UNIVERSITI PUTRA MALAYSIA

EVALUATION OF MICROALGAE AS QUORUM-SENSING INHIBITORS TO PROTECT GNOTOBIOTIC Artemia franciscana (Kellogg, 1906) AGAINST Vibrio campbellii

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By

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November 2020

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Diseases remain as one of the major constraints in aquaculture industry. The use of microalgae as quorum sensing inhibitor (QSI) to disrupt communication in pathogenic bacteria could be another disease control alternative as regulation of virulence factors in bacterial pathogens are regulated by quorum sensing (QS). Thus, the present study evaluated different microalgae isolated from shrimp pond as quorum sensing inhibitors in *in vitro* and in *in vivo* settings. In this study, the effects of nine microalgae species on acyl-homoserine lactone-regulated phenotypes of two QS reporter strains (*Chromabacterium violaceum* CV026 and *Escherichia coli* JB523) and anti-Acute Hepatopancretic Necrosis Disease (anti-AHPND) were investigated. Five microalgae pellet extracts; *Picochlorum eucaryotum*, *Monoraphidium* sp., *Amphora coffeiformis*, *Desertifilum tharense* and *Bulboplastis apyrenoidosa* inhibited violacein production of quorum sensing reporter strain CV026 with the largest inhibition zone of *P. eucaryotum* (6.85 ± 0.15 mm). Further tests showed that the supernatant of algae extracts also inhibited quorum sensing using the QS reporter strain JB523. In JB523 reporter strain, a significant decrease in QS-regulated GFP production ($p < 0.05$) were observed in all microalgae species except for *Spirulina platensis*. The highest percentage of inhibition of JB523 were demonstrated by *P. eucaryotum* (1 ± 0.02 %) respectively, *Monoraphidium* sp. (1 ± 0.04 %) and *Chlorella* sp. (1 ± 0.02 %). The growth of all reporter strains was found to be unaffected by the microalgal samples. The most promising micro-algal strains as bacterial disease control agents were found to be *P. eucaryotum*, *A. coffeiformis* and *D. tharense*. Meanwhile, screening of anti-AHPND activities revealed that three green algae *P. eucaryotum*, *Monoraphidium* sp. and *Chlorella* sp. were able to inhibit two AHPND strains of *Vibrio parahaemolyticus*. The best three microalgae species with QSI activities; *A. coffeiformis*, *D. tharense* and *P. eucaryotum* were selected for *Artemia* challenge assay. A significant ($p < 0.05$) survival were observed in *Artemia* bioencapsulated with *A. coffeiformis* (95 ± 0 %), *D. tharense* (85 ± 10 %), and *P. eucaryotum* (60 ± 0 %) even after challenged with *Vibrio campbellii*, BB120 (12.5 ± 5 %). The results in this study indicated that quorum

quenching microalgae has the potential to be used as biocontrol tool to manage disease outbreak particularly vibriosis in aquaculture farms.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENILAIAN MIKROALGA SEBAGAI PERENCAT PENDERIAAN KUORUM UNTUK MELINDUNGI *Artemia franciscana* (Kellog, 1906) TERHADAP *Vibrio campbellii*

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Penyakit kekal sebagai salah satu kekangan utama dalam industri akuakultur. Penggunaan mikroalga sebagai perencat penderiaan kuorum (QSI) untuk mengganggu komunikasi di antara bakteria patogen boleh dijadikan sebagai alternatif dalam kawalan penyakit kerana faktor kevirulenan bakteria patogen dikawalatur oleh penderiaan kuorum (QS). Oleh itu, kajian ini telah menilai kebolehan mikroalga yang dipencilkan daripada kolam udang sebagai perencat penderiaan kuorum secara *in vitro* dan *in vivo.* Dalam kajian ini, kesan sembilan spesies mikroalga terhadap fenotip aruhan-lakton homoserin asil bagi dua strain QS (*Chromabacterium violaceum* CV026 dan *Escherichia coli* JB523) serta terhadap penyakit akut nekrosis hepatopankreas (anti-AHPND) telah disiasat. Lima ekstrak pelet mikroalga; *Picochlorum eucaryotum*, *Monoraphidium* sp., *Amphora coffeiformis*, *Desertifilum tharense* dan *Bulboplastis apyrenoidosa* telah merencat penghasilan violasin oleh strain penderiaan kuorum CV026 dengan perencatan terbesar oleh *P. eucaryotum* (6.85 ± 0.15 mm). Ujian seterusnya menunjukkan ekstrak supernatan mikroalga juga mengganggu kuorum dalam strain penderiaan JB523. Dalam strain penderiaan JB523, pengurangan ketara ($p < 0.05$) dalam penghasilan Protein Fluoresen Hijau (GFP) aruhan-QS diperhatikan dalam semua spesies mikroalga kecuali *Spirulin platensis.* Peratus perencatan tertinggi bagi JB523 telah ditunjukkan oleh *P. eucaryotum* (1 ± 0.02 %), diikuti *Monoraphidium* sp. (1 ± 0.04 %) dan *Chlorella* sp. (1 ± 0.02 %). Pertumbuhan semua strain QS yang dilaporkan tidak dipengaruhi oleh sampel mikroalga. Strain mikroalga yang paling berpotensi sebagai ejen kawalan penyakit adalah *P. eucaryotum*, *A. coffeiformis* dan *D. tharense.* Sementara itu, penyaringan aktiviti anti-AHPND menunjukkan tiga spesies alga hijau *P. eucaryotum*, *Monoraphidium* sp. dan *Chlorella* sp. boleh merencat pertumbuhan dua strain *Vibrio parahaemolyticus*-positif AHPND. Tiga spesies mikroalga dengan aktiviti QSI terbaik; *A. coffeiformis*, *D. tharense* and *P. eucaryotum* telah dipilih untuk ujian cabaran *Artemia*. Kadar hidup yang ketara ($p < 0.05$) diperhatikan dalam *Artemia* yang diperkayakan dengan *A. coffeiformis* (95 ± 0 %), *D. tharense* (85 ± 10 %) dan *P. eucaryotum* (60 ± 0 %) walaupun selepas dicabar dengan *Vibrio campbellii*, BB120 (12.5 ± 5 %). Hasil kajian ini menunjukkan bahawa

mikroalga perencat kuorum berpotensi digunakan sebagai alat kawalan biologi untuk mengatasi masalah penyakit di ladang akuakultur terutamanya yang melibatkan vibriosis.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree Master of Science. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS / SYMBOLS

v/v	Volume per volume
µL	Microlitre
mL	Millilitre
µm	Micrometre
m^{-2} s^{-1}	Metre square per second
bp	Base pair
min	Minute
%	Percent
°C	Degree centigrade
g	Gram
h	Hour
rpm	Rotation per minute
spp.	Species
mm	Millimeters
LBA	Luria-Bertani agar
LBB	Luria-Bertani broth
dNTP	Deoxyribonucleotide triphosphate
$MgCl_2$	Magnesium chloride
CFU	Colony forming unit
mt	Metric tonne

CHAPTER 1

INTRODUCTION

1.1 Background of study

Microalgae are unicellular aquatic plants that are usually found in freshwater and marine water systems grow in both water column and sediment which absorbs light and carbon dioxide for growth. In aquaculture, microalgae play an important role in aquatic food chain and act as major supplement for various stages of shrimp, molluscs, fishes and zooplankton such rotifer and *Artemia* (Muller-Feuga, 2000; Roy & Pal, 2012). Some microalgae contain high protein, lipid, carbohydrate, vitamins C, E and biotin that are essential nutrients for aquaculture species (Croft et al., 2006; Spolaore et al., 2006). The most popular genus are *Chlorella*, *Tetraselmis*, *Chaetoceros*, *Nannochloropsis*, and *Thalassiosira* (Hemaiswarya et al., 2011; Sirakov et al., 2015). Moreover, microalgae also demonstrate anti-pathogenic activities for bacterial control, improve water quality and enhance aquatic organisms resistance toward diseases (Tendencia et al., 2013).

Farmers have a great interest in culturing microalgae in hatchery as live feed for zooplankton and larvae. Roy et al. (2014) found *Tetraselmis tetrahele* and *Isochrysis galbana* improve the nutritional value of rotifers (*B. plicatilis*) as live food for the milkfish fry. In other research, the genus of *Tetraselmis* also demonstrated a significant growth rate of rotifers in intensive culture system compared to *Nannochloris* sp., *Chlorella* sp., *Isochrysis* sp. and *Nannochloropsis* sp. (Abd-Rahman et al., 2018). Meanwhile, several reports showed that the growth of culture animals fed with mixture of algae species is better than single species, probably due to the fact that particular algae may absence of some nutrient, while other algae possess one (Patil et al., 2005).

Today, aquaculture is one of the fastest growing food producing sectors worldwide. Despite of the progressive growth, emerging microbial diseases hinder the growth of aquaculture and cause significant global economic losses (Santhakumari et al., 2016). For example, Indonesian hatcheries lose up to US$100 million due to luminescent vibriosis (Defoirdt et al., 2007). The common species of *Vibrio* include *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus V. anguillarum*, *V. salmonicida* and *Vibrio campbellii* (Adams & Boopathy, 2013; Santhakumari et al., 2016, You et al., 2016).

As diseases in aquaculture intensify, the uses of antibiotics are no longer effective for cultured animals. The misuse and over dosage of antibiotic has contributed to the expansion and development of antibiotic-resistant bacteria and antibiotic residues in food-producing animals that cause long term adverse health effect in humans and animals (Adams & Boopathy, 2013; Santhakumari et al., 2017). Therefore, a numbers of method have been implemented to control diseases in aquaculture. One of the alternative strategies to replace antibiotics are through the disruption of cell-to-cell

communication of pathogenic bacteria or known as quorum sensing (Defoirdt et al., 2004; Natrah et al., 2011a). Hence, it is interesting to develop potential quorum sensing inhibitor isolated from the aquaculture systems for prevention of bacterial diseases in aquaculture.

1.2 Problem statement

Aquaculture is become one of the important industry worldwide. However, the greatest constraint in aquaculture was reportedly due to bacterial diseases occurring in many ponds caused huge loss of production in aquaculture (Assefa & Abunna, 2018). The infectious disease that resposible in reduction of aquaculture industry is vibriosis caused by *Vibrio* spp bacteria such as *V. harveyi*, *V. campbellii*, *V.vulnificus*, *V. parahemolyticus* and *V. alginolyticus* (Adam & Boopathy, 2013). This disease has extremely affected shrimp aquaculture industry with estimated losses more than USD 1 billion (Zorriehzahra & Banaederakhshan, 2015).

Farmers use various types of antibiotics to control diseases in aquaculture. These antibiotics could harm the aquaculture environment which led to antibiotic resistant microbes that could also be transferred to the consumers. Recent studies found that microalgae are able to inhibit pathogenic bacteria through disruption of bacteria-bacteria communication. However, to our knowledge, the information on extracts of local microalgae as anti-quorum sensing (anti-QS) are still scarce in literature thus, in this study we focused on the local microalgae as disease control agents in aquaculture.

1.3 Significant of study

There are many studies by various researches emphasizing role of algae in agriculture, bio-fuel, medicine, pharmaceutical, cosmetics and others. The application of microalgae in aquaculture has also been one of the major research subjects due to their capability to supply nutrients in aquatic farms. In future, microalgae can be considered as promising live feed and disease control for aquaculture species. One of the effective ways to protect aquaculture species against disease is through bio-encapsulation of *Artemia* with microalgae as the *Artemia* can act as vector to deliver nutrients to the cultured animals. In this study, quorum sensing degraders were screened from microalgae isolated from shrimp ponds. This include investigations on the microalgal extracts against acute hepatopancreatic necrosis disease. Furthermore, the protective effect of microalgae encapsulated in *Artemia* towards *Vibrio campbellii* was also observed.

1.4 Objectives of study

Hence, the objectives of this study were:

1. to isolate and identify different microalgal group (Chlorophyta and Bacillariophyta) from shrimp ponds,

2. to screen microalgae and its associated bacteria with anti-quorum sensing and anti- acute hepatopancreatic necrosis disease (anti-AHPND) against *Vibrio parahaemolyticus*, and

3. to evaluate the capability of potential anti-quorum sensing microalgae encapsulated in *Artemia* and its protection effect against *Vibrio campbellii.*

1.5 Hypothesis of study

The hypothesis of the study:

Null hypothesis: There is no anti-quorum sensing activity from local microalgae species thus did not protect *Artemia franciscana* from vibriosis.

Alternate hypothesis: Local microalgal species have anti-quorum sensing activity and protect *Artemia franciscana* from vibriosis.

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