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Supernatant of Actinomycetes isolates with antiquorum sensing and antibiofilm activity against food spoilage bacteria

GLORIA ANNE, DIANA ELIZABETH WATURANGI♥
Faculty of Biotechnology, Universitas Katolik Indonesia Atma Jaya. Jl. Raya Cisauk-Lapan No. 10, Sampora, Cisauk, Tangerang 15345, Banten, Indonesia. ♥email: diana.waturangi@atmajaya.ac.id

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Abstract. *Anne G, Waturangi DE. 2023. Supernatant of Actinomycetes isolates with antiquorum sensing and antibiofilm activity against food spoilage bacteria. Biodiversitas 24: 5189-5197.* High processing food facilities occur to remove spoilage bacteria that can risk the food safety. However, bacteria can form biofilms as a survival strategy in extreme conditions. They generally use autoinducer signals in communication called quorum sensing as their modulation mechanism. Chemical agents usually treat the biofilm matrix, but side effects may occur in most cases. Therefore, it is necessary to explore natural metabolite compounds to overcome it, one of which comes from Actinomycetes. This study was conducted to determine the anti-biofilm activity of Actinomycetes supernatants, which had previously been performed using their crude extracts. The transition to using supernatant production was motivated by its higher yield and practical application. Supernatants from six Actinomycetes isolates showed positive inhibition of quorum sensing assay using *Chromobacterium violaceum* wild-type and mutant 026 as indicator bacteria through agar well diffusion techniques and validation assay using absorbance measurement of violacein pigment production. Based on the quantification of antibiofilm activity assay, the supernatant showed the ability to inhibit and destroy biofilm from *Bacillus cereus*, *Bacillus subtilis*, and *Shewanella putrefaciens* with various percentages of antibiofilm activity, with the highest antibiofilm activity of 88.34% observed for isolate SW03 specifically targeting *B. subtilis* biofilm destruction. It also appeared in the light microscopy and scanning electron microscopy (SEM) observation that the addition of supernatant showed inhibition and dispersion of biofilm visually compared with the untreated ones. In conclusion, Actinomycetes supernatants have the capability to be used as antibiofilm agents, therefore potential and efficient to be applied in food industry equipment and environment to increase food safety.

Keywords: Bioactive compounds, biofilms, food safety, microplate assay, *Streptomyces*

INTRODUCTION

Biofilms pose significant challenges in the food industry, as they provide a protective environment for bacteria to survive harsh conditions and resist traditional cleaning methods applied in food industries environment. Despite employing various techniques such as chemical compounds, heat, acid treatment, and UV exposure to eliminate harmful bacteria, biofilms can still form due to the bacteria's dynamic survival strategies (Lu et al. 2019). Biofilms are extracellular polymer matrices created by microbial communities that flocked to solid surfaces through a communication mechanism known as quorum sensing (QS) (Majed et al. 2016). Through quorum sensing, bacteria use small signal molecules called autoinducers, which are produced during early bacterial growth and increase until reaching a certain level. This triggers gene expression, leading to bioluminescence, secretion of extracellular hydrolytic enzymes, and ultimately biofilm formation (Urvoy et al. 2021). Biofilm formation begins with planktonic bacteria adhering to surfaces through locomotion, but this initial binding is not strong enough (Verderosa et al. 2019). To initiate biofilm formation, cells communicate with one another intracellularly, sending signals for other cells to attach and express relevant genes. As signal molecules accumulate, adjacent cells recognize them and gather to form microcolonies (Köse-Mutlu et al. 2019). Increased cell density in these microcolonies results in the production of extracellular polymeric substrates (EPS), which contain exopolysaccharide derivatives or carbohydrates, proteins, lipids, nucleic acids, and other components (Di Martino 2018). Once the biofilm reaches its highest density, it facilitates biofilms dispersal, enabling bacteria to find new environments (Lu et al. 2019).

Biofilms form naturally, and in certain instances, the resulting exopolysaccharides can have practical industrial applications (Gangalla et al. 2021a). Exopolysaccharides from *Bacillus aerophilus* rk1 showed promising as an antioxidant. Additionally, the secreted polysaccharides from *B. amyloliquefaciens* RK3 have potential medicinal value, displaying anti-Alzheimer's disease effects in mouse models (Gangalla et al. 2021b). Although, when proper hygiene is not adequately maintained in equipment such as tanks, pipes, and pumps, the presence of biofilm can become a significant concern in the food industry. These can give rise to a range of serious issues, including equipment damage, post-process contamination, reduce product shelf life, and the potential transmission of diseases through horizontal gene transfer (Carrascosa et al. 2021). Moreover, biofilm formed by food spoilage bacteria such as *Bacillus cereus*, *B. subtilis*, and *Shewanella putrefaciens* present a formidable challenge to eradicate due to their protective nature, safeguarding the bacteria from both host cell attacks and traditional disinfectants.

There are ways to combat biofilm, such as preventing bacterial cells adherence microcolonies formation by modifying the physicochemical properties of the substrate and breaking down EPS biofilm component through enzymatic reactions (Azman et al. 2019). Traditional cleaning with chemical agents can have side effects on both products and the environment. This resilience calls for effective antibiofilm strategies to maintain food safety and quality standards in the industry. Thus, it is crucial to explore alternative solutions involving natural metabolite compounds to disrupt biofilms. One such compound comes from Actinomycetes. They are Gram-positive bacteria and recognized as an intermediate group between bacteria and fungi, unicellular, generally form spores, and can be found in soil or marine environments (Azman et al. 2019). Many groups of Actinomycetes are explored their bioactive compounds in search of beneficial products such as novel antibiotics, bioremediation agents, enzymes, enzymes inhibitor, and antibiofilm agents that are useful in the pharmaceutical, agricultural, biotechnology, and food industry (Saleem et al. 2015; Azman et al. 2019; Jagannathan et al. 2021). These metabolites have potential in combating biofilms associated with food industry.

In a previous study conducted by Mulya and Waturangi (2021), Actinomycetes crude extracts from isolates 12 AC, SW03, and KP110 demonstrated anti-biofilm activity against food spoilage bacteria including, *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, and *Shewanella putrefaciens* ATCC 8071, which isolate 12 AC exhibited a remarkable inhibition activity of 89.60% against *B. cereus*, and from isolate SW03 that exhibited 93.06% inhibition against *Shewanella putrefaciens*. Setting it apart from the focus on the crude extract in the last study, further investigation is required to explore the potential of Actinomycetes bioactive compound in another form as an antibiofilm agent. Thus, in this study we determined various antibiofilm activities in Actinomycetes supernatant for higher yield production and cost-effective application in food industry equipment.

MATERIALS AND METHODS

Bacterial cultivation

Six Actinomycetes that were isolated from different origins (12 AC: Ancol Beach, North Jakarta; CW02: Cunca Wulang River, West Flores; SW03, SW14, and SW16: Paddy Field at Gancahan 8 Village, Sleman; and KP110: Kulon Progo River, Yogyakarta) from previous studies were cultured on yeast malt extract agar (YMEA; Oxoid) and incubated at 28°C for seven days (Andreas 2011; Vidyawan 2012). Meanwhile, food spoilage bacteria such *B. cereus* ATCC 10876 and *B. subtilis* ATCC 6633 were cultured on Luria-Bertani agar (LA; Oxoid) and incubated at 37°C for overnight. The *S. putrefaciens* ATCC 8071 was inoculated onto LA and incubated at 28°C overnight. *Chromobacterium violaceum* wild type and mutant *C. violaceum* CV026 were cultured on LA and incubated at 28°C overnight to 48 h for selection of quorum sensing indicator bacteria.

Production of supernatant

A single colony of Actinomycetes isolates was inoculated into 100 mL of tryptic soya broth (TSB; Oxoid) media supplemented with 1% (w/w) glucose (Merck). The cultures were incubated at 28ºC, 125 rpm, for seven days (Mulya and Waturangi 2021; Balachandar et al. 2018). After that, the supernatant was taken by centrifuging culture at a speed of 7000 rpm for 25 min in 50 mL of conical tubes. The supernatant was concentrated 5 × using vacuum oven at 50ºC for two days (Mulya and Waturangi 2021), then stored at -20ºC till used in the next assays.

Detection of quorum quenching activity

The quorum quenching activity of Actinomycetes isolates was detected using agar well diffusion method referred to Mulya and Waturangi (2021). *C. violaceum* wild type was inoculated into LB broth (LB; Oxoid) and incubated overnight using rotary shaker at 120 rpm until the absorbance reaches OD_{600} = 0.132. After that, 100 µL of this culture was streaked continuously in three different directions onto LA. Wells were made using sterile cork borer and as much as 100 µL of Actinomycetes supernatant was added into the wells, then incubated at 28°C for 24 h. Streptomycin 10 mg/mL was used as a positive control whereas 1% of dimethyl sulfoxide (DMSO) was used as a negative control. The activity was determined in triplicates and observed through the translucent violacein pigment around the wells.

Validation of quorum sensing inhibition

The assay was performed based on Rajivgandhi et al. (2018) method with few modifications. *C. violaceum* 026 was inoculated in LB and incubated at 28°C overnight using rotary shaker at 125 rpm before measuring their absorbance (OD_{540} = 0.1). The culture was transferred into micro vials tubes and added with Actinomycetes supernatant with a 1:1 ratio. After that, the mixture was added with 1 µmol/mL of hexanoyl homoserine lactone (HHL; Oxoid) and incubated at 28ºC overnight. After incubation, all micro vials were centrifuged at 1000 rpm speed for 20 min followed with adding 1 mL of 1% DMSO into micro vials after the supernatant was discarded. The mixture was resuspended and centrifuged again with the same condition as before to remove unwanted cells. The absorbance of the supernatant was measured at 540 nm by spectrophotometer. *C. violaceum* CV026 and HHL (100 µL/mL) without Actinomycetes supernatant were used for a positive control. This assay was performed in triplicates.

Biofilm inhibition and destruction assay

The assays were divided into two assay, which were inhibition and destruction assays based on Waturangi et al. (2016). In inhibition assay, food spoilage bacteria were inoculated into brain heart infusion broth (BHIB; Oxoid) supplemented with 2% of glucose (w/v) and incubated at 37ºC, at 150 rpm for 24 h except for *S. putrefaciens* at 28ºC. These bacterial cultures were diluted until the absorbance reaches OD_{600} = 0.132. After that, as much as 100 µL of these cultures was transferred into 96 wells of polystyrene microplate followed by adding 100 µL of

Actinomycetes supernatant and incubated overnight with the optimum temperature. In destruction assay, after the bacteria were incubated overnight, as much 100 µL of Actinomycetes supernatant was added to the well and continuously incubated overnight at the same temperature. Overnight culture of food spoilage bacteria was used as a positive control, and sterile BHIB + 2% glucose (w/v) was used as a negative control. The planktonic of food spoilage bacteria and the media was discarded, rinsed away using sterile water twice and allowed them to air-dry for about 30 min before adding 0.4% of crystal violet (w/v) for 30 min. After discard the dye, rinsed away 5 times with sterile water and allowed it to air-dry once again before adding as much as 200 µL of ethanol. The absorbances was measured at 595 nm microplate reader (TECAN M200 PRO). This assay was performed in triplicates. The percentage of both assays was calculated using the formula:

$$\% \text{ inhibition or destruction} = \frac{\text{OD positive control - OD sample}}{\text{OD positive control}} \text{x } 100\%$$

Observation of biofilm structure

Several biofilm structures from the highest activities of inhibition and destruction biofilm assays were observed by light microscope. Later scanning electron microscopy (SEM) was observed for the highest inhibition and destruction towards the biofilm of two food spoilage bacteria, combined with energy dispersive spectroscopy (EDS) analysis for the destruction assay. The preparation of light microscopy observation was conducted with the similar steps as biofilm inhibition and destruction assay with 0.4% crystal violet staining except the biofilm was grown out on the sterile cover glass and samples can be seen in 40x magnification after rinsing the dye (Tram et al. 2013).

SEM observation was prepared by gently rinsing the grown biofilm on cover glass using sterile distilled water after incubation, and later fixated in 2% (w/v) glutaraldehyde at 4ºC overnight. The cover glass was carefully dehydrated the next day with alcohol in concentrations of 30% (v/v), 50% (v/v), 70% (v/v), 96% (v/v), and 100% (v/v) for 15 min each. Next, the cover glass was dried in the incubator at 37ºC for 10 min (Bucher et al. 2016). Then, the samples were coated with gold (Au) and observed under SEM with EDS feature at BRIN (Badan Riset dan Inovasi Nasional), Tangerang, Indonesia.

Molecular identification of bacteria

The total genomic DNA of each isolate was prepared by using Zymobiomics miniprep Kit. Furthermore, the 16S rRNA gene was amplified using forward IDX primer 63F (5´-CAGGCCCACTAAGTCATGCAA-3´) and IDX reverse primer 1387R (5´-GGGCGGGTAWGTCAAGGC-3´) (Sipriyadi et al. 2016). The PCR reaction was contained of 12.5 µL of GoTaq, 10 mM of each primer, 200 ng/µL of DNA template and top up with ddH_2O up to 25 µL. PCR condition was set to preheat at 94ºC for 2 min, followed by 25 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, elongation at 72ºC for 1 min and the final extension at 72ºC for 20 min. The PCR product was visualized using 0.7% agarose gel electrophoresis for 60 min, 90V before submitted to Genetika Science, Tangerang for DNA sequencing. Data analysis was performed using Seq Trace and aligned using database from GenBank followed by submission to NCBI GenBank.

RESULTS AND DISCUSSION

Detection of quorum quenching activity

The supernatants of the six Actinomycetes isolates (12 AC, CW02, SW03, SW14, SW16, and KP110) gave positive results for having quorum quenching activity against *C. violaceum* wild type, as indicated by the formation of clear zones around the wells, with respective diameters of 2 cm (12 AC), 1.3 cm (CW02), 1.5 cm (SW03), 1.8 cm (SW14), 2 cm (SW16), and 1.5 cm (KP110), which demonstrated effective inhibition of violacein pigment production (Figure 1).

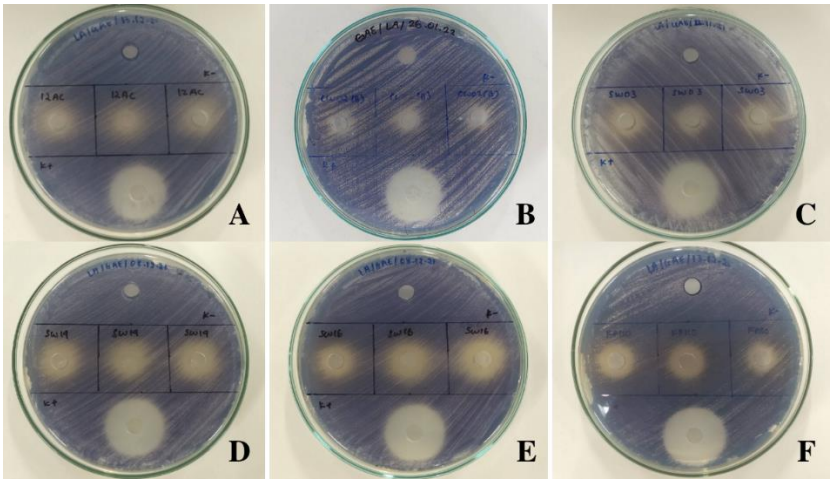

Figure 1. Anti-QS activity of Actinomycetes supernatant in agar well method. A. isolate 12 AC, B. isolate CW02, C. isolate SW03, D. isolate SW14, E. isolate SW16, F. isolate KP110

Validation of quorum sensing inhibition

According to the result, Actinomycetes supernatant showed QS inhibition indicated by the lower absorbance compare with positive control, which was untreated CV026 culture (Figure 2). Supernatants from SW03, SW14, and SW16 performed the highest quorum sensing inhibition with absorbance differences of 0.388, 0.39, and 0.404 compared with control, respectively (Figure 2).

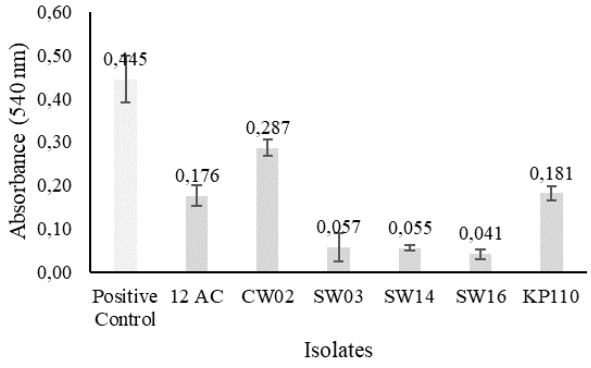

Figure 2. Validation of anti-QS activity of Actinomycetes supernatant using CV026 as bacteria indicator

Biofilm inhibition and destruction assay

Based on the quantification of antibiofilm activity, the supernatant showed the ability to inhibit and destruct mature biofilm from the three pathogens used in this study. KP110 supernatant had the highest activity in inhibiting *B. cereus* and *B. subtilis* biofilms, while SW14 supernatant had the highest inhibition activity on *S. putrefaciens* biofilm formation (Table 1). It was also found that SW14 had the highest destructive activity on *B. cereus* and *B. subtilis* biofilms. In addition, SW03 supernatant appeared to have the highest activity in knocking down *S. putrefaciens* biofilm (Table 1).

Observation of biofilm structure

Light microscopy observation of the biofilm structure was conducted on several isolates which performed high activities. The addition of KP110 and CW02 supernatants on *B. cereus* showed positive biofilm inhibition compared with untreated one (Figure 3). KP110 and SW03 supernatant treatments are also able to inhibit *B. subtilis* and *S. putrefaciens* biofilm, respectively (Figure 3). The addition of SW14 had succeeded in destructing the mature biofilm of *B. subtilis* and *B. subtilis* along with SW03 supernatant towards the biofilm structure of *S. putrefaciens* and *B. subtilis* (Figure 4).

Table 1. Inhibition and destruction activity against *B. cereus*, *B. subtilis*, and *S. putrefaciens* biofilm

Isolates	*B. cereus*		*B. subtilis*		*S. putrefaciens*	
	% Inhibition	% Destruction	% Inhibition	% Destruction	% Inhibition	% Destruction
12 AC	72.47 ± 3.52	67.33 ± 3.55	34.72 ± 3.62	47.13 ± 1.37	59.23 ± 4.11	32.93 ± 7.53
CW02	78.90 ± 2.85 [a]	54.11 ± 2.65	47.41 ± 3.36	59.65 ± 6.29	23.59 ± 1.14	56.98 ± 7.24
SW03	70.43 ± 2.98	41.68 ± 4.17	46.85 ± 1.27	87.80 ± 1.06 [a]	41.22 ± 5.24	63.71 ± 5.58 [a]
SW14	78.31 ± 5.05	77.28 ± 2.44 [a]	41.49 ± 2.70	88.34 ± 1.80 [a]	61.90 ± 4.10 [a]	48.61 ± 4.31
SW16	78.09 ± 3.15	70.28 ± 4.27	53.38 ± 3.18	77.17 ± 2.57	59.30 ± 3.67	31.56 ± 5.03
KP110	82.29 ± 1.95 [a]	52.78 ± 6.79	59.89 ± 4.20 [a]	79.62 ± 0.38	24.74 ± 3.59	46.72 ± 4.89

Note: [a]The selected activities for biofilm observation through light microscope

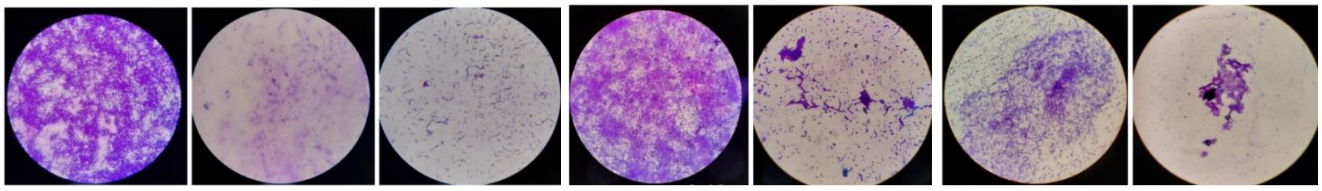

Figure 2. The biofilm structure of. A. *B. cereus* control (left), KP110 (middle), and CW02 (right) after treatment. B. *B. subtilis* control (left) and KP110 (right) after treatment; C. *S. putrefaciens* control (left) and SW14 (right) after treatment in inhibition assay through light microscope

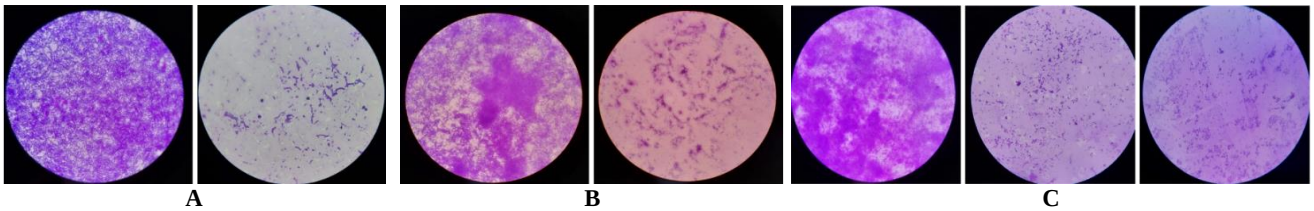

Figure 3. The biofilm structure of: A. *B. cereus* control (left) and SW14 (right) after treatment; B. *S. putrefaciens* control (left) and SW03 (right) after treatment; C. *B. subtilis* control (left), SW03 (middle), and SW14 after treatment in destruction assay through light microscope

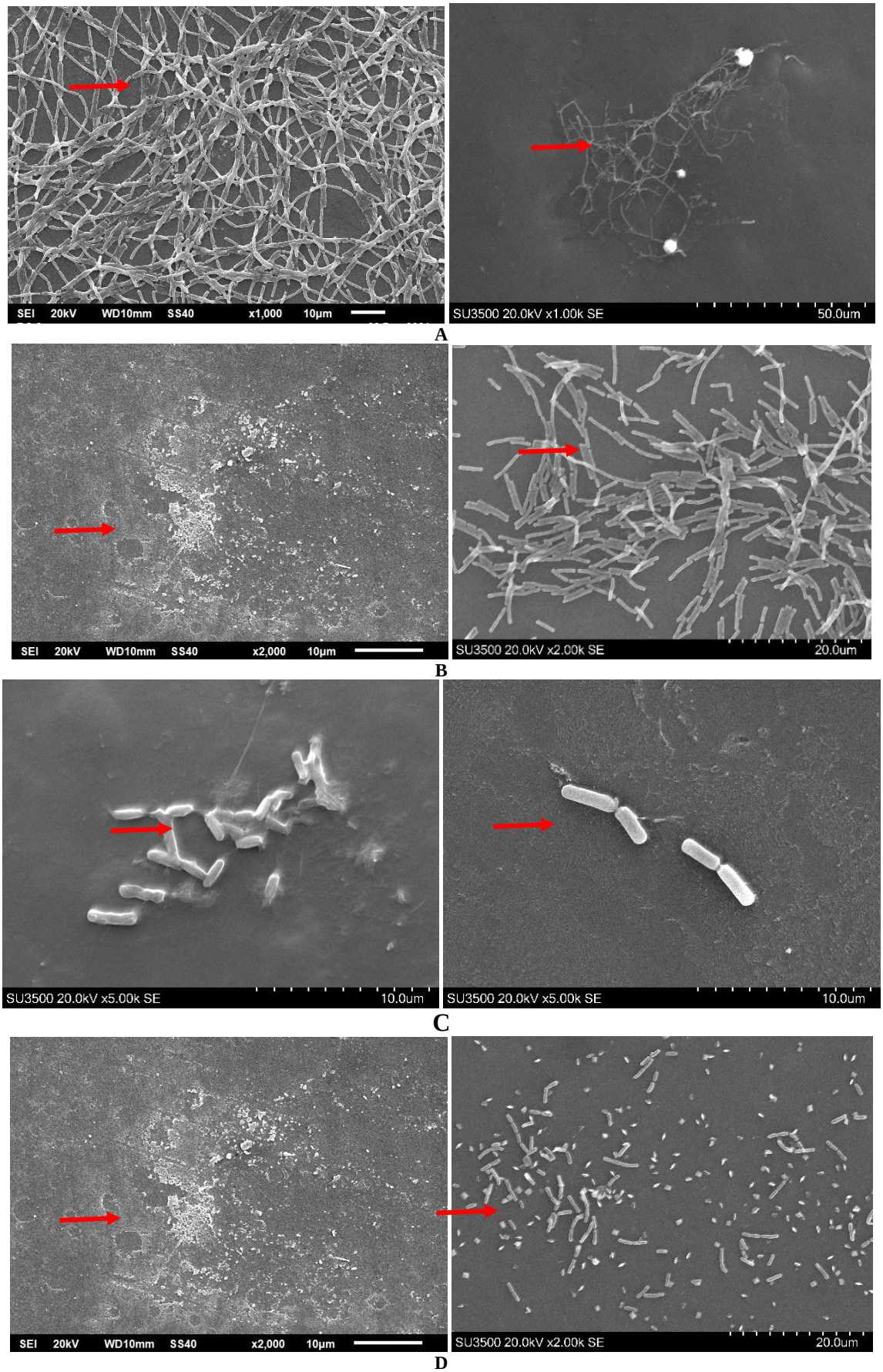

Figure 5. The biofilm structure in (a-b) inhibition and (c-d) destruction assay through SEM. (a) *B. cereus* control (left) and inhibited with KP110 (right). (b) *B. subtilis* control (left) and inhibited with KP110 (right). (c) *B. cereus* control (left) and destructed with SW14 (right). (d) *B. subtilis* control (left) and destructed with SW14

Table 2. Biofilm element composition detected by EDS

Component (% weight)	Bacteria			
	B. cereus	***B. cereus* + SW14**	***B. subtilis***	***B. subtilis* + SW14**
C	10.29	8.89	13.20	39.38
N	ND	ND	ND	5.50
O	45.66	50.03	48.23	29.93
Na	5.83	6.41	6.02	3.05
Mg	1.85	0.33	1.80	1.12
Al	0.42	0.33	0.77	0.21
Si	30.50	26.14	25.92	17.3
P	ND	ND	ND	0.19
S	ND	ND	ND	0.06
K	0.20	0.18	0.29	0.14
Ca	5.25	4.73	3.78	3.12

Note: ND: Not Determined

SEM observation towards inhibition and destruction assay revealed that KP110 and SW14 supernatant which exhibited the highest activity, successfully inhibited and destroyed the biofilm surface of *B. cereus* and *B. subtilis* (Figure 5). Meanwhile, the positive control gave a thick biofilm visually (Figure 5). We selected SW14 supernatant with destruction activity of *B. cereus* and *B. subtilis* to be analyzed for EDS spectra (Table 2). It was performed that treatment with SW14 showed changing in mineral composition compare with control, and the element detected were correlated with biofilm formation. In *B. cereus* + SW14, the composition of C, Mg, Al, Si, K, and Ca showed decreased compared with control, but for O and Na showed increased (Table 2). While the O, Na, Mg, Al, Si, K, and Ca components decreased, C was increased, and N, P, and S were detected after treatment of *B. subtilis* biofilm with SW14 supernatant (Table 2).

Identification of bacteria

Molecular characterization for bacterial identification or 16S rRNA gene analysis showed that 12 AC, CW02, SW03, SW14, SW16, and KP110 were similar to *Streptomyces griseorubens* K5 (Accession number: OM004058), *S. paulus* CA01 (OL960385), *Arthrobacter* sp. (JX434841), *Streptomyces* sp. MCCB 402 (OM004066), *Streptomyces* sp. AHA8 (OM033631), and *Streptomyces matensis* R6 (OL960583) respectively. These isolates had similarity of more than 97% and were submitted to GenBank regarding their accession numbers, except for SW03, which was sequenced before (Waturangi et al. 2016).

Discussion

Naturally, biofilms are formed as an act of adaptation to its environment. In some studies, the derived exopolysaccharides from potential biofilm possess practical industrial uses. As per Gangalla et al. (2021a), exopolysaccharides from *Bacillus aerophilus* rk1 demonstrates potential as an antioxidant, while exopolysaccharides from *B. amyloliquefaciens* RK3 exhibits medicinal potential by showing anti-Alzheimer's disease effects in mouse models (Gangalla et al. 2021b). In spite of the fact, biofilm formation by food spoilage bacteria in the food industry can often cause high economic loss and trigger outbreaks that impact public health. Biofilm is formed in a complex structure as a strategy for self-defense against chemical cleaning agents, often used to clean industrial equipment (Giaouris et al. 2014). It is also known that microorganisms in biofilms can communicate and cooperate through quorum sensing, leading to horizontal gene transfer and making them more difficult to eradicate (Giaouris et al. 2014). One of the natural metabolites known to fight against these problems comes from Actinomycetes. They are Gram-positive bacteria with high G+C content in their DNA, generally form spores, and can be found in soil or marine environments (Azman et al. 2019). Many Actinomycetes are explored for their bioactive compound in search of beneficial products such as novel antibiotics, bioremediation agents, anti-quorum sensing and antibiofilm agents that are useful in the pharmaceutical, biotechnology, and food industry (Jagannathan et al. 2021; Azman et al. 2019). In this study, Actinomycetes supernatant was obtained from six isolates, 12 AC, CW02, SW013, SW14, SW16, and KP110, to quantify their antibiofilm activities against *B. cereus*, *B. subtilis*, and *S. putrefaciens*.

Six Actinomycetes supernatants showed quorum quenching activity, indicated by a clear zone around the well. These supernatants have pigment inhibition against violacein pigment produced by *C. violaceum* wild type that comes from the initiation of gene expression on the *vio* operon mediated by the quorum-sensing mechanism (Abinaya et al. 2023). The quorum quenching activity of six Actinomycetes isolates supernatant was later validated using *C. violaceum* mutant 026 as indicator bacteria. Mutant 026 has autoinducer synthase dysfunction from the insertion *cvi*I gene by Tn5 double transposon, which requires an additional autoinducer (HHL) as a quorum sensing signaling compound to produce violacein pigment (Kothari et al. 2017). The higher QS occurs, the higher value of violacein pigment absorbance (540 nm) is detected, and vice versa. The supernatants of the six Actinomycetes isolates had QS inhibitory activity (Figure 1). Anti-QS mechanisms are mainly divided into four types based on their target: obstructing signal synthesis in the cell by inactivation of the protein signal synthase, inhibiting the QS signal transport, degrading signal molecules by enzymatic reaction, and interfering with the signal receptor by using analog molecules as competitors to prevent the receptor from binding with the real ones (Köse-Mutlu et al. 2019). According to this result, it can be stated that Actinomycetes isolates have antibiofilm activity because of their modulating inhibition mechanism of QS.

Antibiofilm activity assays were divided into biofilm inhibition and destruction test Isolates have inhibitory and destructive biofilm activity of three pathogens used in this study (Table 1). Several strategies of biofilm inhibition come from inhibiting cell attachment to the surface, disrupting the QS system or other signaling pathways for biofilm expression, and hindering biofilm maturation (Ghosh et al. 2020). Meanwhile, the destruction activity is generally in the form of degradation of the extracellular polymeric substances (EPS). The inhibition and destruction

activity of the six isolates supernatant varied between pathogens (Table 1). This may happen because molecular regulations of biofilm are diverse and specific between different species and strains of bacteria (Hall and Mah 2017). It is also found that biofilm structure is influenced by its nutritional content in its surroundings and the substances it utilizes. This is because nutrient metabolism depends on the availability and activity of enzymes, and an increased nutrient intake can lead high levels of biofilm formation (Sharan et al. 2022). Therefore, the antibiofilm activity of bioactive compounds in supernatants might work differently for each biofilm formed (Hall and Mah 2017).

Bacillus cereus and *B. subtilis* are Gram-positive bacteria that utilize autoinducer peptide or AIP as QS signal to form biofilm. In *B. cereus*, PlcR, PapR, and NprB proteins are required forming biofilm, and the EPS components consist of specific extracellular DNA (eDNA), exopolysaccharide, and protein (TasA) (Majed et al. 2016). The biofilm-forming mechanism of *B. subtilis* is regulated by set an ATP-binding cassette ComQXPA complex that leads to protein phosphorylation to initiate biofilm expression (Kalamara et al. 2018). The matrix of *B. subtilis* biofilm consists of exopolysaccharides, TasA, TapA, and BslA proteins (Majed et al. 2016). Furthermore, *S. putrefaciens* are Gram-negative bacteria that use AI-2 as autoinducer and *bpfA* operon to form biofilm with their EPS are mainly composed of protein rather than polysaccharides (Castro et al. 2017; Cheng et al. 2017).

The main bioactive compound of antibiofilm is derived from polysaccharides, proteins, and nucleic acid components (Waturangi et al. 2016). Many studies found that the antibiofilm compounds of Actinomycetes contain polysaccharides that act as surfactant agents as they alter the binding interactions between cells and cells to the surface by modifying their physicochemical properties (Waturangi et al. 2016; Dos Santos Goncalves et al. 2014). Other strategies may include interfering with biosynthesis or assembly of cell attachment factors (Asadi et al. 2019). The extracellular polysaccharides produced by Actinomycetes, particularly in the form of hyaluronic acid-like polysaccharides, may have the potential to serve as anti-adhesive coating agents on industrial equipment. This coating can help prevent bacterial adhesion, which is one of the initial phases of biofilm formation (Champion et al. 2022).

Some Actinomycetes can also produce eDNA, inhibiting initial biofilm formation by binding to the cell's attachment structure to avoid adhering to the surface (Raissa et al. 2020). Thus, they often act as anti-adhesive agents that inhibit biofilm formation (Dos Santos Goncalves et al. 2014). Extracellular enzymes from *Streptomyces* sp. play a role in degrading matrix polymers such as proteins and polysaccharides from biofilm structure (Waturangi et al. 2016; Azman et al. 2019). In addition, antibiofilm agents can also be obtained from crude extracts. The activity tends to be higher than the supernatant because the extract requires a solvent extraction step which increases the purity and usually higher the activities (Mulya and Waturangi 2021). However, some supernatants also had higher activity due to the possibility of the bioactive components from crude extract being carried away by the solvent during the extraction process (Truong et al. 2019). Further identification of the antibiofilm activity from Actinomycetes supernatants was conducted. Several isolates with high antibiofilm activity were then selected and observed using light microscope and SEM analysis with EDS feature. Untreated biofilm displayed a dense and thick appearance, while adding supernatant enabled visualization of dispersed biofilm. SEM results also confirmed that the addition of supernatant can inhibit biofilm formation, resulting in a more scattered after the treatment compared with the control (Figure 2).

The EDS results from the biofilm destruction assay were done to analyze the difference of elements after the treatment. Some elements may be correlated in forming biofilms, either in the early stages or in the maturation and dispersion stages which often require several metals to play a role (Wille et al. 2020). Those correlated elements include C, O, Na, Mg, Al, K, and Ca. C and O are versatile and contribute to forming the backbone of EPS structures such as polysaccharides. The lack of oxygen concentration may also activate the formation of biofilms (Thomas and Fey 2017). Na in the forms of sodium chloride and sodium pyruvate can help the attachment and formation of aggregates in some bacteria (Philips et al. 2017). In small amounts, Mg can play a role in initial attachment, structure development, and biofilm stability (Demishtein et al. 2019). The role of Al (III) is most often at the attachment phase through charge neutralization, precipitation, and promotion of hydrophobic proteins in assisting the initiation of biofilm formation (Cui et al. 2018). It is also stated that bacteria may utilize potassium ion channels as intracellular communication pathways (QS) (Prindle et al. 2015). Ca element also helps biofilm stability in *B. subtilis* by strengthening exopolysaccharide polymers and stabilizing the expression of biofilm matrix-forming genes (Nishikawa and Kobayashi 2021). Elements such as Si may not be correlated with biofilm but unintentionally detected by EDS, for it may be one of the materials from the cover glass.

Molecular identification of the six Actinomycetes isolates was conducted to determine the species of the isolates through DNA sequencing of 16S rRNA gene. The five isolates belong to the genus *Streptomyces* which are the largest genus of Actinobacteria (Law et al. 2019). It is found that *S. griseorubens* can synthesize silver nanoparticles (AgNPs) as an antibiofilm agent against biofilm formation in *Staphylococcus aureus* and *Pseudomonas aeruginosa* under microplate biofilm assay (Baygar and Ugur 2017). The *Arthrobacter* sp. supernatant is also known to contain extracellular amylase and protease activity that can eradicate *S. aureus* and *P. aeruginosa* biofilms (Solihin et al. 2021). *Streptomyces matensis* produces Laminaripentaose-production β-1,3-glucanase (LPHase), a glycoside hydrolase family that cleaves beta-1,3-glucan polysaccharides into pentasaccharide fragments (Woo et al. 2014). LPHase and amylase are known to be able to hydrolyze the polysaccharide backbone of EPS (Miller et al. 2022). Unfortunately, the exploration of

antibiofilm activity for *Streptomyces paulus*, *Streptomyces* sp. strain MCCB 402, and strain AHA8 remains unknown. However, as Pusparajah et al. (2021) stated, supernatant from *Streptomyces* sp. is known to have protease activity that can inhibit the formation and attachment of biofilm of *S. aureus*.

In conclusion, Actinomycetes supernatants examined in this study exhibited significant antibiofilm activity, as demonstrated by their ability to modulate biofilm formation through anti-quorum sensing mechanisms. Furthermore, these supernatants displayed varying percentages of inhibition and destruction activity against biofilm associated with food spoilage bacteria. Given their promising antibiofilm properties and ease of production, they hold considerable potential for application in the food industry, offering a potential solution to high-cost challenges. To elucidate the precise components responsible for the antibiofilm effect, further characterization and identification of the antibiofilm profile are essential. Additionally, conducting toxicity assays is imperative to ensure the safety of these agents for downstream food processing applications.

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