Mutualistic interactions of green microalga *Chlorella* sp. with bacterial quorum sensing degrader *Bacillus* spp isolated from biofloc and freshwater prawn *Macrobrachium rosenbergii*

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Abstract

Aim: Bacterial quorum sensing degrader isolated from biofloc and giant freshwater prawn, *Macrobrachium rosenbergii* were investigated for mutual interaction with green microalga, *Chlorella* sp. for potential use of disease control in aquatic environment.

Methodology: Two potential strains of quorum sensing (QS) degrader were successfully isolated from biofloc and giant freshwater prawn, *Macrobrachium rosenbergii*. Meanwhile, *Chlorella* sp. was made axenic using 13 to 500 ppm of different antibiotic mixtures (chloramphenicol, tetracycline, enrofloxacin, kanamycin, and neomycin), where interaction of algae with different QS degrader strains, different microbial communities and pathogenic bacterium *Aeromonas hydrophila* were investigated.

Results: The QS degrader strains were identified as *Bacillus subtilis*, BP-BFT/1b and *Bacillus cereus*, BP-MBRG/1b. Growth of *Chlorella* sp. added with *Bacillus subtilis*, BP-BFT/1b was higher compared to *Bacillus cereus*, BP-MBRG/1b. The results showed that the growth of axenic *Chlorella* sp. improved in the presence of bacteria, including bacterial QS degraders.

Interpretation: There is a potential to incorporate both *B. subtilis* BPBFT/1b and *B. cereus* BPMBRG/1b in aquaculture systems to overcome invasion of pathogenic bacteria using bacterial QS degrading mechanism.

Key words : Bacterial Quorum sensing, Biofloc, Green microalgae, *Macrobrachium rosenbergii*

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Introduction

In aquaculture, microalgae are the primary food source for a large number of aquatic organisms and they play an important role in their development. Microalgae are cultured as live food for zooplankton, crustaceans, mollusks and fish larvae because they act as source of energy as well as provide essential vitamins and polyunsaturated fatty acids (PUFAs) (Wells *et al.*, 2016). Moreover, microalgae also possess anti-pathogenic activities for bacterial control.

There are several methods to control bacterial diseases in aquaculture such as immunostimulants, vaccines, water disinfection, probiotics including green water technique. Green water can also be a potential technique to control diseases where it improves water quality and enhances aquatic organism resistance to diseases (Chithambaran *et al.*, 2017). Furthermore, previous researches have reported that bacteria greatly affect the growth of microalgae (Grossart *et al.*, 2005), because the organic carbon such as dissolved and particulate particles from live and cell lysis of dead microalgae can promote bacterial growth. In return, bacteria can also enhance the metabolism of microalgae by releasing growth-promoting factors (Fakami *et al.*, 1997). Thus, the use of the consortium may improve aquaculture productions.

Currently, disruption of bacterial cell-to-cell signaling or quorum sensing (QS) is one of the treatments to prevent bacterial disease. Quorum sensing is a mechanism where bacteria are able to coordinate the expression of certain genes in response to the presence of small signal molecules (Hense *et al.*, 2007). It is a type of bacterial cell-to-cell communication that monitors the auto inducer concentration in the environment and control their activity (Miller and Bassler, 2001). Several bacterial pathogens use this mechanism through different signal molecules to regulate virulence gene expression (Pande *et al.*, 2013). To date, the acylated homoserine lactone (AHL) are the most studied bacterial QS signal. Since QS play an important role in pathogenicity towards aquatic host organisms, organisms with QS interference (QSI) activities or Quorum Quenching (QQ) can be used as an alternate source controlling diseases, for which is an ideal choice for aquaculture sector. The aim of this study was to investigate the presence of mutualistic interactions between the green microalga *Chlorella* sp. with bacterial QS degraders isolated from the biofloc and freshwater prawn *Macrobrachium rosenbergii*. The positive interactions between both organisms could later be used as one of the biocontrol strategies to control diseases in aquaculture.

Materials and Methods

Enrichment of QS degraders bacterial strains: Microbial communities from biofloc and gut of freshwater prawn, *Macrobrachium rosenbergii* were used as the source to isolate degrading strain through enrichment techniques. All samples

were incubated for three days in 20 ppm hexanoyl homoserine lactone. Treatment without acylated homoserine lactone served as a control. Each medium was then spread on Trypticase Soy Agar and the cycle was repeated once in three days until bacterial growth in the control medium was totally absent. Colonies isolated from AHL medium were considered as potential QS degrader.

AHL degradation assay: The potential QS degrader was examined for AHL degradation activity using *Chromobacterium violaceum* (CV026) as reporter strain (McClellan *et al.*, 1997). *Chromobacterium violaceum* act as a generic biosensor that detects and responds to the AHLs signal molecule by inducing the synthesis of purple pigment violacein (Defoirdt *et al.*, 2011). Briefly, QS degrader strains (10^7 CFU ml⁻¹) were mixed with 10 ppm AHL and incubated on the shaker (120 rpm) for 24 hrs at 30°C. The filtrates (10 µl) were then spotted on TSA plate spread with CV026 with 20 ppm kanamycin. The samples were checked at 0, 3, 6, 12, 24 and 48 hrs to observe the degradation level of AHL concentration. A series of standard solution were also prepared with different AHL concentrations (0, 1, 2.5, 5, 7.5, 10 and 15 ppm). After 24 hrs, the diameter of purple violacein zone was observed and measured.

Molecular identification of potential QS degrader: Strains were cultured in 10 ml Tryptic Soy Broth (TSB) for 24 hrs. Genomic DNA was extracted using GeneJET genomic DNA purification kit (Thermo Scientific, Europe) following the manufacturer's protocol for Gram positive bacteria. DNA quantification and purity check was conducted using BioPhotometer plus (Eppendorf, Germany). Polymerase Chain Reaction (PCR) amplification targeting a 1500 base pair fragment of 16S rRNA gene using universal primer, forward 5'- AGA GTT TGA TCC TGG CTC AG-3'; reverse 5'- GGT TAC CTT GTT ACG ACT T-3' (First Base Laboratories, Malaysia) was conducted. The final 25 µl reaction volume of PCR mix contained 1 µl genomic DNA, 1 µl of each forward and reverse primer, 1 µl of dNTPs, 5 µl of PCR buffer (green), 1 µl of 25mM MgCl₂, 0.5 µl of Taq DNA polymerase and 14.5 µl of sterile distilled water (Thermo Scientific, Europe). The PCR reactions were performed on Eppendorf Mastercycler DNA Thermal Cycle (Eppendorf, Germany) with the cycling conditions: initial denaturation at 94°C for 10 min, followed by 34 cycles of denaturation at 94.0°C for 1 minute, annealing at 54°C for 1 min, extension at 72°C for 90 sec and final extension at 72°C for 10 min. Then, 15 µl of PCR products were electrophoresed in 1% agarose gel (90 V, 60 min) and was subsequently visualized by UV illumination (Alpha Innotech, USA) after gel red staining. The purified product was sequenced by 1st BASE, Malaysia. The nucleotide sequence result was searched in the GenBank DNA database by using the Basic Local Alignment Search Tool (BLAST) program accessible at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Preparation of axenic microalgae *Chlorella* sp.: *Chlorella* sp. was isolated from a pond located in the University Agriculture Park, Puchong Selangor Malaysia. *Chlorella* sp. was prepared as axenic microalgae in Bold Basal Medium by adding five antibiotic mixtures (chloramphenicol, tetracycline, enrofloxacin, kanamycin and neomycin) at three different concentrations (Table 1). The solution was shaken evenly and placed under fluorescent light at of 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ intensity for a photoperiod of 12 hr light and dark cycle and the pH was adjusted to 8. After 24 hr, the microalgae with antibiotic mixtures were spread on Trypticase Soy Agar and Potato Dextrose Agar to check for bacterial and fungal contaminations. The best antibiotic concentrations were used to obtain axenic *Chlorella* culture. The residual antibiotics were removed through washing steps for at least three times with centrifugation at 3000 rpm for 10 min. Algal cells were then re-suspended in fresh BBM and incubated for at least one week for further growth of axenic *Chlorella*.

Interaction of bacterial QS degrader with axenic *Chlorella* sp.: Interaction of axenic *Chlorella* and QS degraders were tested using QS degrading strains isolated from freshwater prawn, *Macrobrachium rosenbergii* and biofloc. Xenic *Chlorella* sp. and pathogenic *Aeromonas hydrophila* AH-1N mixed with axenic *Chlorella* sp. as positive and negative controls were used. Axenic *Chlorella* sp. was harvested at exponential growth phase and diluted 100X with sterile BBM to obtain 10^4 cells ml^{-1} suspension. Bacterial samples were then added to the microalgal cell at 10^5 CFU ml^{-1} . All culture flasks were placed on a shaker at 25 °C at 120 rpm under continuous 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ illumination at 12 hr : 12 hr light: dark cycle. Bacterial concentration and microalgal cell density were monitored every fourth day for 12 days through plate counting and haemocytometer, respectively.

Statistical analysis: Cell density of microalgae and bacteria were analyzed by One-way ANOVA. All data were tested at 5% level of significance using SPSS (Statistical Package for Social Science) version 21. Significant difference between means were analyzed using Tukey post hoc analysis. The difference was marked by alphabetical notations.

Results and Discussion

Bacterial QS degraders were isolated from AHL enrichment medium (+AHL) when no bacterial growth was

observed in the control medium (-AHL). Two bacterial strains as potential degraders were successfully isolated each from *M. rosenbergii* and biofloc. Both strains showed AHL degradation activity indicated by loss of purple pigmentation controlled by QS in less than six hours (Fig. 1). This showed that 10 ppm AHL can be fully degraded by the strains. Meanwhile, the concentration of AHL in control was constant. Both strains were identified as *Bacillus subtilis* BP-BFT/1b and *Bacillus cereus* BP-MBRG/1b (Table 2). Different QS degrading bacteria have been isolated from fish and prawn gut (Hong and Cutting, 2009; Tinh et al., 2007) *Bacillus* sp. are frequently isolated for their use as quorum sensing degrader and probiotic. In the present study, *B. subtilis* BP-BFT/1b and *B. cereus* BP-MBRG/1b bacteria successfully isolated from biofloc and prawn gut showed full AHL degradation activity as indicated by total loss of purple pigmentation. Different species of *Bacillus* has been identified as QS degraders (Defoirdt et al., 2011; Czajkowski and Jafra, 2009) and species such as *B. cereus*, *B. mycoides* and *B. thuringiensis* have been shown to degrade AHL through production of AHL lactonase enzyme encoded by gene *aiiA* (Dong et al., 2000; Bai et al., 2008).

The green microalga *Chlorella* is among the most common algal species used in aquaculture (Ahmad et al., 2020). Axenic *Chlorella* sp. can be obtained by mixtures of different antibiotics at the concentration from 13 to 500 ppm. No bacterial and fungal growth can be observed after 24 hrs incubation on TSA and PDA media. Meanwhile, bacteria and fungi grew on the agar media inoculated from *Chlorella* sp. with low concentration antibiotic mixtures (6.3 - 250 ppm and 3.1 – 125 ppm) and without any antibiotic mixture after 24 hrs (Table 3).

Axenic microalgae are used for varied applications like in production of biofuels and food grade microalgae (Jichang et al., 2015). Antibiotics treatment have been used to effectively remove bacterial contamination from microalgal cultures (Katoh et al., 2012). As bacterial communities associated with different microalgal strains may vary, hence, different kinds and amount of antibiotics are crucial for effective purifying purposes (Jichang et al., 2015). Shishlyannikov et al. (2011) reported that high concentration of antibiotics for a short time can be used to get pure axenic microalgae. Neomycin (Jones et al., 1973), enrofloxacin (Khao and Thao, 2012) and tetracycline are few antibiotics (Xu et al., 2013). However, high concentrations that

Table 1 : Concentration of antibiotic (ppm) used for axenic *Chlorella* culture.

Series of antibiotic	Concentration (ppm)			
	A	B	C	Control
Chloramphenicol	15	7.5	3.75	0
Tetracycline	12.5	6.25	3.125	0
Enrofloxacin	320	160	80	0
Kanamycin	100	50	25	0
Neomycin	500	250	125	0

Table 2 : Identification of the isolates based on NCBI BLAST

Strain	Isolate	Source	Accession n ^{o1}	Similarity (%)
BP-BFT/1b	<i>Bacillus subtilis</i>	Biofloc	JN128239	92%
BP-MBRG/1b	<i>Bacillus cereus</i>	<i>M. rosenbergii</i>	JF343136	86%

¹ GenBank accession numbers**Table 3** : Different antibiotic treatments to obtain axenic *Chlorella* sp. after 24 hours

Series of antibiotic	Number of bacterial single colony (CFU ml ⁻¹)	Number of fungus single colony (CFU ml ⁻¹)
A (13 ppm – 500 ppm)	-	-
B (6.3 ppm – 250 ppm)	>300	>300
C (3.1 ppm – 125 ppm)	>300	>300
Control (0 ppm)	6.5 x 10u	>300

-No colony observed

Fig. 1 : AHL Concentration (ppm) of BP-BFT/1b and BP-MBRG/1b samples within 15 hours in Luria Bertani containing 10 ppm AHL. **Fig. 2** : Growth performance of *Chlorella* sp. inoculated with single and mixtures of QS degraders (BP-MBRG/1b and BP-BFT/1b) and pathogenic bacteria (*A. hydrophila*) during 12 days of culture period. **Fig. 3** : Specific Growth Rate of *Chlorella* sp. inoculated with and without single and mixtures of QS degraders (BP-MBRG/1b and BP-BFT/1b) and pathogenic bacteria (*A. hydrophila*) during 12 days of culture period. **Fig. 4** : Bacterial plate count in *Chlorella* sp. inoculated with and without single and mixtures of QS degraders (BP-MBRG/1b and BP-BFT/1b) and pathogenic bacteria (*A. hydrophila*) during 12 days of culture period.

have been tested not only affect the associated microorganisms but can be toxic to algal cells (Khao and Thao, 2012; Xu *et al.*, 2013).

The co-culture between microalgae and both QS degrader *Bacillus* strains BP-BFT/1b and BP-MBRG/1b gradually increased the growth of *Chlorella* sp. during 12 days culture period. However, co-culture of *Chlorella* sp. with BP-BFT/1b strain showed higher growth compared to BP-MBRG/1b strain. However, mixtures of both strains showed slightly lower *Chlorella* sp. growth than BP-BFT/1b alone. On the other hand, co-culture with pathogenic *A. hydrophila* AH-1N increased algae growth in the beginning of culture, followed by a sharp decrease from eighth day onwards (Fig. 2). In contrast to all treatments, the highest *Chlorella* sp. cell densities were from xenic algal culture (data not shown). Similarly, the specific growth rate (SGR) of *Chlorella* sp. was also higher in xenic culture (24%) compared to other treatments (less than 14% SGR) (Fig. 3). In parallel, the highest bacterial cell densities were obtained in xenic culture, which increased gradually from Day 0 to Day 12. No bacterial colony was observed in axenic *Chlorella* sp. culture. Meanwhile, the density of all bacterial QS degraders dropped two logs below the initial inoculum. The density, however, stagnated throughout the experiment. Initially, *A. hydrophila* showed slight increase in the growth later it was noted that the density started to decline from Day 4 onwards (Fig. 4).

Existence of BP-BFT/1b and BP-MBRG/1b significantly enhanced the specific growth of xenic *Chlorella* sp. when compared with axenic conditions. The results are in agreement with the findings of Hernandez *et al.* (2008), who quantified the enhanced growth in *Chlorella* sp. and combined it with rhizobacteria *Bacillus pumilus* in a short-term incubation. This enhanced the absorption of nitrogen and phosphorus through different metabolic pathways by microalgal cells. Qu *et al.* (2014) also reported similar results. The growth of *C. vulgaris* was promoted when co-cultured with *Bacillus* sp., where the maximum cell concentration was observed on day 8 and thereafter decreased due to resource competition. In nature, bacteria and microalgae co-exists together and interact symbiotically. Bacteria utilizes products from microalgae cells while bacteria contributed organic nutrient rich in carbon, nitrogen and other growth factors for microalgae (Fukami 1997; Grossart and Simon, 2007; Grossart *et al.*, 2005). However, the growth of microalgae can be inhibited due to several factors like bacteria producing antibiotics (Rooney-Varga *et al.*, 2005), algicidal extracellular metabolites (Munoz and Guieysse, 2006), breaking respiratory chain or inhibiting cell wall synthesis (Zhao *et al.*, 2012) and competition of nutrients (Trabelsia and Rassoulzadegana, 2011).

It was observed that co-existence of bacteria and microalgae showed both positive and negative interactions during culture. High bacterial density in xenic culture was

probably due to availability of organic matter released from *Chlorella* sp. that promoted bacterial growth. Microalgae excrete carbon sources or other products that enhance bacterial activity. Conversely, bacterial growth can be suppressed due to secretion of antibiotics from *Chlorella* sp. (Grossart and Simon 2007) and also due to competition for resources (Qu *et al.*, 2014). According to Lekunberri *et al.* (2011), bacteria and microalgae may also compete for limiting nutrients such as phosphate. In the natural environment, microalgae are always in association with bacteria, fungi and other microorganisms (Tujula *et al.*, 2010). The relationship between microalgae and bacteria can be either symbiotic or inhibitory.

This research showed that axenic *Chlorella* sp. can be obtained using combination of 13 ppm to 500 ppm of chloramphenicol, tetracycline, enrofloxacin, kanamycin and neomycin mixtures. This study also showed that there is a mutualistic interaction between microalga *Chlorella* and bacterial quorum sensing degraders. The presence of bacteria stimulated the microalgal growth and vice versa. However, since inhibitory activities were also observed, the use of consortium needs to be further investigated. This study is important to understand how associated bacteria and microalgae influence the growth of each other. The right combination of algae and bacteria could significantly improve their productivity and efficiency as biocontrol agents against pathogen in aquaculture.

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