CONTROLLING AQUACULTURE PATHOGEN BIOFILM BY CRUDE EXTRACT AND SUPERNATANT OF PHYLLOSPHERE BACTERIA

Gisela Ignacia Tjandra¹, Alexandra Silvia Mulyanti¹, Diana Elizabeth Waturangi*¹

Address(es):
Alma Jaya Catholic University of Indonesia, Faculty of Biotechnology, Department of Biotechnology, Jl. Jenderal Sudirman No. 51, Jakarta 12930

*Corresponding author: diana.waturangi@atmajaya.ac.id

ABSTRACT

Some infectious-aquaculture pathogen species, such as Aeromonas hydrophila, Vibrio harveyi, and Streptococcus agalactiae are able to form biofilm, a protective structure that increases bacterial resistance to some external exposures, for example the antibiotic therapy, making the antibiotic becomes less effective. This ability is supported by the quorum sensing mechanism. Therefore, disruption in quorum sensing mechanism may be a potential way to treat the biofilm formation. The present study was conducted to assess the ability of supernatant and crude extract of some Actinobacteria and phyllosphere bacteria isolates to overcome the biofilms of several aquaculture pathogens. In general, this study consists of several steps, such as primary screening of anti-quorum sensing activity, extraction of bioactive compounds, antibiofilm assay, validation of anti-quorum sensing activity, toxicity assay, and microscopic observation of biofilm. The phyllosphere bacteria isolates showed higher antibiofilm activity compared to the Actinobacteria isolate, where the crude extract had the most ability instead of supernatant. The supernatant and crude extract of all isolates also performed the anti-quorum sensing activities, both qualitatively and quantitatively. Overall, all isolates were potential to be natural sources of antibiofilm agents to control the biofilm of all pathogens used in this study through quorum sensing disruption.

Keywords: Actinobacteria, antibiofilm, aquaculture, phyllosphere bacteria, quorum quenching, quorum sensing

INTRODUCTION

Pathogen attack is one of the main problems that often occurs in the aquaculture system. Some infectious-aquaculture pathogen species, such as Aeromonas hydrophila, Vibrio harveyi, and Streptococcus agalactiae are known as the main causes of several acentric diseases, including motile Aeromonas septicemia (MAS) and vibriosis (Zhang et al., 2016; Assefa and Abunna, 2018). This infectious is also supported by their ability to form biofilm (Arunkumar et al., 2020).

Biofilm is a microbial aggregate encased in a matrix that consists of exopolysaccharides, nucleic acid, and protein (Jamal et al., 2018). It provides cell protection against some unfavourable conditions, such as stress, antimicrobial agent and antibiotic exposure, and host defense mechanism (Cai et al., 2013; Oberoi et al., 2020). Quorum sensing is a cellular communication promoted by autoinducer, responsible for the biofilm formation (Borges and Simões, 2019).

The frequent use of antibiotics to overcome pathogen tends to decrease its effectiveness, since cells are more likely resistant to external exposure when they are in the biofilm (Santhakumari et al., 2015; Vestby et al., 2020). Therefore, other methods are needed to treat biofilms. Research have discovered various natural sources of antibiofilm agents, including those from Actinobacteria, group of filamentous Gram-positive bacteria, and phyllosphere bacteria, group of bacteria that live on the plant’s surface (Ma et al., 2013; Waithaka et al., 2019).

Our previous studies reported that crude extracts of several Actinobacteria and phyllosphere bacteria isolates demonstrated high activities against A. hydrophila, V. harveyi, and S. agalactiae biofilms (Raissa et al., 2020; Nathalia and Waturangi, 2021). Compared to the crude extract, supernatant was considered easier to be produced since it did not require further purification. However, its antibiofilm activity has not yet been assessed. Therefore, here we further explored the ability of supernatant and crude extract of Actinobacteria and phyllosphere bacteria to control biofilm of several aquaculture pathogens.

MATERIAL AND METHODS

Bacterial Cultivation

An Actinobacteria isolate, namely Streptomyces variabilis 16PM and phyllosphere bacteria isolates, namely Morganella morganii JB 8F and Proteus myxofaciens JB 17B were obtained from Atma Jaya Catholic University. S. variabilis 16PM was cultured on yeast malt extract agar at 28 °C for 7 days, while M. morganii JB 8F and P. myxofaciens JB 17B were cultivated on King’s B agar at 28 °C for 48 h. A. hydrophila, S. agalactiae, and V. harveyi were obtained from the Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. A. hydrophila was cultured on Luria agar (LA) at 28 °C for 24 h and S. agalactiae was maintained on LA at 37 °C for 48 h. Meanwhile, V. harveyi was grown on LA supplemented with 2% (w/v) of NaCl at 28 °C overnight.

Primary Screening of Anti-quorum Sensing Activity

S. variabilis 16PM was streaked onto LA and incubated at 28 °C for 72 h, while M. morganii JB 8F and P. myxofaciens JB 17B were streaked onto King’s B agar before incubation at 28 °C for 48 h. Separately, Chromobacterium violaceum wild type as the indicator strain was grown in Luria broth (LB) at 28 °C and agitation at 120 rpm for 48 h. Up to 100 µl of the culture was mixed into 5 ml semisolid agar, then poured onto streaked agar. Plates were re-incubated for 48 h (McLean et al., 2004). This assay was performed in triplicate.

Extraction of Bioactive Compounds

S. variabilis 16PM was cultured in 100 ml of tryptic soy broth (TSB) supplemented with 1% (w/v) glucose at 28 °C for 7 days, meanwhile M. morganii JB 8F and P. myxofaciens JB 17B were cultured in 100 ml of LB at 28 °C for 2 days with agitation at 125 rpm.

After incubation, the supernatant was harvested through centrifugation at 5332 x g for 25 min. To obtain crude extract, supernatant was mixed with ethyl acetate at equal volume (1:1) and shaken at 28 °C and 120 rpm overnight. The upper phase was collected prior to evaporation using a rotary evaporator (55 °C), later dried in vacuum oven to remove the remaining solvent. The dried-crude extract was diluted in 1% (v/v) dimethyl sulfoxide (DMSO) to obtain the final concentration (20 mg/ml) (Ma et al., 2018; Theodora et al., 2019).

Antibiofilm Assay

All pathogens were grown in brain heart infusion broth (BHB) according to their optimum conditions described above. For biofilm inhibition assay, 100 µl of pathogen (OD600 = 0.132) and 100 100 µl of supernatant or crude extract were transferred into a 96-well microplate, then incubated at 28 °C for 24 h. Conversely, for the biofilm destruction assay, pathogen was grown first in 96-well microplate to let the biofilm form, then treated with supernatant or crude extract before reincubation for another 24 h. Sterile medium was used as a negative control, while untreated pathogen culture was used as a positive control.

After incubation, wells were rinsed to remove unwanted cells, then air-dried. The formed biofilm was stained with 0.4% (w/v) crystal violet for 30 min, then rinsed and air-dried. The biofilm was resuspended with 99% (v/v) ethanol before its absorbance value being measured by a microplate reader at 595 nm (Balasubramanian et al., 2017). This assay was performed in triplicate.
Validation of Anti-quorum Sensing Activity

C. violaceum CV026 as the indicator strain was cultivated in LB at 28 °C overnight with agitation of 120 ppm. The bacterial culture (OD₆₀₀ = 0.132) was treated with supernatant or crude extract at the equal volume (1:1) and N-hexanolyl-homoserine-lactone (1 µmol/ml), later incubated at 28 °C for 24 h. Untreated culture was used as a positive control.

After 24 h, pellet was obtained through centrifugation at 1000 x g for 15 min, later diluted in 1 ml of 1% (v/v) DMSO and re-centrifuged at 1000 x g for 15 min. The absorbance value of supernatant was measured at 540 nm. This assay was performed in triplicate (Rajivgandhi et al., 2018).

% Inhibition or destruction activity = \frac{\text{positive control absorbance} - \text{sample absorbance}}{\text{positive control absorbance}} \times 100%

Toxicity Assay

This assay was performed using the brine shrimp lethality assay (BSLT). All crude extracts and supernatants were diluted to various concentrations (50, 100, 500, and 1000 ppm). Artemia salina eggs were hatched in artificial seawater (3.8 g of NaCl in 100 ml of aquadest) for 48 h until they reach the nauplii stage. About 10 nauplii were transferred into sterile test tube contained 4.5 ml of artificial water along 0.5 ml of diluted sample. Untreated nauplii were used as the negative control.

After 24 h, the number of dead nauplii was counted to obtain the mortality value, which was later converted to the LC₅₀ value using the Probit analysis (Syahmi et al., 2010). This assay was conducted in triplicate.

Microscopic Observation of Biofilm

The biofilm structure was visualized using a light microscope. Pathogen (OD₆₀₀ = 0.132) was grown first on a 2 x 2 cm cover glass for 24 h. Biofilm was treated with selected crude extract and re-incubated for another 24 h, then stained using 0.4% (w/v) crystal violet before being observed at a magnification of 400 x (Thenmozhi et al., 2009).

RESULTS AND DISCUSSION

RESULTS

Primary Screening of Anti-quorum Sensing Activity

All isolates showed positive results of anti-quorum sensing inhibition, represented by a non-purple zone around the streaked area (Fig 1).

Antibiofilm Assay

Crude extracts of all phyllosphere bacteria isolates showed higher antibiofilm activities than the supernatants (Tab 1). The addition of crude extract of P. myxofaciens JB 17B could inhibit the biofilm formation by A. hydrophila up to 71.97% as the highest inhibition. It also had the most ability to inhibit and destroy the biofilm of S. agalactiae, up to 59.94% and 54.45%, respectively. Furthermore, crude extract of M. morganii JB 8F performed the greatest biofilm destruction against A. hydrophila up to 64.43%, compared with supernatant. It also demonstrated the highest biofilm inhibition and destruction activity against V. harveyi.

Conversely, biofilm of V. harveyi was highly inhibited and destroyed by the supernatant of S. variabilis 16PM, rather than its crude extract that even showed no destruction activity. However, both showed lower antibiofilm activity against all pathogens compared to phyllosphere bacteria isolates.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anti-quorum sensing activity (%)</th>
<th>A. hydrophila</th>
<th>V. harveyi</th>
<th>S. agalactiae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibition</td>
<td>Destruction</td>
<td>Inhibition</td>
<td>Destruction</td>
</tr>
<tr>
<td>M. morganii JB 8F – S</td>
<td>18.97</td>
<td>22.66</td>
<td>10.59</td>
<td>16.23</td>
</tr>
<tr>
<td>M. morganii JB 8F – E</td>
<td>61.78</td>
<td>64.43*</td>
<td>33.35*</td>
<td>37.99*</td>
</tr>
<tr>
<td>P. myxofaciens JB 17B – S</td>
<td>26.7</td>
<td>13.41</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>P. myxofaciens JB 17B – E</td>
<td>71.97*</td>
<td>62.48</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>S. variabilis 16PM – S</td>
<td>17.82</td>
<td>8.8</td>
<td>31.08</td>
<td>18.36</td>
</tr>
<tr>
<td>S. variabilis 16PM – E</td>
<td>32.1</td>
<td>15.94</td>
<td>25.07</td>
<td>0</td>
</tr>
</tbody>
</table>

*Value marked with * represented highest activity among the others; All values were measured in %

Legend: S = supernatant; E = crude extract

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anti-quorum sensing activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. morganii JB 8F – S</td>
<td>49.05</td>
</tr>
<tr>
<td>M. morganii JB 8F – E</td>
<td>61.67</td>
</tr>
<tr>
<td>P. myxofaciens JB 17B – S</td>
<td>55.71</td>
</tr>
<tr>
<td>P. myxofaciens JB 17B – E</td>
<td>71.43</td>
</tr>
<tr>
<td>S. variabilis 16PM – S</td>
<td>15.55</td>
</tr>
<tr>
<td>S. variabilis 16PM – E</td>
<td>28.96</td>
</tr>
</tbody>
</table>

Legend: S = supernatant; E = crude extract

Validation of Anti-quorum Sensing Activity

The violacein production of C. violaceum CV026 was highly inhibited by crude extract of P. myxofaciens JB 17B (Tab 2). Overall, all phyllosphere isolates showed higher anti-quorum sensing activity instead of Actinobacteria isolate.

Toxicity Assay

Among the other samples, supernatant of P. myxofaciens JB 17B was much less toxic, indicated by a higher LC₅₀ value than others (Tab 3). However, all samples were also considered non-toxic based on their LC₅₀ value, which was greater than 1000 ppm.

Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>LC₅₀ value (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. morganii JB 8F – S</td>
<td>3438.03</td>
</tr>
<tr>
<td>M. morganii JB 8F – E</td>
<td>6547.80</td>
</tr>
<tr>
<td>P. myxofaciens JB 17B – S</td>
<td>8664.40</td>
</tr>
<tr>
<td>P. myxofaciens JB 17B – E</td>
<td>6025.29</td>
</tr>
<tr>
<td>S. variabilis 16PM – S</td>
<td>6025.60</td>
</tr>
<tr>
<td>S. variabilis 16PM – E</td>
<td>2493.71</td>
</tr>
</tbody>
</table>

Legend: S = supernatant; E = crude extract

Microscopic Observation

Figure 2 showed that treated biofilm was smaller in structure compared to the untreated biofilm after the addition of supernatant and crude extract.
autoinducer production and transport, degrading the autoinducer, or competing with the autoinducer to bind with the receptor. The LC₅₀ value is defined as the concentration of a compound that killed half of the test organism after a certain time of exposure (Osman and Osman, 2019). According to Meyer et al. (1982), a compound is considered non-toxic if its LC₅₀ value is less than 1000 ppm, and vice versa. A higher LC₅₀ value means a less toxicity property. Most supernatants in this study demonstrated higher LC₅₀ values than the crude extracts. This probably happened because the crude extract might contain some bioactive compounds that showed more toxicity properties toward the test organism used in this study (Ullah et al., 2013). Microscopic observation visualized the massive reduction in biofilm mass of pathogen after treatment (Fig 2). This indicated that the addition of crude extract could disrupt the biofilm (Vyas et al., 2016).

CONCLUSION

All isolates were potential to be natural sources of antibiofilm agents to control the biofilm of all pathogens used in this study through quorum sensing disruption. Most crude extracts performed higher antibiofilm activity instead of supernatants, thus they were still considered more effective to overcome the biofilm of tested pathogens although require further purification. However, more studies need to be conducted to explore their activity toward other aquaculture pathogens as well as to understand the exact mechanism of quorum sensing inhibition.

Acknowledgments: The authors thank everyone for all their support and contribution during this research.

Funding: This research was supported by the Indonesian Ministry of Education and Culture through Competitive National Research Grant 2020-Fundamental Research.

Conflict of Interest: The authors declare that they have no competing interests.

REFERENCES


Ictalurus punctatus.


