IMPROVEMENT OF GROWTH RATES AND NUTRITIONAL CONTENTS OF FRESHWATER MICROALGAE THROUGH BACTERIA-MICROALGAE INTERACTION

By

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Chair : Professor Fatimah binti Md Yusoff, PhD
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Microalgae have been utilized extensively for decades in various applications, including aquaculture nutrition, to boost the growth and health of aquatic species. Although microalgae have high biomass production per unit area, their growth rate and biochemical compositions are heavily influenced by abiotic and biotic variables, including contamination by microorganisms. Nonetheless, bacteria are no longer seen as mere contamination to the cultures, as the symbiotic microalgae-bacterial interactions may benefit both populations and be exploited for aquaculture use. This study was conducted to develop a bacteria-microalgae consortium with improved nutritional content and biomass production to be used as live feed. Ten freshwater microalgae strains (*Chlorella* sp. (UPMC-A0088), *Chlamydomonas reinhardtii* (UPMC-A0054), *Scenedesmus obliquus* (UPMC-A0057), *Scenedesmus communis* (UPMC-A0061), *Oocystis* sp. (UPMC-A0084), *Poteriochroomonas malhamensis* (UPMC-A0073), *Pavlova noctivaga* (UPMC-A0072), *Navicula permitis* (UPMC-A0071), *Nitzschia palea* (UPMC-A0058), *Cyclotella meneghiniana* (UPMC-A0070)) obtained from microalgae culture collection of Laboratory of Aquatic Animal Health and Therapeutics, Institute of Bioscience, Universiti Putra Malaysia were grown in Bold Basal medium at pH 6.8 ± 0.2 under a light intensity of 120 µmol photons $m^{-2}s^{-1}$ at 25 ± 2°C. The growth rate assessment of the microalgae was carried out, followed by biochemical analyses of total carbohydrate, protein, lipid, and fatty acid compositions. Among the ten microalgae, *Scenedesmus communis*, *Navicula pemitis* and *Cyclotella meneghiniana* showed significantly ($P < 0.05$) higher specific growth rates (0.23-0.37 day^{-1}) and biochemical contents (19.96-52.05% DW carbohydrate, 32.27-46.94% DW protein and 17.22-19.72% DW lipid). Subsequently, to isolate microalgae growth-promoting bacteria (MGPB) from the phycosphere, microalgae cells of the ten species were collected at exponential phase, washed, and subjected to centrifugation and sonication to separate bacteria from microalgae colonies. The samples were cultured onto five different medium agar which suited bacterial growth; Alkaline Nutrient (AN), Mueller Hinton (MH), Glucose Peptone Water (GPW), Thiocitrate Bile-salt Sucrose

(TCBS), De Man and Rogasa (MRS). Morphologically distinct colonies were selected, purified, and stored at −20°C for further use. A total of 80 bacterial isolates were screened for their microalgal growth-promoting traits, whereby indole acetic acid (IAA) production, phosphate solubilizing, and nitrogen-fixing abilities were tested. Only seven isolates were recorded to have the multiple MGP traits, with three strains; CY-2, CY-4, CY-5 showing the highest ($P < 0.05$) record for indole acetic acid (IAA) production, as well as phosphate solubilizing and nitrogen-fixing abilities. These seven potential MGPB were molecularly characterized using 16S rRNA approach and the data sequences were deposited into Genbank to get the accession number (MW301667-MW301673). Subsequently the co-cultivation effect of the best three MGPB on the growth and nutritional content of the best three microalgae strains were assessed. *Ochrobactrum haematophilum* was discovered to considerably ($P < 0.05$) improve the specific growth rate (0.38-0.64 day^{-1}) and nutritional contents (17.76- 33.40% DW carbohydrate, 30.53-37.49% DW protein, 22.09-22.50% DW lipid) of microalgae *S. communis, N. permitis* and *C. meneghiniana* up to two-fold increase. This study has shown that bacterium isolated from the phycosphere of microalgae *Cyclotella meneghiniana, Ochrobactrum haematophilum* may have microalgae growth-promoting traits that aid in the development of bacteria- microalgae consortium that can be utilized as a high-quality live feed in aquaculture.

Keywords: Bacteria; growth rate, microalgae; nutritional quality

SDG: No poverty; zero hunger; life below water

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

PENINGKATAN KADAR PERTUMBUHAN DAN KANDUNGAN PEMAKANAN UNTUK MIKROALGA AIR TAWAR MELALUI INTERAKSI BAKTERIA-MICROALGA

Oleh

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Mikroalga telah digunakan secara meluas selama beberapa dekad dalam pelbagai aplikasi, termasuk pemakanan akuakultur, untuk meningkatkan pertumbuhan dan kesihatan spesies akuatik. Walaupun mikroalga mempunyai pengeluaran biojisim yang tinggi bagi setiap unit kawasan, kadar pertumbuhan dan komposisi biokimianya banyak dipengaruhi oleh pembolehubah abiotik dan biotik, termasuk pencemaran oleh mikroorganisma. Walau bagaimanapun, bakteria tidak lagi dilihat sebagai pencemaran kepada kultur semata-mata, kerana interaksi simbiotik mikroalga-bakteria boleh memberi manfaat kepada kedua-dua populasi dan dieksploitasi untuk kegunaan akuakultur. Kajian ini dijalankan untuk membangunkan konsortium bakteria-mikroalga dengan kandungan nutrisi yang lebih baik dan pengeluaran biojisim untuk digunakan sebagai makanan hidup. Sepuluh strain mikroalga (*Chlorella* sp. (UPMC A0088), *Chlamydomonas reinhardtii* (UPMC A0054), *Scenedesmus obliquus* (UPMC A0057), *Scenedesmus communis* (UPMC A0061), *Oocystis* sp. (UPMC A0084), *Poteriochroomonas malhamensis* (UPMC A0073), *Pavlova noctivaga* (UPMC A0072), *Navicula permitis* (UPMC A0071), *Nitzschia palea* (UPMC A0058), *Cyclotella meneghiniana* (UPMC A0070)) yang diperolehi daripada koleksi kultur mikroalga Makmal Kesihatan dan Terapeutik Haiwan Akuatik, Institut Biosains, Universiti Putra Malaysia telah dikultur dalam medium basal Bold pada pH 6.8 ± 0.2 di bawah keamatan cahaya 120 µmol foton $m^{-2}s^{-1}$, pada suhu 25 ± 2°C. Penilaian kadar pertumbuhan mikroalga telah dijalankan, diikuti dengan analisis biokimia bagi jumlah komposisi karbohidrat, protein, lipid dan asid lemak. Di antara sepuluh mikroalga, *Scenedesmus communis*, *Navicula pemitis* dan *Cyclotella meneghiniana* menunjukkan secara signifikan ($P < 0.05$) kadar pertumbuhan spesifik (0.23-0.37 day^{-1}) dan kandungan biokimia yang lebih tinggi (19.96-52.05% DW carbohydrate, 32.27-46.94% DW protein and 17.22-19.72% DW lipid). Seterusnya, untuk mengasingkan bakteria penggalak pertumbuhan mikroalga (MGPB) daripada fikosfera, sel mikroalga daripada sepuluh spesies dikumpul pada fasa eksponen, dibasuh, disentrifugasi dan disonikasi untuk

memisahkan bakteria daripada koloni mikroalga. Sampel dibiakkan pada lima medium agar berbeza yang sesuai dengan pertumbuhan bakteria; Nutrien Beralkali (AN), Mueller Hinton (MH), Air Pepton Glukosa (GPW), Thiocitrate Bile- salt Sukrosa (TCBS), De Man dan Rogasa (MRS). Koloni yang berbeza secara morfologi telah dipilih, disucikan, dan disimpan pada suhu -20°C untuk kegunaan selanjutnya. Sebanyak 80 pencilan bakteria telah disaring bagi ciri-ciri penggalak pertumbuhan mikroalga, di mana pengeluaran asid indole asetik (IAA), pelarutan fosfat, dan kebolehan penetapan nitrogen telah diuji. Hanya tujuh pencilan direkodkan mempunyai pelbagai ciri MGP, dengan tiga strain; CY-2, CY-4, CY- 5 menunjukkan rekod tertinggi ($P < 0.05$) untuk pengeluaran asid indole asetik (IAA), serta kebolehan pelarutan fosfat dan penetapan nitrogen. Tujuh potensi MGPB ini telah dicirikan secara molekul menggunakan pendekatan 16S rRNA dan jujukan data telah didepositkan ke dalam Genbank untuk mendapatkan nombor penyertaan (MW301667-MW301673). Selepas itu, kesan penanaman bersama tiga MGPB terbaik terhadap pertumbuhan dan kandungan pemakanan tiga strain mikroalga terbaik telah dinilai. *Ochrobactrum haematophilum* didapati dengan ketara ($P <0.05$) meningkatkan kadar pertumbuhan spesifik (0.38-0.64 day^{-1}) dan kandungan nutrisi (17.76-33.40% DW carbohydrate, 30.53-37.49% DW protein, 22.09-22.50% DW lipid) mikroalga *S. communis*, *N. permitis* dan *C. meneghiniana* sehingga dua kali ganda. Kajian ini telah menunjukkan bahawa bakteria yang diasingkan daripada fikosfera mikroalga mungkin mempunyai ciri penggalak pertumbuhan mikroalga yang membantu dalam penghasilan makanan hidup berkualiti tinggi dengan kadar pertumbuhan yang pesat. Kajian ini telah menunjukkan bahawa bakteria yang diasingkan daripada fikosfera mikroalga *Cyclotella meneghiniana*, *Ochrobactrum haematophilum* mungkin mempunyai ciri-ciri penggalak pertumbuhan mikroalga yang membantu dalam pembangunan konsortium bakteria-mikroalga yang boleh digunakan sebagai makanan hidup berkualiti tinggi dalam akuakultur.

Kata kunci: Bakteria; kadar pertumbuhan, mikroalga; kualiti nutrisi

SDG: Tiada kemiskinan; sifar kelaparan; kehidupan bawah air

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
DHA	Docosahexaenoic acid
EDTA	Ethylene-Diamine-Tetra-Acetic
EPA	Eicosapentaenoic acid
g	Gram
h	Hour
IAA	Indole acetic acid
kg	Kilogram
L	Liter
m	Meter
MGPB	Microalgae growth-promoting bacteria
mgL^{-1}	Milligram per liter
ml	Milliliter
P	P value
NaOH	Sodium hydroxide
s	Second
SE	Standard error
SGR	Specific growth rate
Sp.	Species
UPM	Universiti Putra Malaysia
°C	Degree Celsius
%	Percentage
<	Less than
>	More than

CHAPTER 1

INTRODUCTION

1.1 General background of the study

As the foundation of the entire aquatic food chain, microalgae aid in fisheries production and are consumed directly or indirectly within the aquaculture artificial environment, through zooplankton enrichment (Brown and Blackburn, 2013). Being the naturally available food for the aquatic organism, it is not a surprise that microalgae role in aquaculture is significantly important (Becker, 2013). Microalgae have received a great deal of interest due to their rapid growth rate and appealing supply of vital components such as carbohydrates, proteins, starch, cellulose, polyunsaturated fatty acids (PUFAs), and carotenoids (Banskota et al., 2018; Han et al., 2019; Ahmad et al., 2022). Many microalgae species produce amino acids and proteins, which are used in diet to defend against many diseases due to the important enzymatic effects (Smee at al., 2008). Similarly, some microalgae species like *Chlorella* and *Spirulina* can provide large levels of protein equivalent to food-rich sources of protein such as milk, soybean, egg and meat (Koyande et al., 2019). Furthermore, certain microalgae are high in polyunsaturated fatty acids that have a range of health advantages, such as omega-3 and omega-6 fatty acids, which are equally significant to humans since the human body is incapable of producing these fatty acids (Becker, 2013). Many microalgae species have been investigated and explored for their ability to synthesise these valuable fatty acids. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the medicinally important omega-3 polyunsaturated fatty acids are essential for the growth and development of aquatic animal (Becker, 2013). EPA and DHA produced by several microalgae species are the sustainable promising source and the only alternative to fish oils (Armenta and Valentine, 2012).

Nonetheless, the greatest obstacle in the growing of microalgae biomass is their high production cost, which is not economically feasible (Barros et al., 2015). Although microalgae have a high biomass production per unit area, abiotic conditions such as temperature, nutrients, pH, and light can have a substantial impact on their growth rates and biochemical compositions (Fu et al., 2019; Becker, 2013; Guedes et al., 2010). Even if these parameters are managed and improved, microbiological contamination by fungus or bacteria might occur, jeopardising microalgae development. Maintaining the axenic mass microalgae culture, on the other hand, may be challenging and impractical. Nonetheless, the situation has altered. Bacteria are no longer seen as only a source of contamination in cultures, but the interaction between microalgae and bacteria has become a focus of biotechnology due to the significant finding of how microalgae and bacteria can impact each other's physiology and metabolism (Amin et al., 2015; Gonzalez and Bashan, 2000; Kim et al., 2014; Seyedsayamdost et al., 2011; Ramanan et al., 2016). Living in the aquatic habitat of the planktonic region with bacteria, they develop

interactions over time and together influence the climate (Ramanan et al, 2016). Therefore, current studies have focused on their relationships and the impact of such interactions on a global scale (Amin et al., 2015; Landa et al., 2015). Several investigations have found that heterotrophic bacteria are important for microalgal growth and survival (Kim et al., 2014; Seyedsayamdost et al., 2011). Similarly, it was demonstrated decades ago in the terrestrial environment that heterotrophic bacteria do not only degrade plant and animal organic matter but also support plant development through sophisticated communication processes and nutrient exchange (Philippot et al., 2013; Cooper and Smith, 2015). Many studies have demonstrated that microalgae- associated bacteria can increase the development of microalgae and impact processes' performance (Hernandez et al., 2009; Choix et al., 2012), and some have sought to replicate these findings. It was also proven that not only microalgae-associated bacteria can enhance the growth of microalgae, but the well-studied plant growth promoting bacteria (PGPB), *Azospirillum brasilense* can significantly enhance the growth of *Chlorella sorokiniana* as well (Amavizca et. al, 2017).

Bacteria play an important role in delivering phytohormones or macro- and micronutrients to microalgae, which results in a variety of physiological changes inside the microalgae, most notably increased growth rate. However, the significance of bacteria in enhancing microalgal development is frequently overlooked. It was discovered that once the phycosphere bacterial communities, particularly those with growth promoting properties, were removed, the microalgal growth rate under phototrophic conditions was exceedingly sluggish (Cho et al., 2015, Watanabe et al., 2005). Recent investigations on microalgae co-cultivation with growth boosting bacteria indicated that bacteria increase microalgal growth rate by at least 10%, and in some cases by up to 70% (Ramanan et al., 2016). Therefore, in bulk production of microalgal cultures, these microalgae growth-promoting bacteria would be extremely beneficial. There has been a rising interest to look into the diversified microorganism communities inhabiting the microalgal phycosphere since the term 'Phycosphere' was first coined in the year 1972 by Bell and Mitchell (1972). Specific terms such as beneficial bacteria, stimulating bacteria, symbiotic bacteria, probiotic bacteria, algal growth enhancer were then suggested. Most recently, the term microalgal growth promoting bacteria (MGPB) has been proposed by Palacios et al. (2022) that defines all sorts of microorganisms capable of enhancing the development and metabolism of microalgae, by focusing only on the positive interactions. Since modifying the phycosphere, such as by adding the chosen MGPB strains may offer a good strategy for enhancing microalgal output and lowering the cost of microalgal production, the potential MGPB from the phycosphere of microalgae should have been bioprospected.

1.2 Problem statements

Malaysia's aquaculture business is expanding to satisfy demand and supply, increasing the demand for microalgae as feed. Therefore, mass culture in

microalgal biotechnology should involve a grasp of the evolutionary and ecological relevance of the connection between microalgae and bacteria, as it influences not only ecosystems but also the growth of the future biotechnology sector (Subashchandrabose et al., 2011; Wang et al., 2014). Additionally, it is critical to have a better knowledge of the bacterial communities associated with nutrient-rich microalgae in order to improve microalgal growth and nutritional contents for use as live feed in aquaculture.

1.3 Objectives

This study was undertaken with the following objectives:

1. To evaluate the tropical freshwater microalgae species with high growth rates and nutritional contents.
2. To isolate, screen and identify the potential phycosphere bacteria with microalgal growth promoting attributes.
3. To study the co-cultivation effects of isolated symbiotic bacteria on the growth and nutritional contents of selected microalgae.

1.4 Hypothesis

For this study, the best three potential microalgae growth-promoting bacteria were co-cultivated with the best three microalgae strains with the highest growth rates and nutritional contents to test the following hypothesis:

Null hypothesis (H_0): There is no difference in growth rate and nutritional content of microalgae upon cultivation with bacteria.

Alternative hypothesis (H_A): There is a difference in growth rate and nutritional content of microalgae upon cultivation with bacteria.

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