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Inhibition of *Burkholderia cenocepacia* H111 quorum sensing system by environmental bacterial isolates

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Abstract. *Burkholderia cenocepacia* H111 is an opportunistic pathogen which is difficult to eradicate due to its virulence factors, specifically biofilm. The ability of *B. cenocepacia* to form biofilm is regulated by quorum sensing (QS) system using a signal molecule. Environmental bacteria often showed the ability to inhibit the QS of other species because they have special and unique metabolites or enzymes. In previous research several environmental bacteria was isolated from few places in Java Island. However, until now the potential of these bacteria have not been investigated yet. This study was conducted with the aim to investigate the effect of these environmental bacterial isolates, i.e. *Bacillus* sp., *Stenotrophomonas maltophilia*, *Enterobacter ludwigii* and *Kosakonia radicincitans* on quorum sensing system of *B. cenocepacia* H111, particularly on quantities of acyl homoserine lactones (AHLs) and biofilm that were produced. The quantity of AHL molecule is measured by biosensor assay using *Escherichia coli* JM109 pSB401 while the quantity of biofilm formation was quantified by crystal violet method. The result showed that supernatants of all these environmental bacteria have no impact in decreasing of AHL nor biofilm. On the other hands, all the crude extracts showed a reduction either to AHL molecule and biofilm formation. The *Bacillus* sp. crude extract revealed the most reducing effect, with 84 % for AHL molecule and 71 % for biofilm. It can be concluded that crude extracts of all environmental bacteria have quorum quenching activity to *B. cenocepacia* H111 cells, specifically in reducing AHL levels and biofilm quantity. Crude extract from *Bacillus* sp. gave the highest inhibitory effect.

Keywords: Acyl homoserine lactones, biofilm, pSB401, quorum quenching.

1. Introduction

Burkholderia cepacia complex (Bcc) is a group of opportunistic pathogenic bacteria that causes lung tissue infections. This group includes at least 22 closely related species [1]. Infection by Bcc species can cause lung tissue dysfunction and cause death. Because of the high mortality rate, infection by this group of bacteria is called "cepacia syndrome". This infection case often occurs in Europe [2]. One of the most studied Bcc species is *Burkholderia cenocepacia* because it is found dominant in clinical isolates and patients with cystic fibrosis [3].

The difficulty in the eradication of *B. cenocepacia* from the patient's body is due to its resistance to therapeutic conventional antibiotic and its persistence in the host's body [1]. In addition, biofilm formation and virulence factors productions increase the difficulty in eliminating this bacterium. The ability of *B. cenocepacia* to infect patients is regulated by a cell communication or quorum sensing (QS) system. The QS of *B. cenocepacia* is mediated by signal molecules, i.e. N-octanoyl homoserine lactone (C8-HSL) and N-hexanoyl homoserine lactone (C6-HSL) [4] which regulate others genes



expressions, such as genes involve in biofilm formation, motility, extra-cellular protease, siderophore, and others. The formation of these virulence factors cause periodic infections and treatment difficulties [2].

The strategy in inhibiting the QS system is expected to stop the production of pathogenic virulence factors without causing the risk of resistance. This process is often referred to as quorum quenching (QQ). The molecular sources of QQ can vary both from natural and synthetic. Some researchers found that plants and bacteria, includes environmental bacteria, have inhibitory abilities in the QS system [5–9]. Bacterial populations from the environment have been shown to have special and unique metabolites or enzymes [10]. This is because the bacteria that is isolated from the environment have good adaptive mechanisms for fighting different species of bacteria.

In previous research, several environmental bacteria were isolated, i.e. *Bacillus* sp. from Gresik area, East Java, Indonesia [11], *Stenotrophomonas maltophilia* from Central Java [12, 13], *Enterobacter ludwigii* [14] and *Kosakonia radicincitans* [15] both from Surabaya area – East Java. Until now the potential of these bacteria has not been further investigated. Based on bioinformatics analysis of complete genomes of similar bacteria, these bacteria are predicted to have a gene encoding AHL acylase and/or AHL lactonase. These enzymes are known to function as quorum quencher (QQ) agents. Based on the prediction, the potential of these environmental bacteria against the *B. cenocepacia* H111 quorum sensing (QS) system was explored.

This study was conducted with the aim to investigate the effect of crude extracts of these environmental bacterial isolates on quorum sensing system of *B. cenocepacia* H111, specifically on quantities of acyl homoserine lactones (AHLs) and biofilm which are produced.

2. Materials and method

2.1. Materials

Equipments used in this study are micropipettes (0.5 to 10) μL , (10 to 100) μL and (100 to 1 000) μL (Gilson), microcentrifuge (Thermoscientific), laminar air flow cabinet (Omega), oven (Mettler), ultrasonicator (Biologic), *FLUOstar Omega Plate Reader* (BMG Labtech), autoclave (All American), shaker incubator (LM-420D), and spektrofotometer UV-Vis (Genesys), glasswares.

Microtips (100 to 1 000) μL , (10 to 100) μL , and (0.5 to 10) μL (Biologic®), filter membrane 0.2 μm , Luria-Bertani Broth (Merck®), Luria-Bertani Agar (Merck®), Simmon's Citrate Agar (Merck®), Tetracycline (Promega), Yeast Extract (Becton & Dickinson), HCl (J. T. Baker), NaCl (Merck), Ethyl Acetate (J. T. Baker), and Methanol (J. T. Baker). Bacteria used here are *B. cenocepacia* H111, *S. maltophilia*, *K. radicincitans*, *Bacillus* sp. dan *E. ludwigii* and *Escherichia coli* JM109 pSB401 [16].

2.2. Methods

2.2.1. Cultures cultivation. All cultures was stored on Luria Bertani Agar. The medium for cultivation of *E. coli* JM109 pSB401 is added with tetracycline at final concentration of 50 $\mu\text{g mL}^{-1}$.

2.2.2. Extraction of acyl homoserine lactone (AHL). *B. cenocepacia* H111 was cultured in 10 mL LB broth at 37 °C for 24 h. Culture was centrifuged at 13 000 rpm (1 rpm = 1/60 Hz) for 15 min. A volume of 900 μL of supernatant was transferred into a tube containing 100 μL of 0.1 M HCl. Acidified supernatant was then incubated at 37 °C with shaking at 250 rpm for 24 h. The AHL was extracted thoroughly from the supernatant using ethyl acetate followed by solvent evaporation [9].

2.2.3. Preparation of crude extracts of environmental bacteria isolates. Cells were refreshed overnight in LB broth at 37 °C with orbital shaking at 250 rpm. Culture was used as inoculums for 100 mL LB broth at final OD₆₀₀ ~0.01. The culture was then re-incubated until cells entered to stationary phase. Cells were harvested by centrifugation at 13 000 rpm for 10 min, followed by washing the pellet twice with 0.1 M phosphate buffer saline (PBS). Cells pellet was resuspended in PBS to concentration of 1 g cells mL⁻¹. Then the cell suspension is lysed using ultrasonicator. The translucent crude extract was separated from cells debris by centrifuging at 13 000 rpm for 30 min at 4 °C. The crude extract was carefully removed and filtered through a 0.2 μm filter membrane.

2.2.4. Quantification of *B. cenocepacia* H111 AHL. The 24 h *Escherichia coli* JM109 pSB401 culture grew in LB-tetracycline at 37 °C was diluted a tenth time with LB broth. Diluted biosensor cultures were then reincubated for 3 h at 37 °C with shaking at 250 rpm.

The quantification method of AHL molecules was performed based on Wahjudi et al. [9] with slight modifications. The AHL extracted from *B. cenocepacia* was dissolved in methanol. Each microplate well was filled with 5 µL of AHL solution and was left in fume hood until all methanol was evaporated. Dried AHL then mixed with 100 µL of 0.1 M PBS containing 10 µL of crude extracts sample. Plate was incubated at 37 °C with 100 rpm shaking for 1 h. At the end of incubation time, 150 µL of *Escherichia coli* JM109 pSB401 culture was added. The signal detection was carried on every 30 min in microplate reader at 30 °C. AHL is detected and measured based on luminescence signal intensity that was read using the FLUOStar Plate Reader.

2.2.5. Determination of *B. cenocepacia* H111 biofilm quantity. The *B. cenocepacia* biofilm was developed in 2 × TY broth. The refreshed culture was diluted with 2 × TY medium to reach a final OD₆₀₀ of 0.001. Then, 10 µL of crude extract was mixed with 100 µL of diluted *B. cenocepacia* culture in each well and was incubated at 37 °C for 48 h in static condition. Every 24 h, the biofilm were rinsed with 0.1 M PBS and renewed with a same volume of fresh sterile 2 × TY broth.

After 48 h incubation, the broth was carefully removed and the biofilm was rinsed with 0.1 M PBS. The biofilm was then fixed with 130 µL of methanol for 10 min. After the methanol was removed, the microplate was air dried, followed by staining the biofilm with 130 µL of 1 % (w/v) crystal violet solution for 30 min. The well is then rinsed with demineralized water thoroughly to rinse the crystal violet that is not attached. Crystal violet was released from the biofilm with 130 µL of destaining solution consisting of 33 % acetic acid solution. The absorbance of crystal violet solution was read at a wavelength of 590 nm. Absorbance of crystal violet is positively correlated with the thickness of the biofilm layer formed.

2.2.6 Data analyses. All the treatment were done at least in triplicate. As independent variable in this study is the type of crude extract added to *B. cenocepacia* H111 culture. Crude extracts were originated from *S. maltophilia*, *K. radicincitans*, *Bacillus* sp., and *E. ludwigii*. The dependent variable are AHL concentration and biofilm quantity. The AHL concentration is represented by luminescence signal (RLU) produced by biosensors. The luminescence signal indicates the amount of AHL detected by *E. coli* JM109 pSB401 as biosensors. The quantity of biofilms that is affected by crude extracts was measured by crystal violet staining. The absorbance of crystal violet was read at λ_{590} which value indicates the amount of crystal violet attached to the biofilm layer. Data was analyzed for normality and homogeneity followed by one-way analysis of variance (ANOVA) and multiple comparisons testing with Tukey test. Data that is normally distributed and homogeneous is feasible to be analyzed by one-way ANOVA test and Tukey's advanced test.

3. Result and discussion

In this research, the effect of extract originated from environmental isolated bacteria on quorum sensing system of *B. cenocepacia* H111 was investigated. According to Christiaen et al. [7], exploration of bacteria from environmental is an appropriate step to find out the potential quorum quencher molecules. Inhibition of quorum sensing is thought to be the best way in related to avoid the risk of bacterial resistance. Bacterial populations in nature will interact with other bacteria and develop a unique and special adaptive mechanism for the defense of stresses in the environment.

Initially an assay was carried out to confirm the method for quantification of AHL amounts using pSB401 biosensors. The confirmation results showed that the luminescent signal increased with increasing AHL concentration (data not shown). This indicates that the assay was working well in this research. Furthermore, the 24 h acidification of supernatant produced a higher luminescence signal than fast acidification which done along with acidified ethyl acetate extraction step. Cultures supernatant must be acidified before the further assay so that ring-opened AHL becomes recircularized. It takes at least hours at 37 °C. This is in accordance with the literature that during cultivation AHL can undergo lactonolysis due to increase in pH. The ring-opened acidification of AHL in supernatant can avoid false negatives for AHL levels measurement using biosensors [17]. The opened-lactone ring is not active as signal molecules.

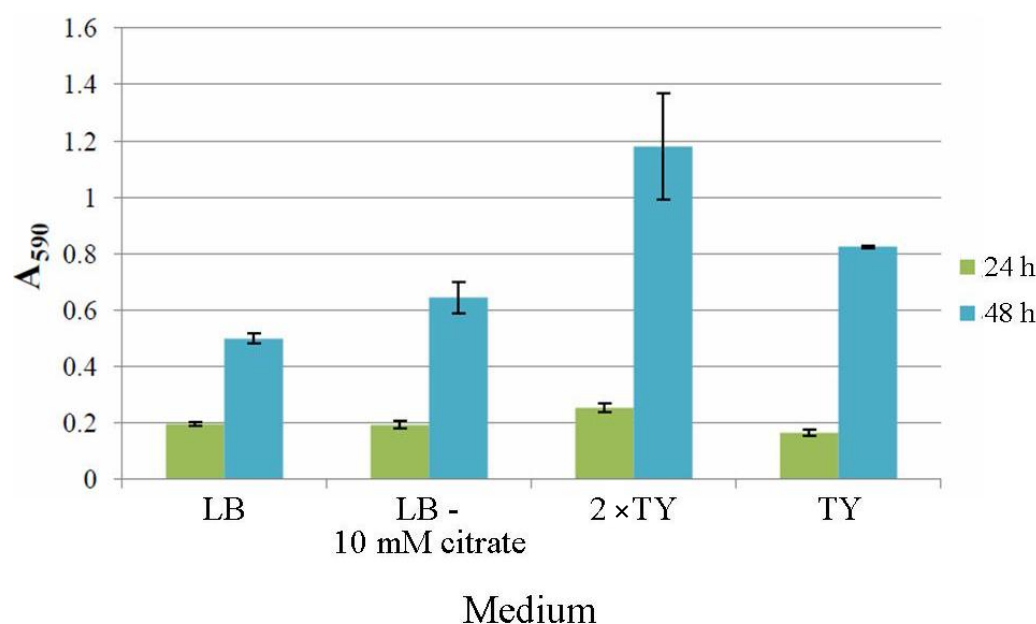


Figure 1. Formation of *B. cenocepacia* H111 biofilm in various media. The 48 h cultivation have more biofilm than the 24 h incubation. A_{590} shows the absorbance of 1 % violet crystals attached to the biofilm layers and is correlated to biofilm quantity. Error bar is a standar deviation of each treatment.

The type of medium and length of cultivation can affect the quantity of biofilm. In figure 1 it can be seen that there are more biofilms formed during 48 h compared to 24 h cultivation in all media, the highest is 1 180 units for biofilm formed in 2 x TY medium. Based on these results, biofilm formation of *B. cenocepacia* H111 was carried out in 2 x TY medium for 48 h. This is to avoid false positive.

The difference in the number of biofilms in each media stated that the growth of *B. cenocepacia* H111 biofilm was affected by the nutrients present in the environment. Componen of medium, such as C and amino acid sources, plays a vital role in providing proper nutrition for the formation of microbial biofilm components [18]. The 2 x TY medium is enriched with sources of peptides and amino acids, such as tryptone, which are needed as raw material for the formation of complex proteins such as BapA and FimA. These proteins are secreted by *Burkholderia cenocepacia* H111 in initiating the attachment of cells on a solid surface [2].

To determine whether extracellular material have activity on AHL and biofilms formation, the next step was to test the effect of cultures supernatants on AHL degradation activity and antibiofilm activity. Addition of supernatant from environmental bacterial culture did not give a difference in the levels of AHL and the quantity of biofilms formed, even the addition of certain crude extracts caused a slight increase in biofilm quantity. This indicates that the supernatant of environmental bacterial might not contains any effectors that influence the quorum sensing *B. cenocepacia* H111 system.

The next step was to investigate the effect of cells crude extract on the quorum sensing system. The efficiency of cell breaking by the ultrasonication method never reaches 100 %. To confirm the presence of living cells in crude extracts, all crude extracts were plated on Luria Bertani agar. It revealed that there is still living cells in post-ultrasonication extract (tabel 1). To avoid interference of living cells on biofilm formation or AHL degradation, the extracts then were filtered through membrane filters.

Data analyses using one way ANOVA and Tukey test showed that AHL concentrations decreased significantly in the samples with crude extracts compared to controls ($\alpha = 5\%$) (table 2). This indicates that crude extracts from environmental bacteria have the ability to degrade AHL molecules from *Burkholderia cenocepacia* H111. Of the four types of extracts given, crude extracts from *Bacillus* sp. showed the greatest AHL degradation compared to the three other crude extracts so that the remaining AHL content was the lowest (16 %) (figure 3).

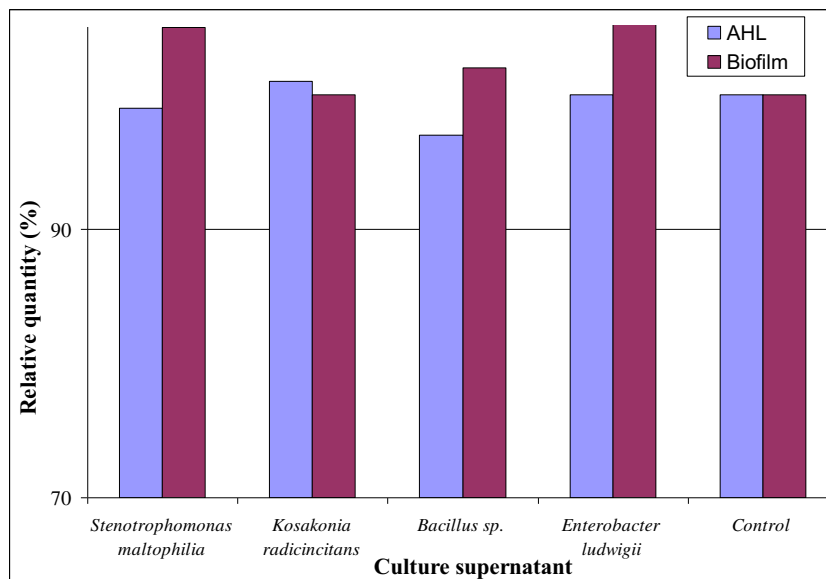


Figure 2. Supernatants of environmental isolates cultures have no effect to AHL concentration and biofilm of *B. cenocepacia* H111. Control is an assay without addition of isolates cultures supernatants. Relative quantity percentage is a percentage of a treatment quantity divided by control quantity. Error bar indicates a standard deviation of each treatment.

Table 1. Total plate count of cells before and after ultrasonication.

Crude Extracts	Total Plate Count (CFU mL ⁻¹)	
	Before ultrasonication	After ultrasonication
<i>Stenotrophomonas maltophilia</i>	2.5×10^8	56
<i>Kosakonia radicincitans</i>	1.0×10^8	98
<i>Bacillus sp.</i>	1.4×10^7	6
<i>Enterobacter ludwigii</i>	8.3×10^7	35

Table 2. The quantities of *B. cenocepacia* H111 AHL and biofilm upon addition of crude extract of environmental isolates.

Crude extract	Relative quantity % age	
	AHL	Biofilm
<i>Stenotrophomonas maltophilia</i>	39 ± 2^b	60 ± 2^b
<i>Kosakonia radicincitans</i>	31 ± 1^c	64 ± 1^b
<i>Bacillus sp.</i>	16 ± 0^e	29 ± 1^d
<i>Enterobacter ludwigii</i>	23 ± 1^d	41 ± 3^c
Control	100 ± 1^a	100 ± 1^a

Note: Numbers followed by different letters in the same column show significantly different according to the Tukey test ($\alpha = 5\%$).

The decrease in AHL levels might be caused by AHL degradation by compound/s in the crude extracts, which no longer be detected by the biosensor. According to Winson et al. [17], degraded AHL cannot attach to the receptor (LuxR) protein so that they are unable to form complexes to activate the *luxCDABE* genes expression.

The same result also occurs for biofilm quantity. Decreasing the quantity of biofilms is seen in all treatments upon addition of crude extracts (table 2 and figure 3). It means that all crude extracts inhibit the formation of *Burkholderia cenocepacia* H111 biofilm. The lowest percentage of the biofilm quantity is found in the sample with crude extract of *Bacillus sp.* (29 %). Interestingly, when the AHL level is low the biofilm levels is also low (figure 3). It seem these extracts cause degradation

of AHL which in turn influence to biofilm formation. Biofilm formation of *B. cenocepacia* cells is regulated by CepIR quorum sensing system mediated by AHL molecules [2].

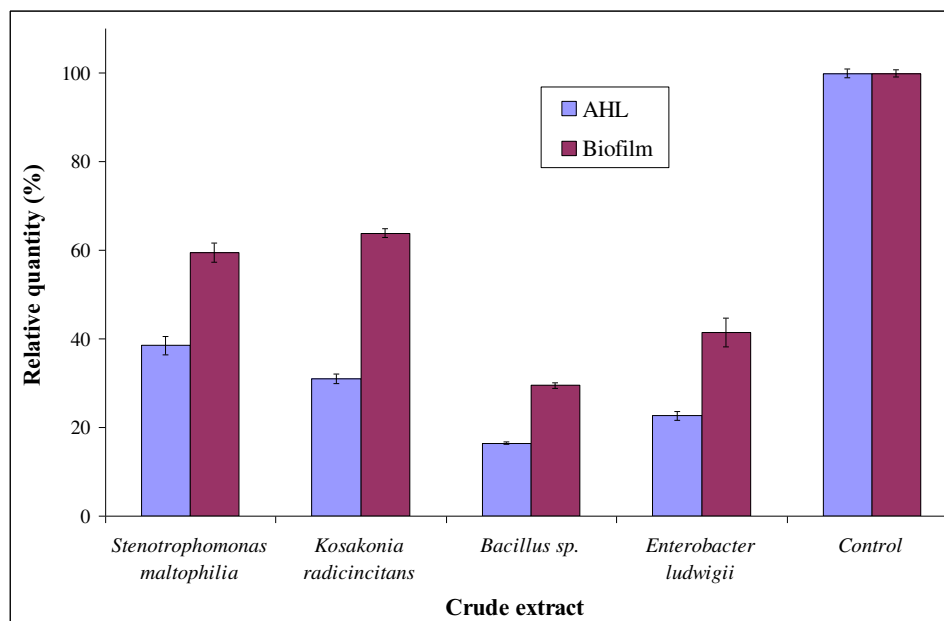


Figure 3 Crude extracts of environmental isolates influence to AHL concentration and biofilm of *Burkholderia cenocepacia* H111. Control is an assay without addition of isolates cultures supernatants. Relative quantity percentage is percentage of a treatment quantity divided by control quantity. Error bar indicates a standard deviation of each treatment.

There are two reasons which might be correlated to the results. This might be caused by AHL or signal molecules degradation or by inhibition of biofilm formation. Molecules, such as amyloglucosidases, have ability to disrupts exopolysaccharide layer (EPS) which causes sloughing in the biofilm formed [19]. According to the genome structures of *Bacillus*, crude extracts of *Bacillus sp.* might be contained glucosidases. This is confirmed in the result that treatment with crude extract of *Bacillus sp.* shows the greatest decrease in the quantity of biofilm (lagging behind 29 %).

Based on others studies, *Bacillus sp.* have the potential to be sources of quorum quencher molecules. Besides its ability to produce glucosidases, *Bacillus* commonly express lactonase which have activity to degrade AHL with 4 to 10 acyl side chains [20]. Lactonases is encoded by *aiiA* gene with high C8-HSL activity. This C8-HSL is one of signal molecules of *B. cenocepacia* H111. It is very likely that the lactonase enzyme found in the crude extract of *Bacillus sp.* which play a role in reducing AHL concentration significantly and subsequently resulted in decreasing in the biofilm quantity. However, this still needs to be confirmed by further research. The extract used here is a crude extract which contains a mixture of various components in it. It is possible that among the components there are also effectors that regulate and modify the AHL or inhibit biofilm formation, in addition to the presence of the suspected acylase/lactonase enzyme.

Furthermore, several factors can influence the fluctuations in the activity of crude extracts from each bacterium such as pH, temperature, cofactor, substrate, the total number of effectors that can vary and differ in each bacterium [21]. Dong et al. [22] found some evidence that the expression of quenching enzymes, such as acylase and lactonase, will increase with the presence of invasive metabolites released by other bacterial populations. This shows that QQ enzyme can be produce insignificantly in community of one species bacterium.

The above results, decreasing AHL levels and quantity of biofilms, are in line with the results of bioinformatics analysis for each environmental bacterium used. The bioinformatics analysis indicate the possibility of these environmental bacteria to degrade AHL signal molecules and biofilm of *B. cenocepacia* H111. Based on the Uniprot database, it was found that all these environmental bacteria had a gene encoding AHL acylase or lactonase. *S. maltophilia* has a *BurJV3_1273* gene that

encodes AHL acylase that belongs to the Ntn-hydrolase domain [20]. The three other bacteria might have lactonases encoding genes. *K. radicincitans* have *attM* genes [23] and *Bacillus* sp. have *aiiA* gene; both encode AHL lactonases which belongs to the B-lactamase domains [24]. Whereas *E. ludwigii* might have a homologous gene *aiiA* [25]. The analysis using the MetaCyc software showed that the AHL acylase enzyme from *S. maltophilia* and the AHL lactonase enzyme from *Bacillus* sp., had broad range activities on the AHL substrates. C8-HSL and C6-HSL, the main AHL molecules in *B. cenocepacia* H111, are also reported to have been successfully degraded by these enzymes via hydrolysis reactions [26]. However, no information yet for the enzymes from two other bacteria.

It is shown in the results above that the reduction ratio of AHL level was lower than the decreasing level of biofilm formation. AHL is an autoinducer (AI) molecule that will trigger the production of virulence factors, one of which is biofilm formation. It is presumed that the ratio of the decrease in AHL levels would be in line with the ratio of the decrease in biofilm due to the two related factors. However, the pathway of *Burkholderia cenocepacia* H111 biofilm formation involves not just one component but varies. According to Fazli et al. [18], *B. cenocepacia* has several inducer compounds for virulence factors production, specifically biofilms, i.e. AHL, BDSF, c-di-GMP, and small-RNAs. If the AHL is damaged or its synthesis is inhibited, the formation of the biofilm layer can still run even though it is not optimal. AHL regulate production of EPS and cell mass while other factors indirectly trigger the maturation of biofilm formation and cell colonization. Further research is needed to obtain quorum quencher molecules present in crude extracts and explore the mechanisms underlying the quenching process.

4. Conclusion

As a conclusion, that the crude extracts of all environmental bacteria have quorum quenching activity to *B. cenocepacia* H111 quorum sensing system, specifically in reducing AHL levels and biofilm quantity. Crude extract from *Bacillus* sp. gave the highest inhibitory effect to the quorum sensing system of *Burkholderia cenocepacia* H111.

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