

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

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

Address (es):

Atma 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<https://doi.org/10.36547/be.493>

ABSTRACT

Some infectious-aquaculture pathogens, 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 pathogens, such as *Aeromonas hydrophila*, *Vibrio harveyi*, and *Streptococcus agalactiae* are known as the main causes of several aquaculture 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 cultivated on yeast malt extract agar at 28 °C for 7 days, while *M. morganii* JB 8F and *P. myxofaciens* JB 17F 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 (BHIB) according to their optimum conditions described above. For biofilm inhibition assay, 100 µl of pathogen (OD₆₀₀ = 0.132) and 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 re-incubation 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.

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

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 rpm. The bacterial culture (OD₆₀₀ = 0.132) was treated with supernatant or crude extract at the equal volume (1:1) and N-hexanoyl-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).

$$\% \text{ Anti-quorum sensing activity} = \frac{\text{positive control absorbance} - \text{sample absorbance}}{\text{positive control absorbance}} \times 100\%$$

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).

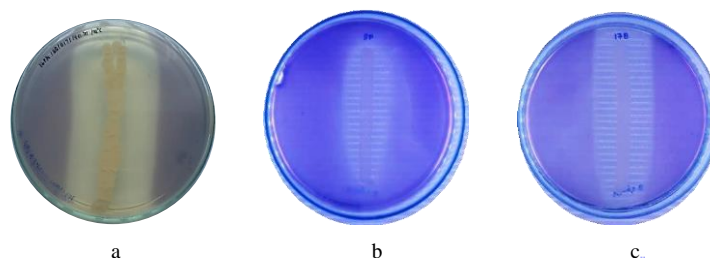


Figure 1 Inhibition of quorum sensing by (a) *S. variabilis* 16PM, (b) *M. morganii* JB 8F, and (c) *P. myxofaciens* JB 17B

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 Antibiofilm activity of supernatant and crude extract of Actinobacteria and phyllosphere bacteria isolates

	<i>A. hydrophila</i>		<i>V. harveyi</i>		<i>S. agalactiae</i>	
	Inhibition	Destruction	Inhibition	Destruction	Inhibition	Destruction
<i>M. morganii</i> JB 8F – S	18.97	22.66	10.59	16.23	25.25	22.14
<i>M. morganii</i> JB 8F – E	61.78	64.43*	33.35*	37.99*	49.28	42.78
<i>P. myxofaciens</i> JB 17B – S	26.7	13.41	X	X	33.78	21.78
<i>P. myxofaciens</i> JB 17B – E	71.97*	62.48	X	X	59.94*	54.55*
<i>S. variabilis</i> 16PM – S	17.82	8.8	31.08	18.36	X	X
<i>S. variabilis</i> 16PM – E	32.1	15.94	25.07	0	X	X

Legend: S = supernatant; E = crude extract; X = not tested due to anti-bacterial activity; Value marked with * represented highest activity among the others; All values were measured in %

Table 2 Anti-quorum sensing activity of supernatant and crude extract of Actinobacteria and phyllosphere bacteria isolates

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

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 LC₅₀ value of supernatant and crude extract of Actinobacteria and phyllosphere bacteria isolates

Sample	LC ₅₀ value (ppm)
<i>M. morganii</i> JB 8F – S	3438.03
<i>M. morganii</i> JB 8F – E	6547.80
<i>P. myxofaciens</i> JB 17B – S	8664.40
<i>P. myxofaciens</i> JB 17B – E	6025.29
<i>S. variabilis</i> 16PM – S	6025.60
<i>S. variabilis</i> 16PM – E	2493.71

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.

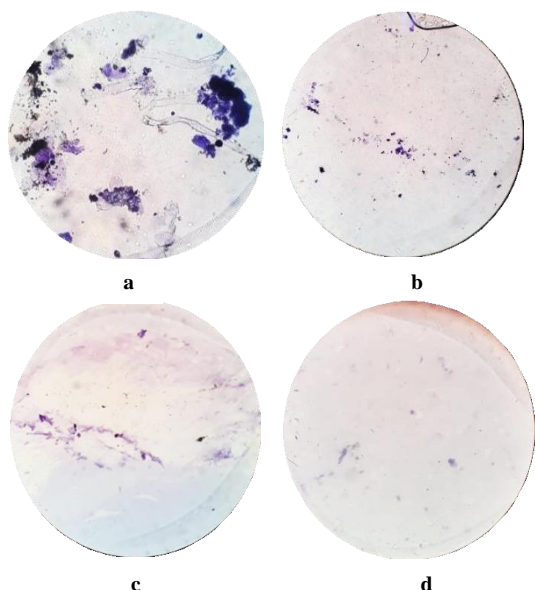


Figure 2 The structure of (a) untreated *A. hydrophila* biofilm, (b) *A. hydrophila* biofilm treated with crude extract of *M. morgani* JB 8F, (c) untreated *S. agalactiae* biofilm, and (d). *S. agalactiae* biofilm treated with crude extract of *P. myxofaciens* JB 17B

DISCUSSION

The use of antibiotics to treat pathogen attack is considered less effective since pathogens are feasible to form biofilm, making them more resistant to antibiotic treatment (Kamaruzzaman et al., 2017). This biofilm-forming ability utilizes quorum sensing (Azman et al., 2019), therefore it is expected that disruption of quorum sensing could be an alternative method to control biofilm formation.

Primary screening of anti-quorum sensing activity showed that all isolates could inhibit the violacein production of *C. violaceum* wild type (Fig 1). Violacein production is regulated by quorum sensing, which requires a signalling molecule called C6-HSL (N-hexanoyl-L-homoserine-lactone). If this molecular signalling is disrupted, the colony would be seen as opaque but still viable, due to the absence of violacein (Tolmacheva et al., 2014).

We used three isolates from previous studies (Raissa et al., 2020; Nathalia and Waturangi, 2021), which do not have antibacterial activity to avoid the chance of false positive. The antibiofilm assay showed various results of supernatant and crude extract of each isolate. This might be due to the specific bioactive compounds present in each sample, which had more characteristically mechanisms to inhibit or destroy certain pathogens (Kalpana et al., 2012). Therefore, a sample would not always show similar results to other pathogens. The purification process also contributed to concentrating the bioactive compounds contained in the crude extract, possibly making them perform higher activities than the supernatants (Truong et al., 2019). Furthermore, various bioactive compounds present might interact each other in either synergism or antagonism way, thereby affecting the mechanism of action of each sample (Bag and Chattopadhyay 2017).

Protein and polysaccharides are antibiofilm compounds that are commonly found. Protein is responsible for biofilm dispersion and destruction since it could weaken and degrade the biofilm matrix (Blackman et al., 2021). Consistent with that, a study conducted by Xie et al. (2018) also mention that extracellular protein from some Actinobacteria isolates can inhibit the biofilm formation of *Staphylococcus epidermidis* at the initial and aggregation stage.

Polysaccharides can interrupt the cell-surface interactions by modifying the surface charge and wettability (Rendueles et al., 2013). It also triggers biofilm dispersal and change cell's hydrophobicity, inhibiting cell aggregation (Valle et al., 2006; Sayem et al., 2011).

A validation test was conducted using *C. violaceum* CV026, the mini-T5-mutant type of wild type strain that only produces violacein if there is compatible external autoinducer, due to lack of AHL (acyl-homoserine-lactone) synthase (Kothari et al., 2017). An external autoinducer called HHL was used to trigger the violacein production through quorum sensing (Delalande et al., 2005). Result showed various percentages of anti-quorum sensing activity of each isolate (Tab 2). Zhang et al. (2019) mention that quorum sensing inhibitor acts through inhibiting the

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 Osmar, 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

- Arunkumar, M., LewisOscar, F., Thajuddin, N., Pugazhendhi, A., Nithya, C. 2020. *In vitro* and *in vivo* biofilm forming *Vibrio* spp: a significant threat in aquaculture. *Process Biochemistry*, 94, 213-223. <http://dx.doi.org/10.1016/j.procbio.2020.04.029>
- Assefa, A., Abunna, F. 2018. Maintenance of fish health in aquaculture: review of epidemiological approaches for prevention and control of infectious disease of fish. *Veterinary Medicine International*, 2018, 1-10. <http://dx.doi.org/10.1155/2018/5432497>
- Azman, A. S., Mawang, C. I., Khairat, J. E., AbuBakar, S. 2019. Actinobacteria - a promising natural source of anti-biofilm agents. *International Microbiology*, 22, 403-409. <http://dx.doi.org/10.1007/s10123-019-00066-4>
- Bag, A., Chattopadhyay, R. R. 2017. Synergistic antibiofilm efficacy of gallotannin 1,2,6-tri-O-galloyl-β-D-glucopyranose from *Terminalia chebula* fruit in combination with gentamicin and trimethoprim against multidrug resistant uropathogenic *Escherichia coli* biofilms. *PLoS One*, 12(5), 1-11. <http://dx.doi.org/10.1371/journal.pone.0178712>
- Balasubramanian, S., Othman, E. M., Kampik, D., Stopper, H., Hentschel, U., Ziebuhr, W., Oelschlaeger, T. A., Abdelmohsen, U. R. 2017. Marine sponge-derived *Streptomyces* sp. SBT343 extract inhibits staphylococcal biofilm formation. *Frontiers in Microbiology*, 8, 1-14. <http://dx.doi.org/10.3389/fmicb.2017.00236>
- Blackman, L. D., Qu, Y., Cass, P., Locock, K. E. S. 2021. Approaches for the inhibition and elimination of microbial biofilms using macromolecular agents. *Chemical Society Reviews*, 50(3), 1587-1616. <http://dx.doi.org/10.1039/d0cs00986e>
- Borges, A., Simões, M. 2019. Quorum sensing inhibition by marine bacteria. *Marine Drugs*, 17(7), 427-452. <http://dx.doi.org/10.3390/md17070427>
- Cai, W., De La Fuente, L., Arias, C. R. 2013. Biofilm formation by the fish pathogen *Flavobacterium columnare*: development and parameters affecting surface attachment. *Applied and Environmental Microbiology*, 79(18), 5633-5642. <http://dx.doi.org/10.1128/AEM.01192-13>
- Delalande, L., Faure, D., Raffoux, A., Uroz, S., D'Angelo-Picard, C., Elasm, M., Carlier, A., Berruyer, R., Petit, A., Williams, P., Dessaux, Y. 2005. N-hexanoyl-L-homoserine lactone, a mediator of bacterial quorum-sensing regulation, exhibit plant-dependent stability and may be inactivated by germinating *Lotus corniculatus* seedlings. *FEMS Microbiology Ecology*, 52(1), 13-20. <http://dx.doi.org/10.1016/j.femsec.2004.10.005>
- Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., Hussain, T., Ali, M., Rafiq, M., Kamil, M. A. 2018. Bacterial biofilm and associated

- infections. *Journal of the Chinese Medical Association*, 81(1), 7-11. <http://dx.doi.org/10.1016/j.jcma.2017.07.012>
- Kalpna, B. J., Aarthy, S., Pandian, S. K. 2012. Antibiofilm activity of α -amylase from *Bacillus subtilis* S8-18 against biofilm forming human bacterial pathogens. *Applied Biochemistry and Biotechnology*, 167, 1778-1794. <http://dx.doi.org/10.1007/s12010-011-9526-2>
- Kamaruzzaman, N. F., Kendall, S., Good, L. 2017. Targeting the hard to reach: challenges and novel strategies in the treatment of intracellular bacterial infections. *British Journal of Pharmacology*, 174(14), 2225-2236. <http://dx.doi.org/10.1111/bph.13664>
- Kothari, V., Sharma, S., Padia, D. 2017. Recent research advances on *Chromobacterium violaceum*. *Asian Pacific Journal of Tropical Medicine*, 10(8), 744-752. <http://dx.doi.org/10.1016/j.apjtm.2017.07.022>
- Ma, A., Lv, D., Zhuang, X., Zhuang, G. 2013. Quorum quenching in culturable phyllosphere bacteria from tobacco. *International Journal of Molecular Sciences*, 14(7), 14607-14619. <http://dx.doi.org/10.3390/ijms140714607>
- Ma, Z. P., Song, Y., Cai, Z. H., Lin, Z. J., Lin, G. H., Wang, Y., Zhou, J. 2018. Anti-quorum sensing activities of selected coral symbiotic bacterial extracts from the South China Sea. *Frontiers in Cellular and Infection Microbiology*, 8, 1-13. <http://dx.doi.org/10.3389/fcimb.2018.00144>
- McLean, R. J. C., Pierson, L. S. 3rd, Fuqua, C. 2004. A simple screening protocol for the identification of quorum signal antagonists. *Journal of Microbiological Methods*, 58(3), 351-360. <http://dx.doi.org/10.1016/j.mimet.2004.04.016>
- Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E., McLaughlin, J. L. 1982. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica*, 45(5), 31-34. <http://dx.doi.org/10.1055/s-2007-971236>
- Nathalia, O., Waturangi, D. E. 2021. Extract from phyllosphere bacteria with antibiofilm and quorum quenching activity to control several fish pathogenic bacteria. *BMC Research Notes*, 14, 1-7. <http://dx.doi.org/10.1186/s13104-021-05612-w>
- Oberoi, J. K., Momin, T., Ande, R., Katkar, N. 2020. Inhibition of bacterial biofilms by *Streptomyces* derived crude extract. *Journal of Biology and Today's World*, 9(1), 211-219.
- Osman, N., Omar, H. 2019. Brine Shrimp Lethality Assay (BSLA) of mixed micro algae extracts from Tilapia fish ponds. *Journal of Algal Biomass Utilization*, 10(1), 8-13.
- Raissa, G., Waturangi, D. E., Wahjuningrum, D. 2020. Screening of antibiofilm and anti-quorum sensing activity of Actinomycetes isolates extracts against aquaculture pathogenic bacteria. *BMC Microbiology*, 20, 1-10. <http://dx.doi.org/10.1186/s12866-020-02022-z>
- Rajivgandhi, G., Vijayan, R., Maruthupandy, M., Vaseeharan, B., Manoharan, N. 2018. Antibiofilm effect of *Nocardia* sp. GRG 1 (KT235640) compound against biofilm forming Gram negative bacteria on UTIs. *Microbial Pathogenesis*, 118, 190-198. <http://dx.doi.org/10.1016/j.micpath.2018.03.011>
- Rendueles, O., Kaplan, J. B., Ghigo, J. M. 2013. Antibiofilm polysaccharides. *Environmental Microbiology*, 15(2), 334-346. <http://dx.doi.org/10.1111/j.1462-2920.2012.02810.x>
- Santhakumari, S., Kannappan, A., Pandian, S. K., Thajuddin, N., Rajendran, R. B., Ravi, A. V. 2015. Inhibitory effect of marine cyanobacterial extract on biofilm formation and virulence factor production of bacterial pathogens causing vibriosis in aquaculture. *Journal of Applied Phycology*, 28, 313-324. <http://dx.doi.org/10.1007/s10811-015-0554-0>
- Sayem, S. M. A., Manzo, E., Ciavatta, L., Tramice, A., Cordone, A., Zanfardino, A., De Felice, M., Varcamonti, M. 2011. Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*. *Microbial Cell Factories*, 10, 1-12. <http://dx.doi.org/10.1186/1475-2859-10-74>
- Syahmi, A. R. M., Vijayaratna, S., Sasidharan, S., Latha, L. Y., Kwan, Y. P., Lau, Y. L., Shin, L. N., Chen, Y. 2010. Acute oral toxicity and brine shrimp lethality of *Elaeis guineensis* Jacq., (oil palm leaf) methanol extract. *Molecules*, 15(11), 8111-8121. <http://dx.doi.org/10.3390/molecules15118111>
- Thenmozhi, R., Nithyanand, P., Rathna, J., Pandian, S. K. 2009. Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of *Streptococcus pyogenes*. *FEMS Immunology & Medical Microbiology*, 57(3), 284-294. <http://dx.doi.org/10.1111/j.1574-695X.2009.00613.x>
- Theodora, N. A., Dominika, V., Waturangi, D. E. 2019. Screening and quantification of anti-quorum sensing and antibiofilm activities of phyllosphere bacteria against biofilm forming bacteria. *BMC Research Notes*, 12, 1-5. <http://dx.doi.org/10.21203/rs.2.15616/v2>
- Tolmacheva, A. A., Rogozhin, E. A., Deryabin, D. G. 2014. Antibacterial and quorum sensing regulatory activities of some traditional Eastern-European medical plants. *Acta Pharmaceutica*, 64(2), 173-186.
- Truong, D. H., Nguyen, D. H., Ta, N. T. A., Bui, A. V., Do, T. H., Nguyen, H. C. 2019. Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of *Severinia buxifolia*. *Journal of Food Quality*, 2019, 1-9. <http://dx.doi.org/10.1155/2019/8178294>
- Ullah, M. O., Haque, M., Urmi, K. F., Zulfiker, A. H. M., Anita, E. S., Begum, M., Hamid, K. 2013. Anti-bacterial activity and brine shrimp lethality assay of methanolic extracts of fourteen different edible vegetables from Bangladesh. *Asian Pacific Journal of Tropical Biomedicine*, 3(1), 1-7. [http://dx.doi.org/10.1016/S2221-1691\(13\)60015-5](http://dx.doi.org/10.1016/S2221-1691(13)60015-5)
- Valle, J., Re, S. D., Henry, N., Fontaine, T., Balestrino, D., Latour-Lambert, P., Ghigo, J. M. 2006. Broad-spectrum biofilm inhibition by a secreted bacterial polysaccharide. *PNAS*, 103(33), 12558-12563. <http://dx.doi.org/10.1073/pnas.0605399103>
- Vestby, L. K., Gronseth, T., Simm, R., Nesse, L. L. 2020. Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics*, 9(2), 59-88. <http://dx.doi.org/10.3390/antibiotics9020059>
- Vyas, N., Sammons, R. L., Addison, O., Dehghani, H., Walmsey, A. D. 2016. A quantitative method to measure biofilm removal efficiency from complex biomaterial surfaces using SEM and image analysis. *Scientific Reports*, 6, 1-10. <http://dx.doi.org/10.1038/srep32694>
- Waihtaka, P. N., Mwaura, F. B., Wagacha, J. M., Gathuru, E. M., Githaiga, B. M. 2019. Purification and cytotoxic assays of four antimicrobial metabolites extracted from Actinomycetes of the soils of Menengai Crater, Kenya. *Novel Research in Microbiology Journal*, 3(3), 351-365. <http://dx.doi.org/10.21608/NRMJ.2019.37208>
- Xie, T. T., Zeng, H., Ren, X. P., Wang, N., Chen, Z. J., Zhang, Y., Chen, W. 2018. Antibiofilm activity of three Actinomycete strains against *Staphylococcus epidermidis*. *Letters in Applied Microbiology*, 68(1), 73-80. <http://dx.doi.org/10.1111/lam.13087>
- Zhang, D., Xu, D. H., Shoemaker, C. 2016. Experimental induction of motile *Aeromonas septicemia* in channel catfish (*Ictalurus punctatus*) by waterborne challenge with virulent *Aeromonas hydrophila*. *Aquaculture Reports*, 3, 18-23. <http://dx.doi.org/10.1016/j.aqrep.2015.11.003>
- Zhang, J., Feng, T., Wang, J., Wang, Y., Zhang, X. H. 2019. The mechanisms and application of quorum sensing (QS) and quorum quenching (QQ). *Journal of Ocean University of China*, 18(6), 1427-1442. <http://dx.doi.org/10.1007/s11802-019-4073-5>