

Anti-Bacterial and Anti-Quorum Sensing Potential of Seaweed-Associated Bacteria from Pulau Bidong, East Coast of Peninsular Malaysia

Siti Nazatul Mohd Yasim¹, Noor Syazwani Omar¹, Shumpei Ichata¹,
Natrah Fatin Mohd Ikhsan², Mohd Effendy Abd. Wahid¹ and Sharifah Noor Emilia^{1*}

ABSTRACT

Bacteria associated with seaweeds are highly diverse and have abundant sources of bioactive compounds. The antagonistic properties of seaweed-associated microbes from Pulau Bidong have not been explored, and few studies have been published on bacteria associated with seaweeds in Malaysia. Six seaweed species (*Caulerpa serrulata*, *C. peltata*, *C. racemosa*, *Lobophora variegata*, *Hypnea pannosa* and *Dictyota dichotoma*) in Pulau Bidong were screened for antibacterial and anti-quorum sensing activities. A total of 26 bacterial strains were isolated from six species of seaweed. It was observed that more bacteria were isolated from the surface of seaweed (epiphytic bacteria) than from within the tissues (endophytic bacteria). Agar well diffusion method was used to screen for antibacterial activity against the marine pathogenic bacterium *Vibrio alginolyticus*. Results showed that isolates LV Epi 1, LV Epi 2, LV Epi 4, CS Epi 1, and CP Epi 3 had significantly higher antibacterial activity against *V. alginolyticus* than other isolates. These isolates were further examined by AHL (acyl homoserine lactone) degradation assay to screen for anti-quorum sensing by using *Chromobacterium violaceum* (CV026) as the biosensor strain. Results showed that isolate LV Epi 2 had the strongest degradation activity. Isolates with positive antibacterial and anti-quorum sensing activities were identified as *Kocuria haloterans* (LV Epi 4), *V. alginolyticus* (LV Epi 2) and *Exiguobacterium indicum* (CS Epi 1) by using 16S rRNA gene sequence. These bacteria associated with seaweed were discovered to be valuable sources of both natural antibacterial and anti-quorum sensing compounds.

Keywords: Acyl homoserine lactone, *Chromobacterium violaceum*, Macroalgae, *Vibrio alginolyticus*

INTRODUCTION

Seaweeds (macroalgae) are a ubiquitous group of photosynthetic organisms that play a crucial part in aquatic ecosystems (Egan *et al.*, 2012). Seaweeds provide many benefits and advantages to other living organisms; for example, they produce a wide variety of metabolites that are beneficial for humans. With high content of bioactive compounds (sterols, terpenoids and brominated phenolic), seaweeds are also known

as a potential source of bioactive natural products (Kolanjinathan and Stella, 2011; Wichachucherd *et al.*, 2019; Klomjit *et al.*, 2021). According to Raghunathan *et al.* (2013), compounds with antiviral, antifungal and antibacterial activity have been detected in green, brown and red seaweeds. Furthermore, according to the review by Natrah *et al.* (2011), numerous aquatic organisms, including microalgae, seaweeds, invertebrates and bacteria, have the potential to disrupt or block quorum sensing (QS) signaling.

¹Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Terengganu, Malaysia

²Faculty of Agriculture, Universiti Putra Malaysia, Selangor, Malaysia

* Corresponding author. E-mail address: emilia@umt.edu.my

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In the past decade, interest in the commercial usage of marine macroalgae has risen, and has led to the screening of macroalgae for beneficial activities that can be applied in various bio-products (Chee *et al.*, 2011; Lafarga *et al.*, 2020; Arguelles, 2021). This includes the screening for antibacterial and anti-quorum sensing activity against bacterial infections in animals and humans (Natrah *et al.*, 2011). Based on the review by Singh *et al.* (2014), the extraction of bioactive compounds from seaweed-associated bacterial communities has recently increased due to their broad-spectrum antimicrobial activity against bacteria, fungi, and viruses. Interestingly, many marine bacteria have been shown to produce secondary metabolites that display antibacterial properties (Singh and Reddy, 2014). Additionally, seaweed extract has been found to control bacterial colonization by interfering with bacterial quorum sensing (QS) systems, which regulate bacterial cell-to-cell communication (Hollants *et al.*, 2012). According to Kanagasabhpathy *et al.* (2009), *Bacillus* sp., *Pseudoalteromonas* sp., *Pseudomonas* sp., and *Vibrio* sp. isolated from the brown seaweed *Calpomenia sinuosa* were capable of quorum sensing inhibition (QSI). These bacteria may prevent biofilm formation caused by fouling bacteria, which leads to macrofouling on the surface of seaweeds. The antibiotics produced by several marine bacteria associated with seaweed make them more suitable for use in the control of fish or shellfish pathogens in marine ecosystems (Egan *et al.*, 2012). The disruption of bacterial communication by QSI is considered a novel and environmentally friendly approach to avoid harmful consequences for algal health and fecundity (Meena *et al.*, 2020).

Malaysia, as a tropical country, has high diversity of seaweeds and seagrasses. It is estimated that there are about 375 seaweed species in Malaysian waters (Phang, 2006). Many studies have related the abundance and diversity of seaweeds to antibacterial activity and bioactive compounds in Malaysia (Phang, 2018). Pulau Bidong is an island located to the northwest of Kuala Terengganu, off the east coast of Malaysia. A previous study by Sidik *et al.* (2012) mainly explored the distribution and diversity of macroalgae on coral reefs in Pulau Bidong. Despite the high abundance and diversity

of seaweeds around the island, little is known about the potential utilization of even the common species as a source of natural bioactive compounds. Accordingly, this study aims to investigate some chemical and functional properties of the seaweed-associated microbial community from Pulau Bidong.

MATERIALS AND METHODS

Collection of samples

Seaweed samples were collected from the intertidal zone of Pulau Bidong (Sidik *et al.*, 2012) during the dry season (February-October). Seaweed samples were collected in situ at 0.5-2.0 m depth by snorkelling and SCUBA diving. These methods give the collector sufficient time to observe and record the seaweeds in their natural habitat and to collect samples (Sidik *et al.*, 2012). Collection was mainly done in rocky, sandy and coral areas. Complete plants including the holdfast were removed from the substrate and placed in plastic bags. Many specimens could be removed from their substrates by hand but those closely adhering to rocks were removed with a knife (Sidik *et al.*, 2012). Overall, a total of six common species were collected: three green seaweeds (*Caulerpa peltata*, *C. racemose* and *C. serrulate*), two brown seaweeds (*Lobophora variegata* and *Dictyota dichotoma*), and one red seaweed (*Hypnea pannosa*).

Bacterial strains

The marine pathogen *Vibrio alginolyticus* ATCC17749, obtained from the Institute of Marine Biotechnology (IMB), Universiti Malaysia Terengganu, was used as the test pathogen to assess antibacterial activity. *Chromobacterium violaceum* (CV026), from the Faculty of Agriculture, Universiti Putra Malaysia (UPM), was used as the biosensor strain to determine anti-quorum sensing activity.

Isolation of endophytic and epiphytic bacteria associated with seaweeds

Isolation of endophytic bacteria was done by cutting the seaweed into small pieces and homogenizing with a sterile cotton swab in a tube

filled with sterile seawater (Hollants *et al.*, 2011). Epibiotic bacteria were obtained by scraping the surfaces of seaweeds with a sterile cotton swab and inoculating into a tube filled with sterile seawater (Kanagasabhpathy *et al.*, 2006). The samples were centrifuged at 3,000 rpm for 5 min. The supernatant was taken and serially diluted with sterile seawater, spread onto a Marine Agar plate and incubated at 28 °C for 24 h. After incubation, the colonies of bacteria on the Marine Agar plates were observed. Pigmented colonies were chosen at random from each plate and streaked onto Marine Agar to obtain pure cultures, and then stored in 40% glycerol as stock in a freezer at -80 °C. Bacterial colonies were further tested for antagonism.

DNA-based identification of isolated bacteria

Genomic DNA of each isolate with positive anti-bacterial and anti-quorum sensing activity was extracted by boiling (Lou *et al.*, 1993). The extracted DNA was further evaluated by PCR for bacterial strain-specific 16S rRNA using the universal primers (forward) 8F (5'-AGAGTTTGA TCATGGCTCAG-3') and (reverse) 1492R (5'-G GTTACCTTGTTACGACTT-3'). Amplifications were carried out in PRO S thermal cycler (Eppendorf, Germany). The cycling conditions were as follows: one cycle at 95 °C for 4 min for initial denaturation, followed by 30 cycles at 95 °C for 30 s for denaturation, 56 °C for 30 s for annealing, 72 °C for 1 min 30 s for extension, and finally elongation at 72 °C for 7 min (Lou *et al.*, 1993). The amplicons were mixed with 1 µL DNA loading dye and separated by agarose gel electrophoresis (1.7% agarose in 30 mL TBE at 100V for 20 min) in parallel with 100 bp DNA ladders (Lou *et al.*, 1993). Finally, the agarose gels were observed through gel documentation machine (Aplegen Omega Lum G) before being photographed. The amplified products were sequenced by FirstBase (Malaysia). The sequences were analyzed using the NCBI BLAST search tool, available online at <http://www.ncbi.nlm.nih.gov>.

Antibacterial assays

Antibacterial activity of 26 isolated bacterial strains was evaluated by agar well diffusion

technique (Manilal *et al.*, 2009). Marine Broth without bacteria was used as negative control and chloramphenicol (30 µg·disc⁻¹) was used as positive control. The assay was performed in triplicate in individual Petri dishes. Marine Agar plates were uniformly spread with bacterial pathogen by using a plate spreader. Thereafter, a sterile cork borer was used to make wells (6 mm diameter) in the agar. After that, 100 µL of the appropriate microbial cell suspension was added to fill each well. A zone of inhibition around the well indicated that antibacterial compound produced by the isolate was able to suppress the growth of the test pathogen (Manilal *et al.*, 2009). The diameter of the inhibition zone after 24 h of incubation at 28 °C was considered to be indicative of bioactivity. Isolates that showed significant activity were then tested by AHL (acyl homoserine lactone) degradation assay.

Detection of acyl homoserine lactones (AHL)

Standard curves of different AHL concentrations (0, 1, 2.5, 5, 7.5, 10 and 15 ppm) were prepared (Tinh *et al.*, 2007). The biosensor strain *Chromobacterium violaceum* (CV026) was cultured for two days in Luria Bertani (LB) broth containing 20 ppm kanamycin (McClellan *et al.*, 1997). The biosensor strain (100 µL) was then spread on LB agar plates. After that, 10 µL of each of AHL concentration was dropped onto the center of an LB agar plate, and then incubated at 28 °C. After 24 h, the diameter of the purple violacein zone was measured and correlated to the concentration of AHL.

AHL degradation assay

Bacterial colonies from the pure culture stock were re-grown overnight in 5 mL of Marine Broth (Tinh *et al.*, 2007). Then, 50 µL of bacterial culture was inoculated into a 50 mL Erlenmeyer flask containing 5 mL of buffered Marine Broth supplemented with 10 ppm AHL and incubated overnight in an incubator shaker. The next day, the inoculums were filter-sterilized with 0.2 micron filter, then 10 µL of the filtrate was spotted onto the middle of an LB plate spread with CV026. After that, the degradation activity was compared with the standard curve. The Marine Broth with 10 ppm AHL only (without any bacteria) served as control.

Statistical analysis

Dependent T-test using SPSS 25.0 was used to analyze the difference between treatments and controls. The test results with $p < 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

Epiphytic and endophytic bacterial isolation from green, red and brown seaweeds

From the six species of seaweed sampled in Pulau Bidong, 26 strains of bacteria were isolated. The highest number of epiphytic bacteria were isolated from the green seaweed *Caulerpa serrulata* (6×10^4 CFU·mL⁻¹) (Figure 1). Epiphytic bacteria isolated from the red seaweed *Hypnea pannosa* (2.4×10^4 CFU·mL⁻¹) were the least abundant (Figure 1). However, isolates from *H. pannosa* contained the most endophytic bacteria (5.5×10^4 CFU·mL⁻¹) compared to other species of seaweed (Figure 1). Meanwhile, endophytic bacteria isolated from the green seaweed *C. peltata* were the least abundant (1.5×10^4 CFU·mL⁻¹) (Figure 1). No endophytic bacteria were isolated from the brown seaweed *Lobophora variegata*, as this species has

a flat surface and also has no thallus (Ahmad, 1995) in which bacteria can live. The other seaweeds collected all have a thallus. It has been described that differentiated parts of the macroalgae form individual morphological niches that have distinct functions; therefore, it is logical to suggest that bacterial associations differ correspondingly (Morrissey *et al.*, 2019). The composition of seaweed-associated bacterial communities is based on the seaweed species, the season and the age of the thalli (Martin *et al.*, 2014).

More bacteria were isolated from the surface of these six species of seaweed than from within the seaweeds. Epiphytic bacterial communities are fast colonizers compared with endophytic bacteria (Singh and Reddy, 2014), because the outer surface of the seaweed provides a favourable substrate (Singh, 2013). Besides, bacterial communities that live on the surface of the seaweeds are dynamic, greatly complex, and comprised of different species of bacteria (Burke *et al.*, 2011). Moreover, the seaweed surfaces also provide nutrients for bacteria to multiply. According to Janaki *et al.* (2013), communities of epiphytic bacteria are crucial for the development of seaweed morphology, and bacteria with antibacterial properties can protect seaweeds from pathogens and surface colonization by competing organisms.

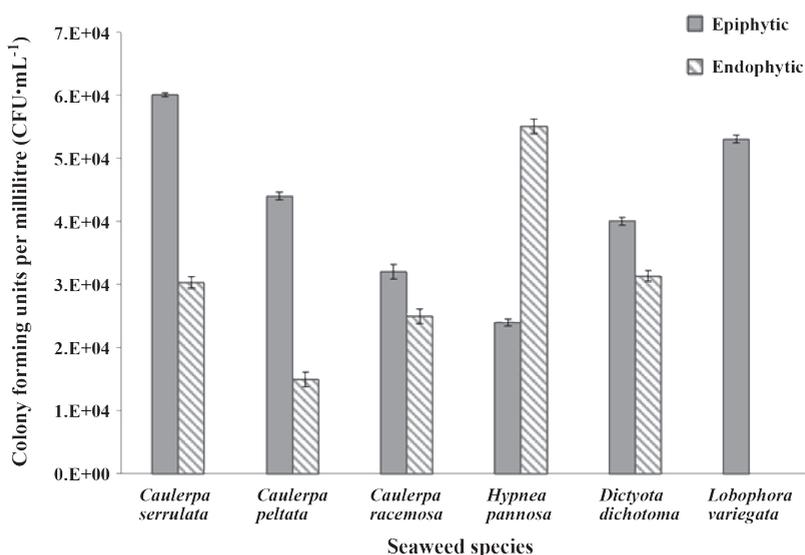


Figure 1. Comparison of colony forming units per millilitre (CFU·mL⁻¹) between epiphytic and endophytic bacteria that were isolated from different species of seaweed.

Antibacterial and anti-quorum sensing activity from bacteria associated with seaweeds

Of the bacteria isolated from seaweeds in Pulau Bidong, the antibacterial assay showed that nine isolates were able to inhibit the bacterial pathogen *Vibrio alginolyticus* (ATCC17749), but only five isolates showed significant difference ($p < 0.05$) from the positive control (Table 1). The bacterial strains isolated from the brown seaweed were more active than the bacteria isolated from the green and red seaweeds. These results are similar to the studies by Wiese *et al.* (2009), Sugathan *et al.* (2012) and Susilowati *et al.* (2015), who all reported that the bacteria associated with brown seaweeds showed higher antibacterial activity than bacteria associated with red and green seaweeds.

The antibacterial activity of the seaweed-associated bacteria observed herein indicates that they produce metabolites that can prevent the attachment and colonization of certain bacterial pathogens in natural environments (Phang, 2018). It has also been shown that epiphytic bacteria from seaweed have the ability to influence the composition of the bacterial community existing on seaweed surfaces by the production of secondary metabolites

with antimicrobial properties (Boyd *et al.*, 1999). Marine bacteria may obtain essential nutrients such as vitamins, polysaccharides and fatty acids from their host, while emitting products such as amino acids, antibiotics and toxins that favor the host's development and metabolism or improve the host's chemical defenses (Armstrong *et al.*, 2004). Seaweeds that are traditionally used for both nutritional and medicinal purposes offer a valuable source of bioactive molecules with a wide spectrum of biological activities (anti-inflammatory, antitumor, antiviral, antimicrobial, neuroprotective), as demonstrated both *in vitro* and *in vivo* (Rima *et al.*, 2022). Interestingly, several researchers have reported that bacteria have the potential to disrupt or block QS signaling (Natrah *et al.*, 2011).

All five isolates that showed better antibacterial activity than the positive control were further investigated for anti-quorum sensing activity. *Chromobacterium violaceum* CV026, a mutant beta-proteobacterium used as the biosensor strain is known to detect and respond to a wide range of AHL molecules by inducing the synthesis of violacein, which is a purple pigment antibiotic (Defoirdt *et al.*, 2011). According to McClean *et al.* (1997), AHL signal molecule is the most active

Table 1. Antibacterial activity of bacteria associated with different seaweed species against *Vibrio alginolyticus*.

Seaweed Species	Zone of Inhibition	
	Bacterial Strain	Diameter (mm)
<i>Caulerpa peltata</i>	CP Epi 1	5.00±0.05 ^{ns}
	CP Epi 2	8.00±0.05 ^{ns}
	CP Epi 3	13.00±0.00*
<i>Caulerpa serrulata</i>	CS Epi 1	15.00±0.10*
<i>Lobophora variegata</i>	LV Epi 1	11.00±0.06*
	LV Epi 2	12.00±0.06*
	LV Epi 3	9.00±0.10 ^{ns}
	LV Epi 4	16.00±0.20*
<i>Dictyota dichotoma</i>	DC Endo 3	5.00±0 ^{ns}
	Chloramphenicol (Positive control)	10.00±0
	Marine Broth (Negative control)	0±0.10

Note: Antibacterial activity was determined as inhibition zone (mm of diameter) measured from the edge of the disc after 24 h±SD; * significant ($p < 0.05$) difference from the positive control; ns = not significant ($p > 0.05$)

molecule in inducing the synthesis of violacein, the natural *C. violaceum* AHL. Only three of the five isolates showed AHL degrading activity (Table 2). Bacterial strains (LV Epi 2, LV Epi 4 and CS Epi 1) with the highest antibacterial activity also showed high AHL degradation activity (Table 2). The LV Epi 2 strain showed the highest degradation activity, with the diameter of purple violacein degraded to 0 cm (equal to AHL concentration of 0 ppm) (Table 2). Romero *et al.* (2010) reported that bacteria isolated from the brown seaweed *Fucus vesiculosus* had the ability to interfere with the AHL biosensor *C. violaceum* (CV026). In our study, the bacteria isolated from the brown seaweed *L. variegata* (LV Epi 2 and LV Epi 4) inhibited and degraded the AHL biosensor *C. violaceum* (CV026). In a study by Kanagasabhpathy *et al.* (2009), 12 % of the bacterial strains that were isolated from the surface of the brown seaweed *Colpomenia sinuosa* showed the ability to produce quorum sensing inhibitors (QSI), which was observed from the pigmentation inhibition on *Serratia rubidaea* JCM 14263 without affecting its growth. Bacterial isolates showing producing of QSI have been found from the genera *Bacillus*, *Pseudomonas*, *Pseudoalteromonas* and *Vibrio*. In our study, AHL-degrader strains LV Epi 2 and LV Epi 4 isolated from the brown seaweed

L. variegata showed the highest antibacterial activity and were able to degrade and inhibit the AHL biosensor CV026 after 24 h. Interestingly, there are no previous reports on the bacteria associated with this seaweed for their capability to produce both quorum sensing inhibitor compounds and antibacterial activity, as presented in our study.

Based on our molecular analyses (Table 3), isolate LV Epi 4 is closely related to the members of the genus *Kocuria*. According to Tang *et al.* (2009), *Kocuria haloterans* (salt tolerating) isolated from saline soil is Gram-positive, coccoid, non-motile and non-endospore-forming. To date, *Kocuria* spp. have been found on the surface of the green seaweed *Caulerpa cylindracea* (Rizzo *et al.*, 2016). However, no *Kocuria* species (including *Kocuria haloterans*) have been reported as having potential as an antibiotic in antibacterial and anti-quorum sensing isolates from seaweed.

The bacterial epiphyte (LV Epi 2) isolated from the brown seaweed *Lobophora variegata* and identified as *Vibrio alginolyticus* was found to show strong quorum sensing inhibition against AHL based on biosensor strain *Chromobacterium violaceum* CV026. *Vibrio alginolyticus* is a halophilic

Table 2. Results of AHL degradation assay.

Seaweed Species	Isolate	Diameter (cm)	AHL concentration (ppm)
<i>Lobophora variegata</i>	LV Epi 2	0	0
	LV Epi 4	1.7	0.08
<i>Caulerpa serrulata</i>	CS Epi 1	2.0	0.08

Table 3. Phylogenetic affiliation of bacterial strains isolated from different species of seaweed based on BLAST analysis.

Seaweed Species	Isolate	Closest relative	Percent of Similarity (%)	Activity
<i>Lobophora variegata</i>	LV Epi 4	<i>Kocuria haloterans</i>	93	Antibacterial and anti-quorum sensing
	LV Epi 2	<i>Vibrio alginolyticus</i>	96	Antibacterial and anti-quorum sensing
<i>Caulerpa serrulata</i>	CS Epi 1	<i>Exiguobacterium indicum</i>	96	Antibacterial and anti-quorum sensing

(salt-tolerant) Gram-negative bacterium that occurs naturally in both temperate marine and estuarine environments. As an opportunistic pathogen, it can cause mild to acute infections in marine animals and humans. Although *Vibrio* spp. are aquatic pathogenic organisms, they have the ability to inhibit or inactivate the QS signals in other bacteria (Kanagasabhpathy *et al.*, 2009). Besides, *Vibrio* spp. are abundant on the surfaces of the green algae *Ulva* sp. and *Porphyra* sp. (Duan *et al.*, 1995). It is also abundant on the red algae *Gelidium* sp. and *Gracilaria* sp. According to Rui *et al.* (2009), the major virulence factor of *V. alginolyticus* is an extracellular alkaline serine protease (ASP) that is closely associated with the quorum sensing system, and the production of ASP occurs in a cell-density-dependent manner. When the expression of LuxR regulator is at a low level, it reduces the expression of ASP, and it is speculated that LuxR activates the transcription of the ASP gene through direct binding to its promoter region. In this study, interestingly *V. alginolyticus* was capable of inhibiting bacterial growth and quorum sensing. Apart from this work, no studies have examined QSI activity in *Vibrio* spp. isolated from the brown seaweed *L. variegata*.

Reports have shown that CS Epi 1 strain is closely related to members of the genus *Exiguobacterium*. Rizzo *et al.* (2016) reported that this genus has been found on the surface of *C. cylindracea*. These bacteria have been applied in the environment as a bioremediation agent to treat organic materials including pesticides and heavy metals. In the present study, *Exiguobacterium indicum* isolated from green seaweed *C. serrulata* surfaces exhibited a wide spectrum of activity against the tested bacterial pathogen. Furthermore, *E. indicum* has not been previously isolated from any species of seaweed.

CONCLUSION

In the present study, six seaweed species collected from Pulau Bidong were subjected to epiphytic and endophytic isolation. In total, 26 bacterial strains were isolated and five of them showed the highest antibacterial activity against the

pathogen *Vibrio alginolyticus* relative to a positive control. Meanwhile, anti-quorum sensing screening revealed that three isolates (*Kocuria haloterans*, *V. alginolyticus* and *Exiguobacterium indicum*) showed the strongest anti-quorum sensing activity. Thus, the present study has indicated that bacteria associated with seaweeds may produce a variety of novel compounds with beneficial activities that could be used in new drugs in the fight against pathogens in the natural marine environment and in aquaculture settings. These could benefit the aquaculture industry, especially in fighting pathogens and disrupting the signals of pathogens.

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