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Antibiofilm activities of crude extract and supernatant of Actinobacteria against biofilm formed by foodborne pathogens and food spoilage bacteria

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Abstract

Bacteria form a biofilm that attaches the microbial population to a solid surface, thus acting as a barrier to protect external stresses from the bacterial community. In the food industry, biofilms are believed to be responsible for food-borne diseases and food spoilages. This research was conducted to characterize anti-quorum sensing (QS), anti-biofilm activity of crude extract, and supernatant from Actinobacteria isolates against biofilm of foodborne, and food spoilage bacteria. Actinobacteria is the group of filamentous spore-forming Gram-positive bacteria, which is well recognized as a source for novel secondary metabolites, such as anti-biofilm and anti-quorum sensing. Two isolates of Actinobacteria utilized in this study were recovered from the Indonesian marine environment in the previous study. Both isolates demonstrated anti-QS activity on early detection assay using the agar overlay method. The Actinobacteria isolates were fermented, and the crude extracts were obtained through extraction using 100 % ethyl acetate and further evaporation, while the supernatant was collected directly following fermentation. The entire Actinobacteria isolates displayed capability as an anti-biofilm agent in crude extracts form at a concentration of 20 $mg.mL^{-1}$, alongside when in its supernatant form. The Actinobacteria isolates in both extracts and supernatants form demonstrated anti-QS activity based on the *N*-Hexanoyl-L- homoserine lactone (HHL) validation assay and were also categorized as non-toxic based on Brine Shrimp Lethality Assay (BSLA).

Introduction

In nature, bacteria grow in polymicrobial communities that coexisted with other species. Thus, bacterial communication between the community members is crucial for bacterial survival in their environments. It has been established that a form of bacterial communication mechanism called quorum sensing (QS) utilizes a unique small diffusible signal generally called autoinducers. Bacterial cells are capable of sensing their population size through QS mechanism. Then, the produced signals affect some gene expression which controls several phenomena, such as virulence, luminescence, motility, sporulation, and biofilm formation (Gilan and Sivan 2013).

With regard to survival strategy, including in the food industry, bacteria form a complex architecture called biofilm that adheres the microbial population to a solid surface such as rubber, polypropylene, plastic, glass, stainless steel, and even food products by exopolysaccharides, proteins, and

nucleic acids. They then perform as a barrier to a bacterial group to protect stresses around the bacterial community. Hence, biofilms are believed to be responsible for contributing to equipment damages, energy costs increase, foodborne disease outbreaks, as well as food spoilage (Alvarez *et al.* 2012). Inhibition of the QS system or known as quorum quenching (QQ) is potentially an alternative way of treating foodborne diseases and food spoilages related to biofilm formation.

A lot of researchers were performed to discover new compounds which have QSI (Quorum Sensing Inhibitory) properties. Various types of QSI compounds have been derived from organic resources, including bacteria, fungi, algae, and plant extracts. Then, these substances subsequently affect microbial communication by preventing biofilm formation or biofilm degradation, so simple drugs or physical treatments could kill the pathogenic bacteria easily. Currently, there are interests in exploring non-toxic biologically active compounds capable of inhibiting biofilms. One of the attractive bioresources of novel bioactive compounds is Actinobacteria. This group of bacteria is the group of filamentous spore-forming Gram-positive bacteria, well-recognized as a source for novel secondary metabolites. They variously produce potential antibiotics, enzymes, enzyme inhibitors, and pharmacological agents (Barka *et al.* 2016). Numerous reports, including our latest research (Mulya and Waturangi 2021) revealed that Actinobacteria produces anti-biofilm agents against numerous pathogenic and food spoilage bacteria; however, research on Actinobacteria and their application to control biofilm formed by foodborne, as well as food spoilage bacteria from Indonesian resources is still inadequate thus far. The present study was carried out for *Bacillus cereus*, *Bacillus substilis*, *Shewanella putrefaciens*, *Vibrio cholerae*, and enterohemorrhagic *Escherichia coli* (EHEC). These bacteria are common culprit for foodborne pathogen and food spoilage. The objectives of this research were to characterize anti-quorum sensing and anti-biofilm activity of compounds from Actinobacteria isolates (crude extract and supernatant) against foodborne and food spoilage bacteria, quantification, microscopy determination, and toxicity determination.

Experimental

Growth of Actinobacteria

Two Actinobacteria isolates utilized in this research were obtained from the previous study (Andreas 2011; Vidyawan 2012) stored in Atma Jaya Culture Collection at -80 ºC. Two Actinobacteria were selected for this study were 1AC and 18PM based on their high activity from previous study (Mulya and Waturangi 2021). Each isolate from the cryopreservation was streaked onto selective medium agar Yeast Malt Extract Agar (YMEA; Oxoid) supplemented with 1 % of $CaCO_3$ using the T-streak method then incubated at 28 ºC for 7 d (Waturangi *et al.* 2016).

Growth of foodborne and food spoilage bacteria

All foodborne pathogens and food spoilage bacteria subjected in this research were *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, enterohemorrhagic *E. coli* (EHEC) ATCC 43890, *Vibrio cholerae* ATCC 14035, and *Shewanella putrefaciens* ATCC 8071. These bacteria were obtained from Atma Jaya Culture Collection. The *S. putrefaciens* was streaked onto Luria Agar (LA; Oxoid) using the T-Streak method then incubated at 28 ºC overnight. The remaining four pathogens were incubated at 37 ºC overnight using the same medium (Waturangi *et al.* 2016).

Growth of quorum sensing indicator bacteria

Chromobacterium violaceum wild type and mutant *Chromobacterium violaceum* CV026 used as strain indicator were obtained from Atma Jaya Culture Collection. Each bacteria was streaked onto LA using the T-Streak method, then incubated at 28 ºC overnight to 2 d (Waturangi *et al.* 2016).

Screening of anti-quorum sensing activity against C. violaceum

This assay was conducted using the agar overlay detection assay. Both Actinobacteria isolates were streaked using the straight-streak method onto LA then incubated at 28 °C for 3 d. Meanwhile, *C. violaceum* wild type was inoculated separately

in Luria Broth (LB; Oxoid) then incubated at 28 ºC overnight. On the third day, *C. violaceum* broth was diluted using semisolid agar (0.75 % w/v) until OD_{600} = 0.132 then 5 mL of the semisolid agar was poured onto LA plates as an agar overlay above the Actinobacteria isolates. These plates were then incubated at 28 °C for 2 d. This screening assay was tested in triplicates. Positive results indicating anti-quorum sensing were observed as an opaque zone near Actinobacteria isolates caused by violacein pigment production inhibition (non-purple zone). Whereas, negative results were seen as a purple colony (Abudoleh and Mahasneh 2017).

Production and extraction of bioactive compound

Both Actinobacteria isolates were inoculated into 100 mL Tryptone Soya Broth (TSB; Oxoid) supplemented with 1 % of glucose (w/v) for carbohydrate aerobic fermentation process. These cultures were then incubated at 28 ºC, 125 rpm speed, for 7 d. Following the fermentation process, these cultures were centrifuged at 7,000 × g speed for 25 min using the 50 mL of conical tubes. Supernatant samples were collected and stored in -20 ºC until used in the next assays. The remaining supernatants were extracted using liquid-liquid extraction with ethyl acetate. The supernatants were added with 100 % ethyl acetate in a 1 : 1 ratio, then shaken at 125 rpm speed overnight. Then, the upper (solvent) phase was collected, evaporated using the rotary evaporator, and dried using the vacuum oven at 50 ºC until crystals were formed. These extracts were then weighted, resuspended with 1 % of dimethyl sulphoxide (DMSO; v/v) until it yields the final concentration of 20 $mg.mL^{-1}$, then kept in -20 ºC for further assays (Hassan *et al.* 2016; Waturangi *et al.* 2016; Leetanasaksakul and Thamchaipenet 2018).

Quantification of anti-biofilm activity

This anti-biofilm assay was divided into two assays: inhibition of biofilm formation and destruction of the biofilm itself (Hassan *et al.* 2016; Waturangi *et al.* 2016).

Inhibition biofilm assay

Five food pathogenic bacteria were inoculated onto Brain Heart Infusion Broth (BHIB; Oxoid) supplemented with 2 % of glucose (w/v) and incubated at 28 ºC for *S. putrefaciens* and 37 °C for the other four bacteria, at 150 rpm speed overnight. The following day, these bacterial cultures were diluted until their absorbance reached OD_{600} = 0.132. Furthermore, 100 µL of these suspensions were transferred into 96 wells of the polystyrene microplate, followed by the addition of 100 µL of Actinobacteria supernatant or extract. Microplates were then incubated overnight with the optimum temperature based on each bacterium.

Destruction biofilm assay

Following the confirmation that the bacterial culture absorbance had reached OD_{600} = 0.132, 100 µL of these suspensions were transferred into another 96 wells of polystyrene microplate and incubated overnight with the optimum temperature based on each bacteria for mature biofilm formed and attached to the well. The following day, 100 µL of Actinobacteria supernatant and extract was added to the well and re-incubated overnight at their corresponding temperature.

Measurement after incubation

Planktonic cells and supernatant were discarded, the attached cells (biofilm) were gently rinsed twice with sterile distilled water, then allowed to air-dry for 30 min. Afterwards, the biofilm was stained using 0.4 % of crystal violet (w/v) for 30 min. The dye was then discarded, washed with sterile distilled water fivefold, and allowed to air-dry before solubilization with 200 µL of ethanol. These absorbances were measured at 595 nm microplate reader (TECAN M200 PRO). Overnight culture of pathogens was used as a positive control, while sterile BHIB medium was used as the negative control. This assay was performed in triplicates. The percentage of biofilm inhibition or destruction activity was calculated using the Eq. 1 below:

$$\% \text{ inhibition or destruction} = \frac{\text{OD positive control} - \text{OD sample}}{\text{OD positive control}} \text{ x } 100\ \% \quad (1)$$

Microscopic determination

Selected biofilm destruction treatments were determined using Scanning Electron Microscope (SEM) at BRIN, Bogor, Indonesia. Biofilm of food pathogenic bacteria was grown with the same conditions as the biofilm destruction assay. Selected food pathogenic bacteria were grown overnight onto BHIB supplemented with 2 % of glucose (w/v) at their corresponding growth temperature, then these cultures were diluted until their absorbance reached OD_{600} = 0.132. Subsequently, the biofilm was allowed to grow in a sterile cover glass overnight. The following day, the biofilm was treated with selected Actinobacteria extract or supernatant by spotting 100 µL of these compounds onto the cover glass, then re-incubated overnight. Following the incubation, the cover glass was gently rinsed with sterile distilled water then fixated in 2 % of glutaraldehyde (w/v) at 4 ^{o}C overnight. The next day, the cover glass was carefully dehydrated with the following steps: 5 min in sterile distilled water, 15 min in 30 % of alcohol (v/v), 15 min in 50 % of alcohol (v/v), 15 min in 70 % of alcohol (v/v), and finally 15 min in 96 % of alcohol (v/v). Afterwards, the cover glass was dried in the incubator at 37 ^{o}C for 30 min. The specimen was then coated with gold (Au), and the surface structure of biofilm was observed under SEM (Bucher *et al*. 2016; Coraça-Huber *et al*. 2020).

Validation of anti-biofilm activity

C. violaceum CV026 was inoculated onto LB, incubated for 28 ^{o}C overnight, then diluted until their absorbance reached OD_{600} = 0.132. This culture was then transferred into vial tubes and added with Actinobacteria supernatant or extract with a 1 : 1 ratio. Lastly, the mixture was added with 1 µmol.mL^{-1} of *N*-Hexanoyl-L-homoserine lactone (HHL; Oxoid) and incubated at 28 ^{o}C overnight. The following day, the vial tubes were centrifuged at 123 × g speed for 15 min. The supernatant was discarded, then 1 mL 1 % of DMSO (v/v) was added, resuspended, and centrifuged again in the same conditions as prior. The supernatant was collected, and their absorbance was measured at 540 nm. The positive control that was used in this assay contained *C. violaceum* CV026 culture and HHL (100 µg.mL^{-1}) without Actinobacteria samples. Total violacein formed, then compared. This validation assay was performed in triplicates. (Rajivgandhi *et al.* 2018).

Toxicity test using Brine Shrimp Lethality Assay (BSLA)

The toxicity of Actinobacteria supernatant and extract was determined using the Brine Shrimp Lethality Assay. This assay was conducted by dissolving 1 mL of Actinobacteria supernatant and extract to make a stock solution (1,000 µg.mL^{-1}). The stock solution was then diluted into a working concentration of 125; 250; 500; and 1,000 µg.mL^{-1}. Briefly, artificial seawater was prepared by dissolving 38 g of sea salt in 1 L of distilled water for hatching of the shrimp eggs. Then, 10 mg of brine shrimp eggs (*Artemia salina*) were incubated in artificial seawater beneath a 60 W lamp, providing direct light and warmth (24 °C – 26 °C). The hatching process required two days for the shrimps to mature as nauplii (larva). On the third day, 4.5 mL of the artificial seawater was moved in test tubes, and ten nauplii were added to each tube. Then, 0.5 mL of diluted Actinobacteria samples were added to the test tubes. The control tube devoid of Actinobacteria samples was substituted by 0.5 mL of 10 mg.mL^{-1} $K_2Cr_2O_7$ for positive control, and 0.5 mL of seawater for negative control. All test tubes were then incubated overnight at ambient temperature. The amount of nauplii alive after 24 h were counted underneath a magnifying glass (Bag and Chattopadhyay 2015). The mortality percentage of brine shrimp nauplii was calculated using Eq. 2 and presented as LC_{50} (lethal concentration, 50 %). This assay was performed in triplicates.

$$\% \text{ Mortalilty} = \frac{\text{Number of nauplii died}}{\text{Number of total nauplii}} \text{ x } 100\ \% \quad (2)$$

Statistical analysis

Statistical analysis was carried out through IBM SPSS Statistics 24. The data were then analyzed using the analysis of variances (ANOVA) and the Tukey test for significant differences, which were determined at $P < 0.05$.

Results

Screening of anti-quorum sensing activity against C. violaceum

Two isolates of Actinobacteria demonstrated positive anti-quorum sensing activity against the bacterial indicator *C. violaceum* wild type. 1AC isolate was recovered from Pantai Ancol, North Jakarta, DKI Jakarta while the 18PM isolate was collected from Pantai Mutiara, North Jakarta, DKI Jakarta. The positive result was indicated by the opaque zone near the Actinobacteria isolates caused by violacein pigment production inhibition (non-purple zone) (Fig. 1).

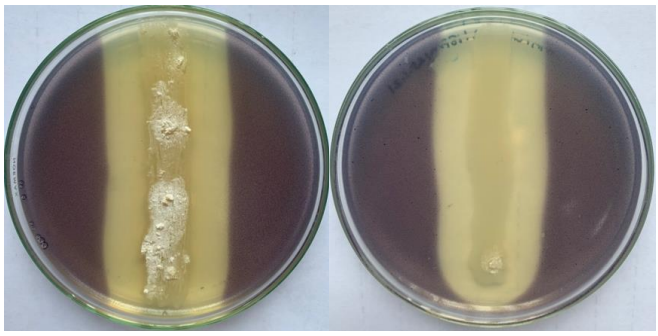

Fig. 1. Anti-quorum sensing activity of Actinobacteria. (**A**) 1AC isolate and (**B**) 18PM isolate.

Quantification of anti-biofilm activity

To determine the spectrum of Actinobacteria extracts and supernatants as anti-biofilm agents, all Actinobacteria samples were tested against biofilm formed by foodborne pathogens and food spoilage bacteria. In reflection to the test results, all Actinobacteria samples were capable of inhibiting and destructing the biofilm formed by tested bacteria (Fig. 2). These results demonstrated that 1AC isolate was capable in preventing biofilm formation and destructing mature biofilm, with the highest activities are both against *B. subtilis* using the crude extract (Fig. 2A and 2B). Contrarily, 18PM isolate performed the highest biofilm inhibition against *V. cholerae* by using the crude extract (Fig. 2C), and intriguingly, supernatant of 18PM isolate showed the highest activity in destructing mature biofilm against *B. subtilis* (Fig. 2D). All activity in this section was quantified using a 20 mg.mL^{-1} concentration.

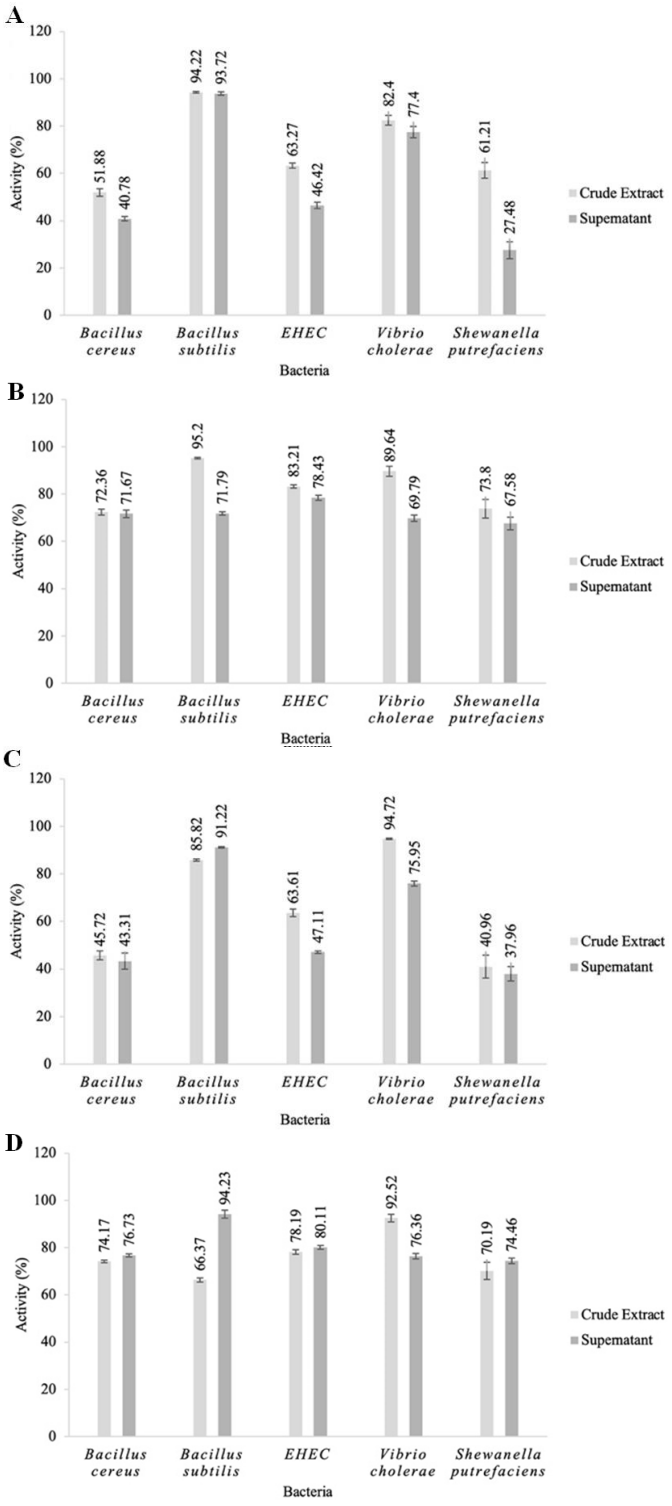

Fig. 2. Inhibition and destruction activity of Actinobacteria samples against food-borne pathogens and food spoilage bacteria. (**A**) 1AC inhibition activity, (**B**) 1AC destruction activity, (**C**) 18PM inhibition activity, and (**D**) 18PM destruction activity.

Microscopic determination using Scanning Electron Microscope (SEM)

Microscopic determination was performed on selected biofilm destruction activities, based on the highest activities of both supernatant and crude extract. The results displayed disruption activity of biofilm formed by both extracts and supernatants isolates. The mature biofilm of *B. subtilis* portrayed a thick 3D structure that adheres with the cover glass. Then the treatment utilizing Actinobacteria compounds demonstrated the ability to disrupt this structure (Fig. 3).

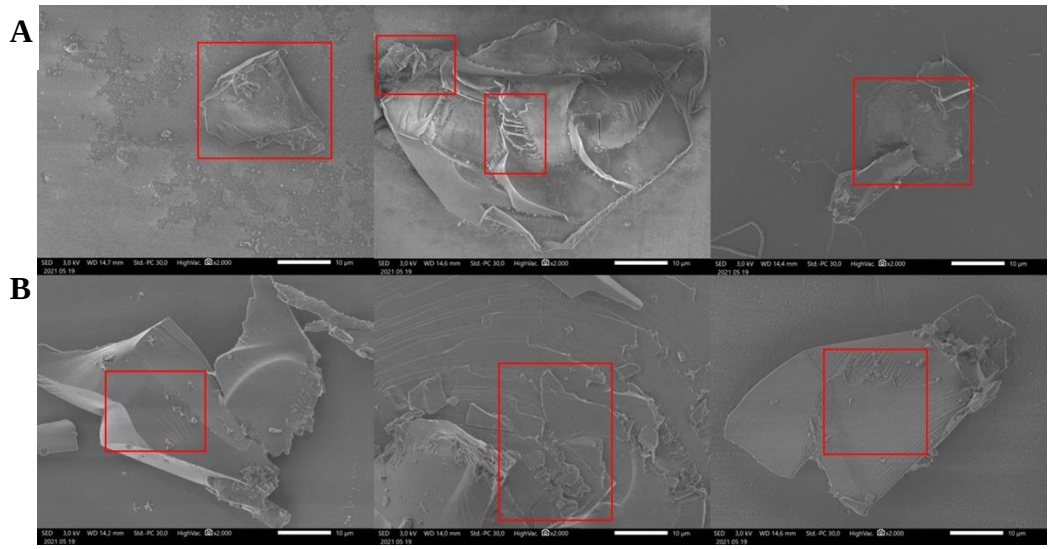

Fig. 3. Biofilm on SEM. **A** – *B. subtilis* biofilms (left to right: untreated biofilm, treated with 1AC extracts, treated with 18PM supernatants); **B** – *V. cholerae* biofilms (left to right: untreated biofilm, treated with 18PM extracts, treated with 1AC supernatants).

Validation of anti-biofilm activity

It is necessary to identify the anti-biofilm mechanism displayed by Actinobacteria. All Actinobacteria isolates demonstrated quorum quenching activity indicated by lower absorbance in comparison to control (Fig. 4). Extract of 1AC isolate performed the highest quorum quenching activity against *C. violaceum* CV026 by 0.471 difference in absorbance.

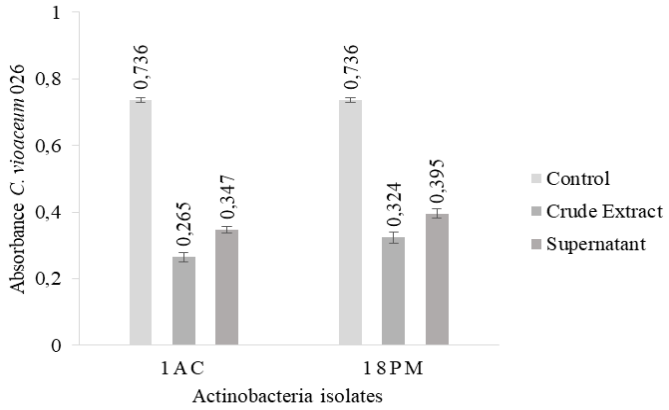

Fig. 4. Validation of anti-quorum sensing.

Toxicity test using Brine Shrimp Lethality Assay (BSLA)

The brine shrimp lethality assay is considered a useful tool for the preliminary assessment of toxicity. Actinobacteria samples were tested for toxicity properties by determining the value of LC_{50}. The results revealed that all Actinobacteria crude extracts and supernatants were considered non-toxic because the value of LC_{50} exceeds 1000 $\mu g.mL^{-1}$ (Table 1).

Table 1. Toxicity of Actinobacteria sample by BSLA.

Isolate	Sample	LC_{50} [$\mu g.mL^{-1}$]
1AC	Extract	2324.5792
	Supernatant	All nauplii survive
18PM	Extract	2366.5401
	Supernatant	All nauplii survive

Discussion

Biofilm greatly jeopardize food safety and food industries. Unfortunately, food pathogenic bacteria

are able to adapt to the selective pressure from food-processing processes through the formation of biofilm. Many food industries are faced with problems including but not limited to suffering of huge losses, food spoilages, and foodborne disease outbreaks due to this. It is well documented that most biofilm formation is through the quorum sensing mechanism through a variety of signal molecules. To overcome these difficulties, identification of an anti-biofilm and/or anti-quorum sensing molecules is of paramount importance.

One of the main objectives of this study is to find unexploited bioresources as a potential candidate bioactive compound with anti-biofilm and/or anti-quorum sensing activities. Marine environments are considered as good resources to explore possible isolates, including Actinobacteria which possess bioactive compound with various properties.

Actinobacteria are Gram-positive bacteria with high G+C DNA content that constitute one of the largest bacterial phyla, and they are ubiquitously distributed in both aquatic and terrestrial ecosystems. On an agar plate, Actinobacteria characteristics displayed as rough, chalky, filamentous, while some also show pigmentation. These microorganisms also possess extensive secondary metabolism and produce approximately two-thirds of all naturally derived antibiotics in the current clinical use, as well as numerous anticancer, antihelmintic, and antifungal compounds. Importantly relating to this research, numerous Actinobacteria possess anti-biofilm and/or anti-quorum sensing properties (Barka *et al.* 2016; Hassan *et al.* 2016).

In a previous study by Mulya and Waturangi in 2021, 10 isolates of Actinobacteria were able to inhibit and destroy the biofilm formation of *B. cereus*, *B. subtilis*, and *S. putrefaciens*. Two Actinobacteria isolates were selected in this study based on their high activities from our previous studies. The isolates were 1AC and 18PM, recovered from marine environments from previous studies (Andreas 2011; Vidyawan 2012).

It is crucial carry out a primary screening of anti-quorum sensing activity from all Actinobacteria isolates. Both, the Actinobacteria isolates showed anti-quorum sensing activity on the agar overlay detection method. These isolates inhibited pigmentation produced by *C. violaceum* through quorum sensing inhibition, which was indicated by the opaque zone near the Actinobacteria isolates caused by violacein pigment production inhibition (non-purple zone) (Kothari *et al.* 2017).

In this study, the Actinobacteria isolates were cultured, then the supernatant was collected, and some were extracted using a multipolar solvent, ethyl acetate to form a crude extract. The ethyl acetate was chosen because they perform as a multipolar solvent with medium polarity and is adequately suitable for food industry due to their low toxicity. In this study, activities from both crude extract as well as supernatant for different purpose was assessed, in relation to their application in treating pathogenic or food spoilage bacteria. Supernatants are more practical and economical to be produced and applied on large surfaces such as disinfection of food equipments, while crude extracts required further purification and further assessment in regard to safety prior to application in food samples.

Referring to Fig. 2, crude extract of 1AC isolate performed higher inhibition and destruction activity against biofilm formed by foodborne pathogens and food spoilage bacteria than in supernatant form. Hence, it can be argued that the bioactive compound against food bacteria used in this research, produced by 1AC was successfully extracted using ethyl acetate. In comparison to the supernatants samples, crude extracts portrayed a higher concentration of bioactive compounds, then resulting in a higher anti-biofilm activity. 1AC isolates performed the best activity in inhibiting and destructing the biofilm of *B. subtilis*. Furthermore, 18PM isolate samples in the form of supernatant showed higher inhibition and destruction activities against biofilm of *B. subtilis* in comparison to crude extracts. This result is as initially expected as 18PM supernatants possess some bioactive compound, specifically against *B. subtilis* that was not able to be extracted with ethyl acetate, resulting in higher activity compared with crude extract.

Anti-biofilm compound operates specifically, different compounds from different isolates have different targets on parts of biofilm. Many researchers reported that some Actinobacteria producing a protease enzyme which is believed to be one of the most effective enzymes in biofilm

eradication via hydrolysis of both matrix proteins and adhesins. Likewise, DNase enzyme is capable of digesting the extracellular DNA which is present within the biofilm structure. Polysaccharide lyases also contribute in an important role in cleaving the exopolysaccharides of biofilm (Baidamshina *et al.* 2017; Roy *et al*. 2018). Some Actinobacteria also produce extracellular DNA. As an anti-biofilm compound, extracellular DNA is able to limit swarmer cell attachment by binding to the adhesive structure of the planktonic cell needed for surface attachment (Berne *et al.* 2010). Polysaccharides produced by Actinobacteria also have an important role as anti-biofilm agents. Polysaccharide operates through interference of cell-surface influence and cell-cell interactions, which is important for biofilm development, and may also lead to the dispersion of the preformed biofilm (Taj *et al.* 2014; Hassan *et al.* 2016). Reflecting from this fact, lower activity of inhibition and destruction activity yields active compound produced by the two isolates being not fully compatible with some bacteria, while contrarily this might be effective with other bacteria.

In reference to Fig. 2, it is also known that the Actinobacteria isolates have a greater ability to destruct the biofilm than inhibits the formation of the biofilm itself. Based on this fact, our Actinobacteria samples may majorly consist of bioactives that is able to destruct biofilm matrix rather than destructing QS signals. While comparing to the Gram of food bacteria, Actinobacteria samples in the form of crude extract seem to be more effective to Gram-negative food bacteria. Meanwhile, in the form of supernatants, our sample is more effective to be applied to Gram-positive food bacteria.

Further biofilm surface observation using SEM confirmed the destruction activity of Actinobacteria samples. Untreated biofilm of *B. subtilis* and *V. cholerae* is pictured as a thick, complex, and dense biofilm, whereas areas of biofilm after Actinobacteria treatments showed as disrupted biofilms (Fig. 3). Eventually, Actinobacteria extracts and supernatants used in this research were able to destruct biofilm. The samples used in this research have some anti-biofilm compounds or any other analog molecules that were able to disrupt the biofilm formation in the maturation phase or destructing even the matured biofilm itself.

To validate that our Actinobacteria sample has anti-biofilm activity and/or anti-quorum sensing activity, an anti-quorum sensing validation assay was done using a standardized AHL molecule such as HHL. This molecule was used by *C. violaceum* as a signaling molecule in quorum sensing mechanism, which is reflected by violacein production. The higher the quorum sensing process yields subsequently higher violacein production, and the higher the detected absorbance. From this assay, it is known that the compound from Actinobacteria samples not only has anti-biofilm activities, but also has anti-quorum sensing activities (Fig. 4).

Similar to the anti-biofilm compounds that work specifically, the anti-quorum sensing compound also works specifically. Our Actinobacteria might produce enzymes including AHL-lactonase, AHL-acylase, and/or paraoxonase that degrades the HHL signal and/or produce some compounds that analogs with HHL so they can competitively bind to LuxR receptor at *C. violaceum* CV026 against HHL itself (Kothari *et al.* 2017; Bundale *et al.* 2018). Apart from degrading signal molecules that play an important role in bacterial cell-to-cell communication, there have been numerous compounds that inhibit or decrease the signal molecule synthesis, thereby interfering quorum sensing. Most of the signal molecule synthesis inhibitors work by indirectly inhibiting precursor molecules, which are important for signal molecule synthesis. To illustrate, small molecule triclosan act on enoyl-ACP reductase, an important precursor of AHL synthesis, and immucillin A (ImmA) inhibits 5-MAT/S-adenosyl-homocysteine nucleosidase (MTAN), both of which are crucial for AHL and AI-2 synthesis. Finally, many small-molecule inhibitors block the binding of a signal molecule to the receptor, thereby severing the quorum sensing signaling cascade. They have been identified such as ursene triterpenes and oroidin (Singh *et al.* 2005; Peach *et al.* 2011; Narendrakumar *et al.* 2018).

For future application regarding food safety, it is crucial to assess toxicity effects of Actinobacteria. Reffering to Table 1, all of Actinobacteria crude extracts and supernatants were considered non-

toxic due to the value of LC_{50} being more than 1000 $\mu g.mL^{-1}$ (Bag and Chattopadhyay 2015). Actinobacteria supernatants did not kill any of the nauplii during the assay. This result was predicted because supernatants have a much lower bioactive concentration compared to crude extracts.

Lastly, the 1AC and 18PM isolate have been sequenced from previous study that has not been published. 1AC showed similarity with *Streptomyces griseorubens* strain NBRC 12780 (submitted GenBank accession number: MW680936) and 18PM showed similarity with *Streptomyces thermocarboxydus* strain NBRC 16323 (submitted GenBank accession number: MW680906). *Streptomyces* is the largest genus of Actinobacteria and is also isolated the most frequently (Arn *et al.* 2020). It was discovered that *Streptomyces griseorubens* was able to produce silver nanoparticles (AgNPs) extracellularly, then these nanoparticles decrease the biofilm formation of *Streptococcus aureus* and *Pseudomonas aeruginosa* in a microplate well assay (Baygar and Ugur 2017). The same nanoparticles might contribute to a role as an anti-biofilm agent in our compound. Meanwhile, *Streptomyces thermocarboxydus* was widely known for having the ability in oxidizing the toxic carbon monoxide gas into innocuous CO_2, thus lowering its atmospheric concentration to safer levels (Kim *et al.* 1998). Unfortunately, the anti-biofilm agents of *Streptomyces thermocarboxydus* were not yet identified.

Ultimately, Actinobacteria samples obtained from this research demonstrated characteristics as anti-biofilm agents against several foodborne and food spoilage bacteria used in this study, while also performing as anti-QS agents. Each component of bioactive compound works specifically by targeting parts of biofilm or inhibiting biofilm formation through disrupting of the QS mechanism. All of them were also considered non-toxic. Therefore, the Actinobacteria extracts and supernatants used in this research showed tremendous potential for application against biofilms in food and food industries respectively.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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