy, S., Balakrishnan, S., Ranjith, S., Husain, F., Sathyan, A., Peter, A. S., Prahalathan, C. and Kumarasamy, A. (2020). Bacteriocin—a potential antimicrobial peptide towards disrupting and preventing biofilm formation in the clinical and environmental locales. *Environmental Science and Pollution Research* 27: 44922-44936.

Famuyide, I. M., Aro, A. O., Fasina, F. O., Eloff, J. N. and McGaw, L. J. (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 complementary and alternative medicine* 19, 1-13.

Famuyide, I. M., Fasina, F. O., Eloff, J. N and McGaw, L. J. (2020). The ultrastructural damage caused by Eugenia zeyheri and Syzygium legatii

e-ISSN 2716-697X

acetone leaf extracts on pathogenic Escherichia coli. *BMC veterinary research* 16(1), 326.

Ghrairi, T., Braiek, O. B. and Hani, K. (2014). Detection and characterization of a bacteriocin, putadicin T01, produced by *Pseudomonas putida* isolated from hot spring water. *Apmis* 123(3), 260-268.

Haney, E. F., M. J. Trimble, J. T. Cheng, Q. Vallé and R. E. W. Hancock. (2018). Critical assessment of methods to quantify biofilm growth and evaluate antibiofilm activity of host defence peptides. *Biomolecules* 8(2), 29.

Haris, Z. and Ahmad, I. (2024). Green synthesis of silver nanoparticles using Moringa oleifera and its efficacy against gram-negative bacteria targeting quorum sensing and biofilms. *Journal of Umm Al-Qura University for Applied Sciences* 10(1), 156-167.

Husain F. M., Ahmad, I. Khan, M. S., Ahmad, E., Tahseen, Q., Khan, M. S. and Alshabib, N. A. (2015). Sub-MICs of Mentha piperita essential oil and menthol inhibits AHL mediated quorum sensing and biofilm of Gram-negative bacteria. *Frontiers in microbiology* 6, 420.

Kamer, A. M. A., Abdelaziz, A. A. Al-Monofy, K. B. and Al-Madboly, L. A. (2023). Antibacterial, antibiofilm, and anti-quorum sensing activities of pyocyanin against methicillin-resistant Staphylococcus aureus: in vitro and in vivo study. *BMC microbiology* 23(1), 116.

Kim, D. H., Kim Y. C. and Choi, U. K. (2011). Optimization of antibacterial activity of *Perilla frutescens var. acuta* leaf against *Staphylococcus aureus* using evolutionary operation factorial design technique. *International journal of molecular sciences* 12(4), 2395-2407.

Kim, Y. J., Yu, H. H., Park, Y. J., Lee, N. K. and Paik, H. D. (2020). Anti-biofilm activity of cell-free supernatant of *Saccharomyces cerevisiae* against *Staphylococcus aureus*. *Journal of Microbiology and Biotechnology* 30(12), 1854.

Liu, H., Zhang, R., Zhang, Q., Tian, M., Ren, X., Wang, L. and Wang, X. (2023). Antifungal Activity of Cell-Free Supernatants from *Lactobacillus pentosus* 86 against *Alternaria gaisen*. *Horticulturae* 9(8), 911.

Liu, J., Madec, J. Y., Bousquet-Mélou, A., Haenni, M. and Ferran, A. A. (2021). Destruction of *Staphylococcus aureus* biofilms by combining an antibiotic with subtilisin A or calcium gluconate. *Scientific reports* 11(1), 6225.

Liu, X., Cai, J., Chen, H., Zhong, Q., Hou, Y., Chen, W. and Chen, W. (2020). Antibacterial activity and mechanism of linalool against *Pseudomonas aeruginosa*. *Microbial Pathogenesis* 141, 103980.

Matijašević, D., Pantić, M., Rašković, B., Pavlović, V., Duvnjak, D., Sknepnek, A. and Nikšić, M. (2016). The antibacterial activity of *Coriolus versicolor* methanol extract and its effect on ultrastructural changes of *Staphylococcus aureus* and *Salmonella Enteritidis*. *Frontiers in Microbiology* 7, 1226.

Meroni, G., Panelli, S., Zuccotti, G., Bandi, C., Drago, L. and Pistone, D.

e-ISSN 2716-697X

(2021). Probiotics as Therapeutic Tools against Pathogenic Biofilms: Have We Found the Perfect Weapon?. *Microbiology Research* 12(4), 916–937.

Mishra, R., Panda, A. K., De Mandal, S., Shakeel, M., Bisht, S. S. and Khan, J. (2020). Natural Anti-biofilm Agents: Strategies to Control Biofilm-Forming Pathogens. *Frontiers in microbiology* 11, 566325.

Mombeshora, M. and Mukanganyama, S. (2017). Development of an accumulation assay and evaluation of the effects of efflux pump inhibitors on the retention of chlorhexidine digluconate in *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *BMC research notes* 10, 1-9.

Mulya, E. and Waturangi, D. E. (2021). Screening and quantification of anti-quorum sensing and antibiofilm activity of Actinomycetes isolates against food spoilage biofilm-forming bacteria. *BMC microbiology* 21, 1-8.

Mukti, K. (2019). Biofilm Inhibition of Pathogenic Bacteria by Mangrove Plant Extracts. Dissertation. Biotechnology Research Institute, University Malaysia Sabah, Malaysia.

Panphut, W., Budsabun, T. and Sangsuriya, P. (2020). In vitro antimicrobial activity of *Piper retrofractum* fruit extracts against microbial pathogens causing infections in human and animals. *International Journal of Microbiology* 2020(1), 5638961.

Raab, N. and Bachelet, I. (2017). Resolving biofilm topography by native scanning electron microscopy. *Journal of Biological Methods* 4(2), 1-5.

Ray, S., Jin, J. O., Choi, I. and Kim, M. (2023). Cell-Free Supernatant of *Bacillus thuringiensis* Displays Anti-Biofilm Activity against *Staphylococcus aureus*. *Applied Biochemistry and Biotechnology* 195(9), 5379-5393.

Sanchez, C. J., Ward, C. L., Romano, D. R., Hurtgen, B. J., Hardy, S. K., Woodbury, R. L., Trevino, A. V., Rathbone, C. R. and Wenke, J. C. (2013). *Staphylococcus aureus* biofilms decrease osteoblast viability, inhibits osteogenic differentiation, and increases bone resorption in vitro. *BMC musculoskeletal disorders* 14, 1-11.

Saptami, K., Arokia Balaya Rex, D., Chandrasekaran, J. and Rekha, P. D. (2022). Competitive interaction of thymol with cviR inhibits quorum sensing and associated biofilm formation in *Chromobacterium violaceum*. *International Microbiology* 25(3), 629-638.

Snopková, K., Dufková, K., Chamrád, I., Lenobel, R., Čejková, D., Kosina, M., Hrala, M., Holá, V., Sedláček, I. and Šmajs, D. (2022). Pyocin-mediated antagonistic interactions in Pseudomonas spp. isolated in James Ross Island, Antarctica. *Environmental Microbiology* 24(3), 1294-1307.

Theodora, N. A., Dominika, V. and Waturangi, D. E. (2019). Screening and quantification of anti-quorum sensing and antibiofilm activities of phyllosphere bacteria against biofilm forming bacteria. *BMC research notes* 12, 1-5.

e-ISSN 2716-697X

van Teeseling, M. C., de Pedro, M. A. and Cava, F. (2017). Determinants of Bacterial Morphology: From Fundamentals to Possibilities for Antimicrobial Targeting. *Frontiers in microbiology* 8, 1264.

Venkatramanan, M., Ganesh, P. S., Senthil, R., Akshay, J., Ravi, A. V., Langeswaran, K., Vadivelu, J., Nagarajan, S., Rajendran, K. and Shankar, E. M. (2020). Inhibition of quorum sensing and biofilm formation in *Chromobacterium violaceum* by fruit extracts of *Passiflora edulis*. *ACS omega* 5(40), 25605-25616.

Wahi, A. K., Singh, A. and Singh, A. K. (2011). Determination of minimum inhibitory concentration (MIC) of some novel triazole derivative. *International Journal of Research in Pharmacy and Chemistry* 1(4), 1108-1114.

Yu, S., Jiang, B., Jia, C., Wu, H., Shen, J., Hu, X. and Xie, Z. (2020). Investigation of biofilm production and its association with genetic and phenotypic characteristics of OM (osteomyelitis) and non-OM orthopedic *Staphylococcus aureus*. *Annals of clinical microbiology and antimicrobials* 19, 1-9.

Zagaglia, C., Ammendolia, M. G., Maurizi, L., Nicoletti, M. and Longhi, C. (2022). Urinary tract infections caused by uropathogenic *Escherichia coli* strains—new strategies for an old pathogen. *Microorganisms* 10(7), 1425.