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EVALUATION OF POTENTIAL PROBIOTIC BACTERIA FOR MICROALGAE PROPAGATION AND Artemia fransiscana (Kellog, 1906) BIOENCAPSULATION

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By

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Thesis Submitted to School of Graduate Studies, University Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Chairman : Murni Marlina Bt Abd Karim, PhD
Faculty : Institute of Bioscience

The emergence of aquaculture industry is in need for non-antibiotic based disease control approach to minimize risk of antibiotic-resistance bacteria. Bacterial infections mainly caused by *Vibrio* spp. have caused mass mortalities to fish especially during larval stages. The potential of microalgae as future feedstocks in aquaculture sector has prompted to a new area of research in finding potential probiotics that can enhance algal biomass and at the same time, suppress fish larval pathogens. The current research was undertaken to discover new potential probiont strains isolated from microalgae and elucidate their interaction. The capability of the potential probionts in protecting *Artemia* from *Vibrio* spp. was also determined in this study.

A total of 27 strains were successfully isolated from different species of fresh microalgae cultures (*Amphora* sp., *Chaetoceros* sp., *Chlorella* sp., *Nannochlorum* sp. and *Spirulina* sp.). All the isolated strains were screened for antibacterial activity against fish pathogens, *Vibrio harveyi* and *Vibrio parahaemolyticus* in *in vitro* study. Results of the agar well diffusion assay showed that 4 out of 27 strains were able to inhibit the growth of *V. harveyi* and *V. parahaemolyticus*. Strains labeled as A-1, A-2 and Cl-3 showed inhibition towards both pathogens tested while strain S-2 showed inhibition towards *V. parahaemolyticus* only. These four potential probionts were identified as *Lysinibacillus fusiformis* strain A-1 (isolated from *Amphora* sp.), *Bacillus* sp. strain A-2 (isolated from *Amphora* sp.), *Lysinibacillus fusiformis* strain Cl-3 (isolated from *Chlorella* sp.) and *Bacillus pocheonensis* strain S-2 (isolated from *Spirulina* sp.) using 16s rRNA molecular method.

Biochemical assay demonstrated that all these probionts had the ability in excreting catalase, gelatinase, lipase, oxidase, protease, and anti-quorum sensing. In biofilm

inhibition assay, the highest percentage of inhibition of *V. harveyi* and *V. parahaemolyticus* were demonstrated by *B. pocheonensis* strain S-2 (70.36 ± 4.84 %) and *L. fusiformis* strain A-1 (67.79 ± 4.70 %), respectively. Through microalgae propagation experiment, the inoculation ratio of bacteria and microalgae was fixed to 1:4, and co-culture incubation was monitored for 7 to 8 days. *Bacillus* sp. strain A-2 and *L. fusiformis* strain Cl-3 were able to promote the growth of *Amphora* sp., and *Chlorella* sp., respectively. *Bacillus* sp. strain A-2 showed a good correlation after co-cultured with *Amphora* sp. evidenced by a steep growth of *Amphora* sp. (8.5×10^5 cells ml^{-1}) in comparison with the control (1.4×10^5 cells ml^{-1})) at day 8 of co-culture. The cell density of *Chlorella* sp. was promoted more than two times due to *L. fusiformis* strain Cl-3 supplementation (9.9×10^6 cells ml^{-1}) compared with the control (4.2×10^6 cells ml^{-1}), observed at day 7 of co-culture incubation.

A preliminary *in vivo* assay was carried out on *Artemia fransiscana* nauplii against *V. harveyi* and *V. parahaemolyticus*. Probionts were supplied to *Artemia* as single strain and in multiple strains known as MIX (combinations of all potential probionts) along with respective microalgae. Results revealed that *Artemia* bioencapsulated with MIX + *Amphora* sp. showed the highest survival rate (60 ± 4 %) when compared with the control (13 ± 2 %), after challenged with *V. harveyi*. A significant reduction of vibrios load was observed in *Artemia* bioencapsulated with MIX + *Amphora* sp. (7.29 ± 0.02 CFU ml^{-1}) compared with the control (7.31 ± 0.05 CFU ml^{-1}).

Meanwhile, for the *Artemia* challenged with *V. parahaemolyticus*, a significant survival was observed in *Artemia* bioencapsulated with *L. fusiformis* strain A-1 + *Amphora* sp. (78 ± 2 %); *Bacillus* sp. strain A-2 + *Amphora* sp. (78 ± 1 %); *L. fusiformis* strain Cl-3 + *Chlorella* sp. (78 ± 2 %) and MIX + *Amphora* sp. (78 ± 2 %) in comparison with the control (32 ± 1 %). The number of vibrios in *Artemia* bioencapsulated with *B. pocheonensis* strain S-2 + *Spirulina* sp. showed significant reduction (7.16 ± 0.09 CFU ml^{-1}) after challenged with *V. parahaemolyticus*. In conclusion, the present study proposed that all the potential probionts isolated from microalgae exhibit essential probiotics properties which worth to be studied for application in aquaculture. Two potential probionts (*Bacillus* sp. strain A-2 and *L. fusiformis* strain Cl-3) were found to be good promoting bacteria for microalgal growth. Moreover, mixture of all potential probionts is beneficial for *Artemia* bioencapsulation in conferring protection to *Artemia* nauplii against pathogenic vibrios in the culture environment.

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

PENILAIAN BAKTERIA BERPOTENSI YANG MEMPUNYAI CIRI-CIRI PROBIOTIK UNTUK PERTUMBUHAN MIKROALGA DAN PENGKAYAAN DIDALAM *Artemia fransiscana* (Kellog, 1906)

Oleh

NUR NATASYA AIN BT ROSLAND

November 2018

Pengerusi : Murni Marlina Bt Abd Karim, PhD
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Kepesatan industri akuakultur memerlukan satu pendekatan dalam usaha mengawal penyakit yang tidak berasaskan antibiotik bagi mengurangkan risiko bakteria yang mempunyai daya tahan yang tinggi terhadap antibiotik. Jangkitan bakteria yang disebabkan oleh spesies *Vibrio* telah menyebabkan kematian yang tinggi terhadap ikan terutama sewaktu peringkat larva. Potensi microalga sebagai stok makanan pada masa hadapan dalam sektor akuakultur telah mendorong kepada bidang penyelidikan baru dalam mencari probiotik yang berpotensi bagi meningkatkan biojisim algal dan pada masa yang sama dapat mengurangkan patogen yang biasa menyerang larva ikan. Penyelidikan semasa dijalankan untuk mengkaji potensi baru probiotik dari microalga dan interaksinya dengan microalga. Kemampuan probiotik tersebut dalam melindungi *Artemia* dari *Vibrio* spp. turut dijalankan.

Sejumlah 27 strain bakteria yang berbeza telah berjaya diasingkan dari pelbagai spesies mikroalga (*Amphora* sp., *Chaetoceros* sp., *Chlorella* sp., *Nannochlorum* sp. dan *Spirulina* sp.). Bakteria tersebut telah menjalani ujian saringan *in vitro* dalam usaha mengenalpasti bakteria yang mempunyai kebolehan menghalang pertumbuhan patogen, *Vibrio harveyi* dan *Vibrio parahaemolyticus*. Hasil ujian *in vitro* menunjukkan bahawa 4 dari 27 strain mampu merencatkan pertumbuhan *V. harveyi* dan *V. parahaemolyticus*. Strain A-1, A-2 dan Cl-3 mampu menghalang pertumbuhan kedua - dua patogen yang diuji manakala strain S-2 tidak menunjukkan sebarang perencatan terhadap *V. harveyi*. Keempat-empat bakteria berpotensi ini dikenalpasti sebagai strain *Lysinibacillus fusiformis* A-1 (diasingkan dari *Amphora* sp.), *Bacillus* sp. strain A-2 (diasingkan dari *Amphora* sp.), *Lysinibacillus fusiformis* strain Cl-3 (diasingkan dari *Chlorella* sp.) dan *Bacillus pocheonensis* (diasingkan daripada *Spirulina* sp.) menggunakan 16s rRNA.

Ujian biokimia menunjukkan bahawa semua bakteria yang berpotensi sebagai probiotik mempunyai kemampuan dalam merembeskan enzim catalase, gelatinase, lipase, oxidase,

protease, dan anti-kuorum. Ujian perencatan biofilem menunjukkan bahawa *B. pocheonensis* strain S-2 mampu merencatkan *V. harveyi* paling tinggi (70.36 ± 4.84 %) manakala *V. parahaemolyticus* direncatkan oleh *L. fusiformis* strain A-1, dengan kadar perencatan tertinggi iaitu 67.69 ± 4.70 %. Melalui eksperimen pertumbuhan mikroalga, nisbah inokulasi bakteria dan mikroalga telah ditetapkan pada 1: 4, dan inkubasi bersama dijalankan selama 7 hingga 8 hari. *Bacillus* sp. strain A-2 dan *L. fusiformis* strain Cl-3 dapat meningkatkan pertumbuhan *Amphora* sp. dan *Chlorella* sp. masing-masing. Ini dibuktikan dengan peningkatan sel densiti yang tinggi oleh *Amphora* sp. (8.5 x 10^5 sel ml^{-1}) berbanding dengan kawalan (1.4 x 10^5 sel ml^{-1}), dilihat pada hari ke-8 pengkulturan bersama. Sel *Chlorella* sp. telah meningkat lebih dua kali ganda disebabkan oleh *L. fusiformis* strain Cl-3 (9.9 x 10^6 sel ml^{-1}) berbanding dengan kawalan (4.2 x 10^6 sel ml^{-1}), diperhatikan pada hari ketujuh pengkulturan bersama. Ujian *in vivo* telah dilakukan terhadap nauplii *Artemia fransiscana* menentang *V. harveyi* dan *V. parahaemolyticus*. Bakteria berpotensi ini dibekalkan kepada *Artemia* dalam satu strain tunggal dan kombinasi semua strain yang terpilih, dikenali sebagai MIX bersama-sama dengan mikroalga. Keputusan *in vivo* menunjukkan bahawa *Artemia* yang diperkayakan dengan MIX + *Amphora* sp. (60 ± 4 %) menunjukkan kadar kelangsungan hidup tertinggi berbanding dengan kumpulan kawalan (13 ± 2 %). Penurunan yang ketara dalam bilangan vibrio diperhatikan dalam *Artemia* yang telah diperkayakan dengan MIX + *Amphora* sp. (7.29 ± 0.02 CFU ml^{-1}) berbanding dengan kumpulan kawalan (7.31 ± 0.05 CFU ml^{-1}).

Dalam kes *Artemia* yang dicabar dengan *V. parahaemolyticus*, kelangsungan hidup yang ketara diperhatikan dalam *Artemia* yang diperkayakan dengan *L. fusiformis* strain A-1 + *Amphora* sp. (78 ± 2 %); *Bacillus* sp. strain A-2 + *Amphora* sp. (78 ± 1 %); *L. fusiformis* strain Cl-3 + *Chlorella* sp. (78 ± 2 %) dan MIX + *Amphora* sp. (78 ± 2 %) berbanding dengan kumpulan kawalan (32 ± 1 %). Bilangan vibrio di dalam *Artemia* yang diperkayakan dengan *B. pocheonensis* strain S-2 + *Spirulina* sp. juga menunjukkan penurunan yang ketara (7.16 ± 0.09 CFU ml^{-1}), selepas dicabar oleh *V. parahaemolyticus*. Kesimpulannya, kajian ini menyarankan agar keempat-empat bakteria berpontensi yang diasingkan dari mikrolaga ini dikaji dengan lebih mendalam kerana ia berpotensi dijadikan probiotik untuk kegunaan dalam akuakultur. Dua bakteria berpotensi (*Bacillus* sp. strain A-2 dan *L. fusiformis* strain Cl-3) didapati mempunyai kebolehan dalam meningkatkan pertumbuhan mikroalga. Kajian ini mencadangkan bahawa kombinasi semua probiotik terpilih boleh menjadi bahan yang baik dalam memperkayakan *Artemia* dan melindunginya dari patogenik *Vibrio* didalam kultur persekitaran tersebut.

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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 of Master of Science. The members of the Supervisory Committee were as follows:

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%	Percentage
$\mathrm{CFU\ ml^{-1}}$	Colony forming units per millilitre
$\mathrm{Cells\ ml^{-1}}$	Cells per millilitre
mmt	Million metric ton
mm	Millimetre
ml	Millilitre
min	Minute
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
$\mathrm{Na_2S_2O_3}$	Sodium thiosulphate
$\mathrm{g\ l^{-1}}$	Gram per litre
L	Litre
h	Hour
ppt	Part per thousand
TSA	Thiosulphate Soy Agar
TCBS	Thiosulphate Citrate Bile Salt
rfu	Relative forming unit
µl	Microlitre
um	Micrometre
°C	Degree Celcius

CHAPTER 1

INTRODUCTION

1.1 Background of study

The extensive of industrial development has caused severe disruption to the worldwide aquatic environment. Various problems have occurred, for instance, the widespread of aquatic diseases and the deterioration of water quality have become a major threat in aquaculture.

The accumulation of drug residues in aquatic products has raising a great concern in term of food safety due to the use of antibiotics in aquaculture practices. Furthermore, several pathogens have also resist to drug. Antibiotics must be replaced or reduced with new and environmental friendly products since the food quality demands keep increasing and the negative impacts of antibiotics have caused a risk to humans (Quesada et al., 2013; Arias-Andres et al., 2014; Siriyappagouder et al., 2014).

Live microalgae have been considered as an essential element in aquaculture diet. For instance, microalgae serve as diet component for marine bivalve, mollusc, larvae of some gastropods, fish and shrimp species which, later on will be used as live feeds to cultivate carnivorous larvae of many shrimp and fish species currently farmed (Muller-Feuga, 2000; Naumann et al., 2013). Several tests on non-living diets of algal or non-algal origin have been studied over the years in an attempt providing cost-effective approaches to live microalgae feeds (Hemaiswarya et al., 2011). Unfortunately, Aji (2011) claimed that non-living diets was not able to completely replace live microalgae as feed in aquaculture. Thus, live microalgae remain as primary choice in marine aquaculture feeding (Hemaiswarya et al., 2011) and the management of microalgae production is fundamental in most aquaculture operations, especially in hatcheries (Naumann et al., 2013).

As aquaculture is facing problem due to massive loss because of spreading of diseases there are numerous approaches available to protect aquatic animals against pathogens. Of these approaches, probiotics have become widely used for disease control. Probiotics are defined as live microorganisms where it can confer a health benefit to the host when consume in adequate amount (Reid et al., 2003). Although probiotics offer a promising alternative in aquaculture, the ways that probiotics are applied need to take into consideration to prevent any negative impacts. Probiotics applications can be either in mono or multiple strains. Probiotics also can be a combination of probiotic strains with immunostimulants or prebiotic (Hai, 2015).

Most of the current outbreaks are due to intracellular pathogens, and the production of vaccines against these pathogens has not been an easy task. Vaccines have been reported only can control a specific disease. Due to this problem, vaccinations alone is not possible

in combating all the diseases that occurred in aquatic environments. Thus, the production of non-toxic and non-polluting biological agents for antibiotics substitutions and vaccines has become the main purpose in aquaculture research and development (Wang et al., 2017).

Therefore, the use of probiotics in replacement to antibiotics could be a great alternative in order to improve the safety and quality of aquaculture products. Probiotics are harmless bacteria that improve the well being of an organism or host and contribute either directly or indirectly in protecting the host against harmful bacterial population (Pandiyan et al. 2013). Numerous studies demonstrated that some bacteria have been used as probiotics in aquatic culture to combat bacterial diseases or to improve growth and survival. Moreover, the alteration of microbial community in culture environment also contribute to the reduction of potentially pathogenic microbial population, and enhancement of microalgae as well as live feed cultures (Planas et al., 2004; Villamil et al., 2003; Zink et al., 2013). Planas et al. (2015) pointed out that the selection and application of bacteria as probiotic in microalgae cultures are required to eliminate potentially inhibiting strains or strains that might cause detrimental effects to the cultures. It is well known that bacteria was able to increase or supress the growth of certain microalgae species.

The symbiotic consortium plays an important role in aquatic ecosytems. A greater insight of these associations between microalgae and bacteria in natural ecosystems is very important aspect that has not been thoroughly investigated (Tandon and Jin, 2017). The effects of single bacterial strain on microalgal growth were well documented in several studies. For instance, *Rhizobium* sp. enhanced the growth of *Botrycoccus braunii* and *Candidatus phycosocius bacilliformis* (Rivas, 2010; Tanabe et al., 2015), *Pelagibaca bermudensis* enhanced the growth of *Tetraselmis striata* (Park et al., 2017), and the growth of *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Scenedesmus* sp. and *B. braunii* was enhanced by *Rhizobium* sp. (Kim et al., 2014). In previous study, de-Bashan (2016) also reported that associations of *Azospirillium brasilense* and *Bacillus pumilus* to the *Chlorella* sp. culture significantly increased its growth rate.

The occurrence of pathogenic and opportunistic bacteria in aquatic culture can be controlled by incorporation of probiotics via *Artemia*. Preventing colonization of pathogenic bacteria with selected probiotic strains has been proposed as a valuable approach for microbial control during *Artemia* culture. Another studies demonstrated that probiotics bacteria (*Aeromonas* sp. strain LVS3 and *V. alginolyticus* strain LVS8) can hinder the growth of *Vibrio proteolyticus* (Verschuere et al., 2000) while lactic acid bacteria can reduce the number of *Vibrio alginolyticus* in *Artemia* culture (Villamil et al., 2002). Application of probiotics in rotifers culture exhibit inhibitory effects towards *Vibrio anguillarum* (Harzevili et al., 1988).

1.2 Problem statement

An utmost bottleneck in aquaculture is the sudden reduction of fish larvae caused by pathogenic bacteria (Reid et al., 2009). Among the groups of diseases in aquaculture, vibriosis is the well-known cause of massive economic losses and responsible for high mortality in cultured shrimp, fish and shellfish. Global losses resulting from shrimp diseases are estimated around US$3 billion where *Vibrio harveyi* has been identified as the main causative agent, in which it caused 100 % losses in larval production in many countries (Lavilla-Pitogo et al., 1990; Vinod et al. 2006). *Vibrio campbelli*, *V. parahaemolyticus*, *V. harveyi* and *V. tubiashii* were found to predominate in the west coast of Peninsular Malaysia (You et al., 2016). According to Ransangan et al. (2013), the three most dominant species found in shrimp hatchery (*P. monodon*) in Sabah, Malaysia were *V. harveyi* (22.2%), *V. parahaemolyticus* (22.2%) and *V. alginolyticus* (19.4%).

In addition, the diseases and sudden change of environmental conditions often lead to serious economic losses. The use of antibiotics to control these crashes however has led to spreading and developing of antibiotic-resistance bacteria. Several alternatives have been implemented to improve the sustainability and quality of aquaculture products. Of those methods, the most promising alternatives is the use of probiotics in aquatic animals (Rekiel et al., 2007).

Low concentration of microalgae biomass is one of the serious problems face by most hatcheries production. Thus, the enhancement of microalgae cultures is one of the crucial things that need to be considered for high production of microalgae.

1.3 Significant of the study

Aquaculture as the science of cultivating aquatic animals, plants and organisms like fish, shellfish, microalgae and seaweed, for human consumption and use. In future, microalgae are considered to be one of the most promising feed stocks for aquaculture. One of the effective way to enhance microalgae biomass is through mutualistic interaction between microalgae-bacteria symbiosis. In this study, probionts were isolated from microalgae and co-cultured again with the microalgae. Beneficial bacteria or probionts with antibacterial properties that able to enhance the growth of microalgae would be valuable in aquaculture industry. Besides increasing the algal biomass, the probionts are able to supress fish larval pathogens in aquaculture system thus decreasing the prevalence of diseases caused by *Vibrio* spp..

1.4 Objectives of the study

Hence, the objectives of this study were :

1. To isolate, screen and identify bacteria with antibacterial activity as probiotics from different species of microalgae against *Vibrio harveyi* and *Vibrio parahaemolyticus*

2. To determine the properties of potential probionts in producing extracellular enzymes, anti-quorum sensing, biofilm inhibition and its effect on the growth of respective microalgae.

3. To determine the capability of potential probionts to colonize in *Artemia* and its protection effect against *Vibrio* spp. in preliminary *in vivo* assay.

1.5 Hypothesis of the study

The hypothesis of the study :

Null hypothesis : The isolated strains are unable to inhibit the growth of *V. harveyi* and *V. parahaemolyticus* in *in vitro* assay, do not show any probiotic properties, do not able to increase the growth of microalgae and do not provide protection to *Artemia fransiscana* from vibrios infection.

Alternative hypothesis : The isolated strains are able to inhibit the growth of *V. harveyi* and *V. parahaemolyticus* in *in vitro* assay, portray probiotic properties, can increase the growth of microalgae and able to protect *Artemia fransiscana* from vibrios infection.

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