In Vitro Antibacterial Activity of Marine Microalgae Extract against *Vibrio harveyi*

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Featured Application: Potential application of *N. oceanica*, *Isochrysis* sp. and *T. weissflogii* as inhibitory bacteria and probiotics in controlling bacterial diseases in the aquaculture industry.

Abstract: Marine microalgae may produce antibacterial substances. At the exponential phase of growth, four species of marine microalgae were examined for their potential to create secondary metabolites that limit the growth of *Vibrio harveyi*: *Nannochloropsis oceanica*, *Chaetoceros gracilis*, *Isochrysis* sp. and *Thalassiosira weissflogii*. *V. harveyi* is a pathogenic bacteria that can cause severe mortality and loss in aquaculture. Disc diffusion assay and co-culture assay were used to determine antibacterial activity. On TSA % NaCl media, the disc impregnated with microalgae and extracted with ethanol, methanol, saline water, and dimethyl sulfoxide (DMSO) was tested against *V. harveyi* at concentrations of $1.0 \times 10^5$, $10^6$ and $10^7$ CFU mL$^{-1}$. The disc diffusion assay revealed that *N. oceanica* extracted with ethanol had the largest inhibitory zone against *V. harveyi*. Meanwhile, only *N. oceanica*, *Isochrysis* sp. and *T. weissflogii* reduced the growth of *V. harveyi* ($10^5$ CFU mL$^{-1}$) in the co-culture assay ($p < 0.05$). The current findings reveal that the hydrophilic chemicals in microalgae extract have antibiotic activity against the highly virulent *V. harveyi*, which causes vibriosis, a serious disease in farmed fish and aquaculture cultivation around the world.

Keywords: microalgae; antibacterial; *Vibrio harveyi*; *Nannochloropsis oceanica*; *Chaetoceros gracilis*; *Isochrysis* sp.

1. Introduction

In recent years, the aquaculture business has grown quickly and is predicted to supply approximately 62% of fish for human demand and consumption by 2030 [1,2]. However, diseases in aquaculture could impact the aquaculture production function by destroying basic resources, lowering the physical output or unit value of a production process, lowering the efficiency of a production process, and ultimately leading to economic losses in the aquaculture sector [2]. Vibriosis is one of the most prevalent bacterial diseases and is claimed to have damaged farmed marine fish in Malaysia, resulting in a USD 7.4 million loss in 1990 [3] and severe economic loss to Asian seabass farmers in 2017 [4].

Vibriosis is associated with infections in fish, such as skin necrosis, ulceration, and scale drops on the abdomen caused by a variety of *Vibrio* spp., including *Vibrio harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. penaeicida*, and *V. splendidus* [5]. *V. harveyi*, the primary bacterium responsible for catastrophic death in fish farming, has led to massive economic loss [6]. Antibiotics and chemicals are frequently used by farmers to combat harmful organisms. However, they have been used sparingly since they are expensive,
non-biodegradable, highly biomagnified, and antibiotic resistance has grown [7]. Antibiotic overuse can result in various environmental problems, including contamination of the culture environment, organism harm, and the development of bacterial resistance that can extend to the food chain [8]. Alternatively, numerous techniques for controlling pathogenic vibriosis have been proposed, including phage therapy, short-chain fatty acid inhibition of bacterial growth, quorum-sensing disruption, probiotics, immunostimulants, vaccinations, and green water [9].

Pathogen management in aquaculture, particularly disease prevention employing herbs and phytochemicals, has garnered a considerable amount of attention in the previous decade [10]. Furthermore, microalgae can produce a variety of bioactive compounds such as carotenoids, polysaccharides, vitamins, and lipids [11]. Recently, microalgae have been frequently utilised in aquaculture as nutritional supplements, immunostimulants to strengthen immune systems, and to increase disease resistance against harmful bacteria [2]. Furthermore, they contain antibacterial and antiviral properties that could be exploited for disease prevention and management in the aquaculture industry [11]. Thus, because the habitat is in a trapped and limited area, such as in fish-rearing tanks or cage culture, the transmission of numerous viral, fungal, parasitic, and bacterial diseases easily occurs among cultured fish, which can further cause co-infections and mortality in cultured fish [12,13]. It has been found that adding microalgae to farmed fish reduces the bacterial load of larval rearing systems by reducing the number of opportunistic bacteria [14].

Furthermore, the growing desire for more ecologically friendly disease control strategies has prompted academics to investigate alternate ways with little negative effects. Thus, the primary goal of this work was to investigate the antibacterial activity effects of several marine microalgae cultures, including *Nannochloropsis oceanica*, *Chaetoceros gracilis*, *Isochrysis* sp. and *Thalassiosira weissflogii*, against *V. harveyi* at different cell viability levels.

2. Materials and Methods

2.1. Cultures and Preparation of Microalgae Extract

The species of unicellular marine microalgae were tested. *N. oceanica*, *C. gracilis*, *Isochrysis* sp. and *T. weissflogii* microalgae strains were collected from the Culture Collection of the Live Feed Culture Laboratory, Borneo Marine Research Institute, University Malaysia Sabah. Each microalgae culture was produced and maintained in 250 mL Erlenmeyer flasks with 100 mL Guillard’s F/2 media [15]. The microalgae culture was reactivated as an inoculum for four days and the starting culture concentration was $1 \times 10^5$ cells mL$^{-1}$. The cultivation conditions were set at 25 ± 1 °C with 24 h of continuous illumination at 1000 µmol photon m$^{-2}$ s$^{-1}$ given by cool white fluorescent lights. The microalgae inoculum culture was put into 500 mL Erlenmeyer flasks with 200 mL of Walney’s media [16]. After 10 days in culture, the cultivation reached the exponential phase of growth and cell density ($1 \times 10^7$ cells mL$^{-1}$). The cell viability of cell microalgae was evaluated using a Malassez haemocytometer on a daily basis. By plating 50 µL samples to tryptic soy agar (TSA) with 2% (w/v) NaCl and Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar, the viability of cell microalgae culture was tested for contamination. The microalgae culture was afterwards recovered and harvested by centrifugation at low speed (2000× g) for 10 min at 15 °C, and the algal pellet was filtered through Whatman no. 1 filter paper [17]. The extraction of microalgae biomass was carried out with four different solvents: methanol, ethanol, Dimethyl sulfoxide (DMSO), and saline water, with a ratio of 0.1 g of algal biomass for 1.0 mL of solvent. Every microalgae extract was fixed into $10^6$ cells mL$^{-1}$. Before further usage, the crude extracts were promptly refrigerated at 4 °C.

2.2. Preparation of Vibrio harveyi Inoculums

Vibrio strains were collected from the Live Feed Culture Laboratory Culture Collection at the Borneo Marine Research Institute, University Malaysia Sabah. *V. harveyi* strains were obtained from an epidemic of vibriosis in Asian seabass, *Lates calcarifer* (Bloch). *V. harveyi* stock culture was subcultured into TCBS agar plates and incubated overnight at 28 °C.
After 24 h of incubation, pure single colonies were selected and subcultured in 1.5 percent NaCl Tryptic Soy Broth (TSB). The bacterium was then cultured for 24 h at an incubator shaker. Then, 1 mL of inoculum was transferred to a 1.5 mL microcentrifuge tube and centrifuged for 5 min at 2000 rpm (10,000 × g). After discarding the supernatant, 700 µL of TSB containing 1.5% NaCl was added to the tube and vortexed to mix the bacterial pellet with the TSB. To obtain varying amounts of inoculum, appropriate dilutions were made, and the viable cell count was validated using a spread plate test using a serial dilution of the bacterial suspension.

2.3. Disc Diffusion Antibacterial Assay

The various microalgae extractions were tested for their antibacterial activity against the pathogenic bacteria strains of *V. harveyi*. A modified agar disc diffusion assay method was used to investigate the antibacterial activity of algal extracts [18]. Petri dishes containing TSA and 2% (w/v) NaCl were seeded with *V. harveyi* inoculum at three different concentrations: $10^5$ CFU mL$^{-1}$, $10^6$ CFU mL$^{-1}$, and $10^7$ CFU mL$^{-1}$. To test the extracts’ activity, sterile filter paper discs (6 mm) were impregnated with 20 µL of the various algal extracts. The discs were allowed to dry at room temperature before being placed on test plates inoculated with *V. harveyi*. As a control, discs with the same volume of extractants (20 µL) were made. The plates were incubated for 48 h at 35 °C. Extracts containing antibacterial components produced distinct, clear, and circular zones of inhibition around the filter discs, and this positive activity was quantified by measuring the growth inhibition zone (mm) surrounding the discs after 24 h and scoring them based on the diameter of the inhibition zone [19].

2.4. Co-Culture Antibacterial Assay

Cultures of four microalgae species, *N. oceanica*, *C. gracilis*, *Isochrysis* sp. and *T. weissflogii*, were utilised at $10^6$ CFU mL$^{-1}$ concentration to evaluate their potential to inhibit the various viability of cell growth of *V. harveyi* by incubating this bacteria in these microalgae cultures (co-culture). The concentration of *V. harveyi* used in this co-culture experiment was determined by the disc diffusion assay result that indicated the broadest inhibitory zone. Co-culture assay samples were collected at 0, 12, 24, 48, 72, 96, and 120 h after the experiment began, and 50 µL of 10-fold dilutions were spread on TCBS agar plates to assess the bacterial content in the samples. All studies were performed in triplicate, and each microalga studied, as well as *V. harveyi*, received a control treatment.

2.5. Statistical Analysis

All data for the concentration of *V. harveyi* at each point of time for co-culture assay were statistically analyzed by one-way ANOVA using software SPSS (version 22), and graphs have been plotted using Microsoft Excel.

3. Results

3.1. Disc Diffusion Antibacterial Assay

According to the findings of this investigation, four microalgae extracts showed varying degrees of inhibitory impact against *V. harveyi*, as shown in Table 1, and the extraction had broad-spectrum activity against pathogenic bacteria. On the other hand, ethanolic extracts inhibited all microalgae with the broadest (+++) range of inhibition against the lowest concentration of *V. harveyi* at $10^5$ CFU mL$^{-1}$. However, at the maximum concentration of *V. harveyi* ($10^7$ CFU mL$^{-1}$), *N. oceanica*, *Isochrysis* sp. and *T. weissflogii* extracts displayed a moderate (++) spectrum of inhibition, whilst *C. gracilis* showed low (+) inhibition. Interestingly, the ethanolic extract of *N. oceanica* demonstrated the broadest (+++) inhibitory zone against all *V. harveyi* concentrations tested; $10^5$, $10^6$, and $10^7$ CFU mL$^{-1}$. 
Table 1. Antibacterial susceptibility test of the microbial extract against *V. harveyi*.

<table>
<thead>
<tr>
<th>Concentration of <em>V. harveyi</em></th>
<th>Extractant</th>
<th>N. oceanica</th>
<th>T. weissflogii</th>
<th>C. gracilis</th>
<th>Isochrysis sp.</th>
<th>Control (Extractant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^5 CFU/mL</td>
<td>Ethanol</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Saline water</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10^6 CFU/mL</td>
<td>Ethanol</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Saline water</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10^7 CFU/mL</td>
<td>Ethanol</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td></td>
<td>Saline water</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>DMSO</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Note: (−): No activity, (+): D < 6 mm, (++): 6 < D < 8.5 mm, (+++): D > 8.5 mm. D: Diameter of the inhibition zone in millimeters.

3.2. Co-Culture Antibacterial Assay

In the co-culture assay, three (3) microalgae species—co-culture assays of *N. oceanica* (Figure 1a), *T. weissflogii* (Figure 1b), and *Isochrysis* sp. (Figure 1d)—demonstrated minimal antibacterial activity by preventing significant (*p* < 0.05) proliferation of *V. harveyi* at different time intervals when compared to the control group culture of *V. harveyi* with no microalgae.

In the co-culture *V. harveyi* with *N. oceanica* (Figure 1a), the concentration of *V. harveyi* was significantly lower (*p* < 0.05) at 24 to 48 h incubation as compared to control. However, there was no significant difference (*p* > 0.05) in *V. harveyi* concentration at 72, 96, and 120 h incubation.

For the co-culture *V. harveyi* with *T. weissflogii* (Figure 1b), the concentration of *V. harveyi* was significantly lower (*p* < 0.05) at 24, 48, and 72 h incubation, followed by no significant difference (*p* > 0.05) at 96 and 120 h incubation as compared to control.

Meanwhile, the co-culture *V. harveyi* with *Isochrysis* sp in Figure 1d demonstrated a significantly lower (*p* < 0.05) concentration of *V. harveyi* at 72, 96, and 120 h, as compared to the control. In comparison to the control, co-culture *V. harveyi* with *C. gracilis* (Figure 1c) exhibited no significant difference (*p* > 0.05) in *V. harveyi* concentration. In fact, no bibliographic data on the antibacterial activity of *C. gracilis* described in the prior study is available. The growth of *V. harveyi* strains increased steadily with time up to 48 h until reaching a plateaued condition and subsequently decreased insignificantly (*p* > 0.05) at the end of the experiment in the control group culture with no microalgae added.
Figure 1. Cont.
Figure 1. (a) V. harveyi concentration was significantly lower (p < 0.05) at 24–48 h incubation in co-culture assay with N. oceanica as compared to control V. harveyi with no microalgae added. (b) V. harveyi concentration was significantly lower (p < 0.05) at 24 to 72 h incubation in co-culture assay with T. weissflogii as compared to control V. harveyi with no microalgae added. (c) V. harveyi concentration was significantly lower (p < 0.05) at 72 to 120 h incubation in co-culture assay with Isochrysis sp as compared to control V. harveyi with no microalgae added. (d) V. harveyi concentration showed no significant difference (p > 0.05) in co-culture assay with C. gracilis as compared to control V. harveyi with no microalgae added. Note: Values are presented as mean ±SD. Mean values of V. harveyi within the same time (hours) with different lowercase letters are significantly different (p < 0.05).
4. Discussion

Many bioactive and pharmacologically active chemicals, particularly antibacterial compounds, are potentially produced by marine microalgae [6,20,21]. Furthermore, microalgae are gaining popularity due to their ability to create bioactive metabolites with anticancer, anti-inflammatory, antibacterial, and antioxidant effects [11,22]. Previous studies have suggested that fatty acids [23], terpenoids, carbohydrates [24], peptides, polysaccharides, and alkaloids are responsible for antibacterial activity in microalgae [25]. Novel antibacterial substances were also discovered in microalgae, with Coccomyxa onubensis fatty acid extracts inhibiting E.coli and P. mirabilis [26]. Furthermore, microalgae have been increasingly treated with other compounds such as cyanovirin, oleic acid, linoleic acid, palmitoleic acid, -carotene, or phycocyanin, which have antioxidant or anti-inflammatory properties, as well as antimicrobial activity, such as against Staphylococcus aureus and methicillin-resistant Staphylococcus aureus (MRSA) [22,23].

Table 1 shows variable degrees of inhibition on several species of microalgae extract against V. harveyi from this investigation. As a result, the generation of antibiotics is heavily reliant on microalgal species [27]. Furthermore, the availability of antibiotic agents can vary greatly between various species within the same class or within a single species, depending on which ecotypes are adapted to certain conditions [27,28]. The green microalgae Dunaliella sp. isolated from severely polluted waters, for example, was found to be more active against bacteria than ecotypes obtained from less polluted waters [29]. Recently, microalgae of the genus Nanochloropsis have been shown to be rich in polyunsaturated fatty acid compounds (PUFAs), carotenoids, polyphenols, and vitamins, and have previously been used in aquaculture [30], while the microalgal strain of Isochrysis sp. was shown to be high in fucoxanthin and DHA [31]. T. weissflogii, on the other hand, has antibacterial properties due to its high presence of substances such as betain, lipids, phospholipid, polyunsaturated fatty acid, fucoxanthin (FX), and eicosapentaenoic acid (EPA) [32]. C. gracilis showed a limited inhibited zone as compared to other microalgae extracts, particularly at higher concentrations of V. harveyi. This could be because the compound composition of C. gracilis is minimal and contains only non-essential amino acids [33].

Apart from microalgae species, the presence of antibacterial chemicals in microalgae extracts is also strongly reliant on the extraction process and solvent utilised. According to the findings of this investigation, ethanol is the best extractant since it demonstrated the broadest inhibition against V. harveyi in the disc diffusion method. Based on prior research, ethanol was also used to extract the thalli of Gracilaria fisheri from India, the extract of which exhibited a high inhibitory effect against V. harveyi [34]. An ethanol extract of Gracilaria corticata from India was found to be highly active against V. cholerae and V. parahaemolyticus, but less active against Pseudomonas aeruginosa and Shigella flexneri [35]. Another study on ethanolic extract of Spirulina also demonstrated more effective against two species of Vibrio [36]. These findings suggest that molecules with antibacterial action in microalgae are generally hydrophobic and might even be extracted more easily with organic solvents [27]. This could indicate that ethanol is a competent extraction method capable of extracting lipids with a specific set of chemicals present during extraction.

The methanolic extract is a well-established and reported method to isolate active antimicrobial components from microalgae [37,38]. In another study, methanol was found to be the best organic solvent to obtain bioactive compounds from Dunaliella sp. [39]. The methanolic extract of freshwater microalgae exhibited antibacterial activity against important human bacterial pathogens [40]. Methanolic extract in the current study inhibited V. harveyi moderately, whereas the antibiotic synergism with the methanolic extract of C. vulgaris exerted enhanced anti-bacterial activity in E. coli [41]. Therefore, the selection of a solvent is also important for the further utilisation of post-extracted microalgal biomass. According to Navarro et al., 2017 [26], it was suggested that fatty acids could be involved in the antibacterial activity due to increased activity from green microalga extracts obtained with non-polar solvents compared to polar solvents.
In the present study, microalgae extraction by saline water and DMSO demonstrated a comparable less effective antibacterial activity against *V. harveyi* based on the concentration of *V. harveyi*. Nevertheless, it was reported that the extraction of antimicrobial compounds from *Scenedesmus subspicatus* using DMSO inhibited the growth of *Klebsiella pneumoniae* and *Escherichia coli* [42]. As observed in previous studies, water extracts of *Dunaliella* sp. had lower bioactive properties than the ones obtained from organic solvents [43]. Hence, the selection of a solvent is important for optimisation of the extraction process and generation of its bioactive compounds, which have diverse applications not only in aquaculture but also against human pathogens. According to Pradhan et al., 2012 [36] no antimicrobial activity was detected in the aqueous extracts, which was probably because of the low polar nature of the active components.

The number of *V. harveyi* increased exponentially, especially in the first 24 h during the experimental period, without the presence of microalgae cells (control) demonstrating that the bacteria cells were able to utilise the growth medium of the microalgae cultures. The inhibition of *V. harveyi* in co-culture assay with *N. oceanica*, *Isochrysis* sp. and *T. weissflogii* indicated the production of antibacterial compounds by these microalgae cultures. Previous research has shown that *Isochrysis* sp. and *Nannochloropsis* exhibit antibacterial action against the majority of vibriosis pathogens, including *V. alginolyticus*, *V. lentus*, *V. splendidus*, *V. scophthalmi*, *V. parahaemolyticus*, and *V. anguillarum* [44]. The microalgae species *Nannochloropsis* sp. and *Isochrysis* sp. have been demonstrated to synthesise short-chain fatty acids, which are implicated in antibacterial activity [45,46]. Thus, microalgae biomass is regarded as a source of valuable chemical elements that can be used in animals, particularly farmed fish, as pharmaceuticals in aquaculture industries.

Another element that could explain these discrepancies is that antibacterial activity is linked to the culture conditions and growth phase of the microalgae cultures. Although all microalgae species were at the same concentration in all trials in this study, the growth phase for each microalgae culture could have been different and may have influenced the results. Previous studies suggested that modifying the culture conditions of green microalgae exhibited differences in antibacterial activity [47–49], whereas modifications on microalgae culture conditions could be stimulated to produce secondary metabolites with antibacterial activity, as well as potentially larger quantities of these secondary metabolites [50]. This may include modifications on media composition, pH, light, and temperature [51,52]. As an example, the highest light intensity (4800 lux) exposure effectively induced the production of antibacterial compounds by *Dunaliella* sp. and suggested that the higher antimicrobial effect was related to the content of bioactive compound produced in stress conditions [39]. Other than the culture environment, the period of microalgae cultivation also plays an important parameter in producing bioactive compounds [39,53]. Therefore, the culture environment could be artificially manipulated in order to allow for increased production of antibacterial compounds in microalgae.

5. Conclusions

Out of four species of microalgae, only *N. oceanica*, *Isochrysis* sp. and *T. weissflogii* demonstrated a significant (*p < 0.05*) inhibitory effect against *V. harveyi* in an in vitro co-culture assay. This indicated a positive effect of the addition of these microalgae in the rearing of fish larvae and implicated the production of antibacterial compounds. Ethanolic extract from *N. oceanica*, *Isochrysis* sp. and *T. weissflogii* demonstrated promising antibacterial activity against *V. harveyi*. Meanwhile, *C. gracilis* inhibited the development of *V. harveyi* with minimal inhibition but no significant difference (*p > 0.05*) in the co-culture assay and disc diffusion method, indicating that it had less potential to limit the growth of *V. harveyi*. Therefore, in the aquaculture industry, *N. oceanica*, *Isochrysis* sp. and *T. weissflogii* act as potential inhibitory bacteria and probiotics in controlling the disease. More comprehensive studies are recommended to optimize its cultivation conditions, the extraction process, and the purification of its antibacterial compounds to unleash their potential.

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