Isolation of macroalgae-associated bacteria and assessment of their quorum sensing inhibition and anti-biofilm activities against *Pseudomonas aeruginosa*

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Received: July 2026 Accepted: September 2026

Abstract

Bacterial biofilms make pathogen control in aquaculture more difficult because the extracellular matrix can protect cells and reduce their response to antimicrobial treatment. Quorum quenching has been studied as a way to reduce biofilm formation and virulence without directly killing bacterial cells. This study screened three cultivable bacteria associated with marine macroalgae for quorum-sensing inhibition and anti-biofilm activity. Strains 1 and 2 were isolated from *Acanthophora* sp., while Strain 3 was isolated from *Caulerpa* sp. Quorum-sensing inhibition was tested using the *Chromobacterium violaceum* CV026 biosensor. Cell-free supernatants (CFSs) were tested against *Pseudomonas aeruginosa* biofilms using crystal violet staining. None of the three isolates produced a detectable response in the CV026 assay across three screening runs. In the biofilm assay, Strain 3 CFS showed a 98.95% reduction in retained *P. aeruginosa* biofilm biomass compared with the untreated control. Strains 1 and 2 showed inhibition values of −24.11% and −3.74%, respectively. These results show that the three isolates exhibited different effects on *P. aeruginosa* biofilm biomass. Strain 3 showed the strongest anti-biofilm effect, and Strains 1 and 2 did not reduce retained biofilm biomass. The different responses also show that biofilm activity may depend on the bacterial isolate and the compounds released into the CFS. Strain 3 can therefore be selected for taxonomic identification and further study to determine the compounds linked to its anti-biofilm activity. Overall, the study identified Strain 3 as the most promising isolate for further research work on biofilm control in aquaculture-related bacterial pathogens and other similar applications.

Keywords: Anti-biofilm activity quorum-sensing inhibition, Macroalgae-associated bacteria, *Chromobacterium violaceu, Pseudomonas aeruginosa*, Aquaculture

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Introduction

Biofilms are surface-associated microbial communities enclosed in an extracellular polymeric matrix containing polysaccharides, proteins, lipids and extracellular DNA. This matrix promotes attachment and creates chemical and physiological heterogeneity that can reduce antimicrobial penetration and support tolerant subpopulations (Di Martino, 2018; Uruén *et al*., 2020). In aquaculture systems, biofilms may harbour opportunistic pathogens on tank walls, pipelines and other wetted surfaces, complicating sanitation and increasing the risk of recurrent exposure. Measures that limit attachment, matrix development or virulence expression are therefore relevant complements to conventional antimicrobial control (Lim *et al*., 2025; Lubis *et al*., 2024; Yin *et al*., 2021).

Quorum sensing is a cell-density-dependent regulatory process in which bacteria produce and detect signalling molecules to coordinate collective phenotypes. Many Gram-negative bacteria use N-acyl homoserine lactones (AHLs) to regulate functions that include virulence-factor production and biofilm development (Papenfort & Bassler, 2016). Quorum-sensing inhibition or quorum quenching can act by limiting signal synthesis, degrading the signal or blocking signal reception. Its value must nevertheless be demonstrated experimentally because activity is signal- and assay-dependent, and resistance or compensatory responses remain possible (Defoirdt, 2025).

Marine host-associated microbiomes are potential sources of quorum-quenching and anti-biofilm metabolites. Macroalgal surfaces support diverse bacterial assemblages shaped by host chemistry and environmental conditions, and signalling interactions can influence community structure (Adouane *et al*., 2024). Marine bacterial isolates and extracts have shown quorum-sensing inhibition or anti-biofilm activity in previous screens, including macroorganism-associated bacteria evaluated against aquaculture pathogens. Recent work with *Bacillus velezensis* D-18 further illustrates that confirmed interference with quorum-regulated phenotypes can coincide with reduced *Vibrio* biofilm formation (Monzón-Atienza *et al*., 2024).

Quorum-sensing inhibition and biofilm inhibition are related but not interchangeable endpoints. Biofilm biomass can decrease through growth inhibition, impaired attachment, matrix degradation or altered surface chemistry without detectable interference in the AHL pathway (Cady *et al*., 2012; Hori & Matsumoto, 2010). Conversely, quorum-sensing biosensors only detect the signals and receptors they are designed to test. Therefore, activity against other signal types may not be detected. Weak activity may also be missed if it is below the detection limit of the assay (Papenfort & Bassler, 2016; Steindler & Venturi, 2007). This study therefore aimed to screen the bacteria isolated from marine macroalgae for

interference with a short-chain AHL response using *C. violaceum* CV026 and evaluate the anti-biofilm activity of their cell-free supernatants (CFSs) against *P. aeruginosa* using a crystal violet assay.

The findings of this study contribute to two of the United Nations Sustainable Development Goals (SDGs). Most directly, the work supports SDG 14 (Life Below Water), as bioprospecting of macroalgae-associated bacteria promotes sustainable use of marine biological resources and provides a route for controlling biofilm-forming pathogens in aquaculture and algal culture systems without the routine discharge of broad-spectrum antibiotics into coastal waters. Quorum-sensing- and biofilm-targeting strategies work on the bacterial phenotype and not on viability, and as such, exert less selective pressure for resistance than conventional bactericidal agents, which is also discussed in this study in relation to SDG 3 (Good Health and Well-being). These approaches form part of the wider agenda to control antimicrobial resistance in the shared environmental reservoir connecting aquaculture, the environment and human health.

Materials and methods

Study design and macroalgal material

Macroalgal material comprising *Halymenia* sp., *Sargassum* sp., *Acanthophora* sp. and *Caulerpa* sp. were collected from Port Dickson, Negeri Sembilan, Malaysia (2°24′50.7″N, 101°51′16.9″E). Samples were collected from the shallow subtidal area at a water depth of about 0.5–1.0 m. Three healthy specimens of each macroalgal taxon were collected, giving a total of 12 specimens. Specimens of the same taxon were collected from separate thalli located at least 1 m apart to reduce repeated sampling of the same individual. The samples were collected using sterile forceps and placed separately in sterile sampling bags. A small amount of sterile seawater was added to prevent the samples from drying during transport. The samples were kept in an insulated container with ice packs at about 4-8 °C and transported to the UCSI Biotechnology Laboratory. All samples were processed within 6 h of collection.

In the laboratory, each specimen was processed separately under aseptic conditions. The macroalgal samples were rinsed three times with sterile seawater to remove sediment, debris and loosely attached microorganisms. Chemical surface sterilisation was not carried out because the study aimed to recover cultivable bacteria associated with the macroalgae and was not limited to endophytic bacteria.

Isolation and phenotypic characterisation of macroalgae-associated bacteria

Mueller–Hinton agar (MHA; Oxoid, Thermo Fisher Scientific, UK), Luria–Bertani (LB) agar (Merck, Germany) and tryptic soy agar (TSA; Merck, Germany) were prepared with sea salt adjusted to about 25 ppt. Marine Agar 2216 (Difco, Becton Dickinson, USA) was prepared according to the manufacturer's instructions. The pH of

the media was adjusted to 6.5–6.8. All media were autoclaved at 121 °C for 15 min.

For direct explant isolation, rinsed macroalgal tissue was cut into pieces of about 1 cm² using sterile scissors and forceps. The tissue pieces were placed directly onto MHA, LB agar, TSA and Marine Agar 2216. For isolation from tissue homogenates, 1.0 g of rinsed macroalgal tissue was placed in a sterile mortar containing 9 mL of sterile seawater adjusted to about 25 ppt. The tissue was homogenised using a sterile pestle until a uniform suspension was obtained. The homogenate was serially diluted tenfold with sterile seawater from 10^{-1} to 10^{-4}. A volume of 100 µL from each selected dilution was spread onto MHA, LB agar, TSA and Marine Agar 2216 using a sterile spreader. Each macroalgal specimen was treated as an independent biological sample. Duplicate plates were prepared for each selected dilution and culture medium.

The plates were incubated aerobically at 30 °C for 48 h. After incubation, colonies were observed based on size, shape, colour, margin, elevation, opacity and surface appearance. Colonies with clearly different appearances and stable growth after subculture were selected as separate isolates. Three bacterial isolates were selected for further study. Two isolates were obtained from homogenised *Acanthophora* sp. tissue and one isolate was obtained from homogenised *Caulerpa* sp. tissue. Each selected colony was streaked onto fresh MHA and incubated at 30 °C for 24-48 h. The streaking step was repeated until colonies with a uniform appearance were obtained. Working cultures were grown in Mueller-Hinton broth (MHB) adjusted to about 25 ppt and incubated at 30 °C until an OD_{600} of at least 0.6 was reached.

For storage, an equal volume of bacterial culture was mixed with sterile 80% (v/v) glycerol to give a final glycerol concentration of 40% (v/v). The cultures were stored at −20 °C until further use. Colony size, shape and colour were recorded from 24-48 h cultures grown on MHA. Gram staining was carried out using a standard staining method. A thin bacterial smear was prepared on a clean glass slide, air-dried and heat-fixed. The smear was stained with crystal violet for 1 min and treated with Gram's iodine for 1 min. The slide was decolourised with 95% ethanol for about 10-20 s and counterstained with safranin for 1 min. The slide was rinsed with distilled water between the required steps and allowed to air-dry. Gram reaction and cell shape were observed using a light microscope with a 100× oil-immersion objective, giving a total magnification of 1000×. Three independently prepared smears were examined for each isolate.

Preparation of cell-free supernatants

Each selected bacterial isolate was inoculated into MHB adjusted to about 25 ppt and incubated at 30 °C with shaking until the culture reached an OD_{600} of at least 0.6. The cultures were centrifuged at 6000 ×g and 4 °C for 10 min. The supernatant was collected without disturbing the pellet and passed

through a sterile 0.-µm syringe filter. For the confirmatory assay, 10 mL of the filtered supernatant was centrifuged again at 6000 ×g and 4 °C for 15 min. The supernatant was collected and used as the cell-free supernatant (CFS). The pH of each CFS was measured before use. When needed for comparison between isolates, the pH was adjusted to 6.5-6.8 using sterile HCl or NaOH. Sterility of the CFS was checked by spreading 100 µL onto MHA and incubating the plates at 30 °C for 48 h. The CFS was considered free of cultivable bacterial cells when no colonies were observed. The CFS was used immediately after preparation or stored at 4 °C for no longer than 24 h before use.

CV026 biosensor validation and quorum-sensing inhibition screening

Chromobacterium violaceum (CV026) was used to screen the bacterial isolates for possible quorum-sensing inhibitory activity. CV026 was grown in LB broth at 30 °C for 18-24 h. The overnight culture was adjusted to about 1×10^7 CFU mL^{-1} before preparation of the assay plates. LB agar containing N-hexanoyl homoserine lactone (C6-HSL; Sigma Aldrich, USA) at a final concentration of 0.5 µM was used for the CV026 assay. The adjusted CV026 culture was spread evenly over the agar surface to form a uniform lawn. Sterile 6-mm filter-paper discs were placed on the agar. A volume of 20 µL of CFS from each bacterial isolate was added to separate discs. The negative control contained 20 µL of sterile MHB that had been processed in the same way as the bacterial cultures. The plates were incubated at 30 °C for 24 h. A normal CV026 response was shown by purple violacein production. A colourless or non-pigmented zone around a test disc within the purple bacterial lawn was recorded as a possible quorum-sensing inhibitory response. A clear zone with no visible bacterial growth was considered possible antibacterial activity rather than quorum-sensing inhibition. The assay was repeated in three independent experiments. The CV026 response was recorded as positive when a non-pigmented zone was present without complete inhibition of bacterial growth. The diameter of the non-pigmented zone, including the 6-mm disc, was measured in millimetres when the zone boundary was clear.

Crystal violet biofilm assay

The effect of CFS from the three selected isolates on biofilm formation by *Pseudomonas aeruginosa* was tested using a crystal violet assay in sterile flat-bottom 24-well plates. *P. aeruginosa* was grown in MHB at 37 °C until the exponential growth phase. The bacterial suspension was adjusted to about 2×10^6 CFU mL^{-1} before use. The medium blank contained 1,000 µL of sterile MHB. The untreated growth control contained 500 µL of sterile MHB and 500 µL of *P. aeruginosa* suspension. Each treatment contained 500 µL of CFS and 500 µL of *P. aeruginosa* suspension. The final volume was 1,000 µL per well. The final *P. aeruginosa* concentration was about 1×10^6 CFU mL^{-1}. A CFS

blank containing 500 µL of CFS and 500 µL of sterile MHB was also included for each isolate.

The plates were incubated without shaking at 37 °C for 24 h. After incubation, the liquid was carefully removed from the side of each well without touching the bottom. Fresh treatment mixtures containing the same volumes of newly prepared *P. aeruginosa* culture and the corresponding CFS were added slowly to the wells. The plates were incubated for another 24 h at 37 °C. After the second incubation, the liquid was carefully removed. Each well was washed three times with 1 mL of sterile distilled water to remove unattached cells. The plates were left to air-dry. Then, 500 µL of 0.1% (w/v) crystal violet was added to each well and left at room temperature for 1 min. The stain was removed, and the wells were washed three times with sterile distilled water. The plates were then air-dried completely. The crystal violet attached to the biofilm was dissolved by adding 500 µL of 30% (v/v) acetic acid to each well. The plates were left at room temperature for 10 min. The solution was mixed gently, and absorbance was measured at 570 nm (OD_{570}) using a microplate reader. Each treatment was tested in three technical replicates. The assay was repeated three times using separately prepared *P. aeruginosa* cultures and CFS preparations. Each separate experiment was treated as one biological replicate. The blank OD_{570} value was subtracted from each sample value before calculation. Biofilm inhibition was calculated as:

$$\text{Biofilm inhibition (\%)} = [(OD_{control} - OD_{treatment}) / OD_{control}] \times 100$$

Where $OD_{control}$ is the mean blank-corrected OD_{570} of the untreated *P. aeruginosa* control and $OD_{treatment}$ is the mean blank-corrected OD_{570} of the CFS treatment.

A positive value showed lower crystal-violet-stained biofilm biomass than the untreated control. A value of 0% showed no difference from the control. A negative value showed higher stained biofilm biomass than the untreated control.

Results

Colony morphology and characterization of bacterial isolates

Three morphologically distinct isolates were selected. Strains 1 and 2 originated from homogenised *Acanthophora* sp. and differed in colony pigmentation. Strain 3 originated from homogenised *C. taxifolia* and produced smaller orange colonies (Table 1).

Strain 1 stained predominantly purple and appeared as short rods, whereas Strains 2 and 3 stained predominantly pink-red. The dense smear initially obtained for Strain 3 reduced confidence in its cellular morphology; later, less-concentrated smears supported a Gram-negative, short-rod interpretation (Table 2).

Quorum sensing inhibition screening of bacterial isolates

No non-pigmented halo was observed around any isolate preparation in the three screening runs. Thus, none produced a detectable inhibitory effect on the AHL 1-induced CV026 response under the conditions tested (Table 3).

Table 1: Macroalgal source and colony morphology of the bacterial isolates.

Isolate	Macroalgal Source	Colony Size	Colony Pigmentation
Strain 1	*Acanthophora sp.*	Medium	Yellow
Strain 2	*Acanthophora sp.*	Medium	Light-yellow
Strain 3	*Caulerpa taxifolia*	Small	Orange

Table 2: Gram-staining appearance and cellular morphology of the bacterial isolates.

Isolate	Gram-Stain Appearance	Observed Morphology	Arrangement	Interpretation
Strain 1	Predominantly purple/violet	Short rod-shaped cells (bacilli/coccobacilli-like)	Mostly dispersed, some small aggregates	Suggestive of Gram-positive bacilli
Strain 2	Predominantly pink/red	Small short rods/coccobacilli-like cells	Numerous cells distributed throughout the field	Suggestive of Gram-negative bacilli
Strain 3	Predominantly pink/red	Very small rounded to short rod-like cells	Densely packed with substantial aggregation	Suggestive of Gram-negative cells; interpretation less certain due to dense smear

Table 3: Qualitative CV026 quorum-sensing inhibition screening results.

Isolate	Replicate 1	Replicate 2	Replicate 3	Overall Interpretation
Strain 1	No halo observed	No halo observed	No halo observed	No detectable QSI response
Strain 2	No halo observed	No halo observed	No halo observed	No detectable QSI response
Strain 3	No halo observed	No halo observed	No halo observed	No detectable QSI response
Neutral control	No halo observed	No halo observed	No halo observed	Expected neutral response

Anti-biofilm activity of cell-free supernatants against p. aeruginosa

The mean crystal violet measurements yielded calculated biofilm-inhibition values of -24.11%, -3.74% and 98.95% for Strains 1, 2 and 3, respectively (Table 4).

Table 4: Descriptive change in retained *P. aeruginosa* biofilm biomass after treatment with isolate CFS.

Isolate (CFS source)	Macroalgal Source	Mean Biofilm Inhibition (%)	Interpretation
Strain 1	*Acanthophora sp.*	-24.11	Numerical increase in biofilm biomass (no inhibition)
Strain 2	*Acanthophora sp.*	-3.74	Negligible / no meaningful inhibition
Strain 3	*Caulerpa* sp.	+98.95	Strong inhibition of biofilm formation

The negative values for Strains 1 and 2 represent greater retained biomass than the untreated control. Strain 3 produced substantially less retained biomass than the control. Overall, the results obtained in this study show that none of the three isolates exhibited detectable AHL-mediated quorum-sensing inhibition activity, yet one isolate (Strain 3) exhibited strong anti-biofilm activity against *P. aeruginosa*.

Discussion

The main finding was the large reduction in retained *P. aeruginosa* biofilm biomass with Strain 3 CFS, which showed a 98.95% reduction compared with the control. This result suggests that Strain 3 should be selected for further testing. However, this finding alone does not prove quorum-sensing inhibition and does not show the reason for the lower crystal violet signal.

The CV026 assay did not show a detectable response for any of the three isolates under the conditions used in this study. Similar results have been reported in previous screening studies, where only a small number of bacterial isolates showed activity in the CV026 assay. Romero *et al*. (2011) screened 166 marine bacterial isolates and found that only 24 isolates, or 14.4%, showed quorum-quenching activity against C6-HSL using CV026. The number of active isolates also differed between sample sources. Only 9.4% of isolates from fish tank sediment and 6.3% from water tank biofilm showed activity. Devaraj *et al*. (2017) screened 147 soil actinobacterial strains and found that only three strains reduced violacein production in CV026. These strains were identified as *Micromonospora*, *Rhodococcus* and *Streptomyces*. These findings show that CV026 activity is not found in all bacterial isolates and can differ between strains. The lack of a detectable response from Strains 1, 2 and 3 in the present study is similar to this pattern.

Strain 3 showed a different result in the biofilm assay, where its CFS strongly reduced retained *P. aeruginosa* biofilm biomass. This shows that the response of Strain 3 differed between the CV026 and biofilm assays. A similar pattern has been reported in studies where only some bacterial isolates showed quorum-sensing activity, while others showed stronger effects on biofilm formation. Wijaya *et al*. (2023) screened 40 marine actinobacterial isolates and found anti-quorum-sensing activity in 10 isolates using *C. violaceum*

ATCC 12472. Eight isolates also showed inhibition in the CV026 assay. Their extracts also reduced biofilm formation in several pathogenic bacteria, including *P. aeruginosa*. These results show that the activity can vary between bacterial isolates and between the responses measured. In the present study, the main effect of Strain 3 was the strong reduction in *P. aeruginosa* biofilm biomass rather than a detectable response in the CV026 assay.

Strains 1 and 2 gave negative inhibition values, which means that more crystal violet was retained than in the control. This result was different from Strain 3, which showed a strong reduction in *P. aeruginosa* biofilm biomass. Similar differences between bacterial strains have been reported in previous studies. Aljohani *et al*. (2023) tested CFS from 25 human commensal and related bacteria against *P. aeruginosa*. Most of the CFS did not reduce biofilm formation. CFS from *Escherichia coli* Nissle 1917 reduced biofilm formation in all tested *P. aeruginosa* strains, but CFS from *E. coli* ATCC 25922, *E. coli* K-12 MG165 and the wound isolate *E. coli* WIBG 2.4 did not show biofilm inhibition. This shows that anti-biofilm activity can differ even between strains of the same bacterial species. Anti-biofilm activity has also been reported for CFS from other bacteria. Drumond *et al*. (2023) tested cell-free spent media from *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactiplantibacillus plantarum* and *Lactobacillus johnsonii* against *P. aeruginosa* ATCC 9027 and ATCC 27853. The supernatants reduced biofilm formation under different treatment conditions, with inhibition ranging from about 40% to 80%. CFS from *Corynebacterium amycolatum* ICIS 99 was also reported to reduce *P. aeruginosa* biofilm formation and exopolysaccharide production, which is an important part of the biofilm matrix (Gladysheva & Cherkasov, 2023). These studies show that the effect of CFS on *P. aeruginosa* biofilm can vary between bacterial strains. A similar pattern was seen in the present study, where Strain 3 showed strong anti-biofilm activity, while Strains 1 and 2 did not reduce the retained biofilm biomass. The different responses may be linked to differences in the compounds released by each isolate. The strong activity of Strain 3 may therefore be a specific property of this strain rather than a common effect of all bacterial CFS.

Future work can focus on confirming the activity of Strain 3 and finding out more about the isolate. Molecular identification using 16S rRNA gene sequencing can be done first, followed by whole-genome sequencing if needed (Chun *et al*., 2018; Janda & Abbott, 2007). More biofilm assays using independent cultures, biological and technical replicates, and suitable statistical analysis can help confirm the result (Aljohani *et al*., 2023). Planktonic growth and cell viability can also be measured to better understand the anti-biofilm effect or bacterial growth (Aljohani *et al*., 2023; Drumond *et al*., 2023). Heat treatment, protease treatment and size fractionation can help

show the type of active compound in the CFS (Aljohani *et al*., 2023). More quorum-sensing tests and direct measurement of signalling molecules can then be used to check whether the activity of Strain 3 is related to quorum quenching (Ortori *et al*., 2014).

Conclusion

Three macroalgae-associated bacterial isolates were screened for quorum-sensing inhibition and anti-biofilm activity. None of the isolates showed a detectable response in the CV026 assay. In the biofilm assay, Strain 3 CFS showed a 98.95% reduction in retained *P. aeruginosa* biofilm biomass, while Strains 1 and 2 did not reduce biofilm biomass. These results show that the three isolates differed in their biofilm activity and that Strain 3 exhibited the strongest anti-biofilm effect. Therefore, strain 3 selected for further identification and study of the compounds linked to its activity. The findings support its potential for further research on biofilm control in aquaculture-related bacterial pathogens.

Acknowledgment

The authors thank the technical staff of the Faculty of Applied Sciences, UCSI University, for assistance with laboratory facilities and equipment maintenance.

Funding

This work was supported by UCSI University through the Research Excellence and Innovation Grant (REIG-FAS-2025/004).

Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

Conceptualization: HTT, MJH, MK; Methodology: HTT, HTJC, JS, YYL; Investigation: HTT, HTJC, JS; Data analysis: HTT, HTJC, JS; Writing – original draft: HTT, HTJC, JS; Writing – review and editing: SMP, MJH; Supervision: YYL, SMP, MJH. All authors read and approved the final manuscript.

Data availability

The data generated and analysed during this study are available from the corresponding author upon reasonable request.

Ethics approval

Ethical approval was not required for this study because the work involved bacterial isolates and did not involve human participants or experimental animals.

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