PRODUCTIVITY, BIOCHEMICAL COMPOSITION AND TOXICITY OF MIXED MICROALGAE IN DIFFERENT OUTDOOR CULTIVATION SYSTEMS

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By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Dedicated to my family

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman : Hishamuddin Bin Omar, PhD
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Cultivated microalgae are considered as one of the largest unexploited global biomass resources for a sustainable production of food and replacement of fossil resources. Microalgae may offer a viable alternative to fossil fuels; but, this technology must overcome a number of hurdles before it can compete in the market and be widely deployed. These challenges include strain selection and improvement, nutrients sources, the production of microalgae in large-scale and to improve the economics of the entire system. Though there is much excitement about the potential of microalgae there are abundant of work still required in the field. The potential of microalgae to solve variety of world's problems was not realized because of bottleneck in microalgal supplies at reasonable cost. Therefore, the objectives of this study are to culture mixed microalgae in different weather conditions; to compare the productivity, proximate compositions, fatty acid profiles and finally the toxicity values of mixed microalgae. The study was conducted in Tapak Ternakan Ikan, Taman Pertanian Universiti and Department of Biology, Faculty of Science, Universiti Putra Malaysia. The temperature and light intensity during the cultivation period were recorded and scored every day. Optical density and dry weight were measured every two days for 10 days. The growth parameters were affected significantly with the differences in weather conditions. The growth parameters showed highest values during hot weather followed by mix and rainy weather conditions. The highest productivity was found during mix weather condition in unsheltered fertilized mesocosm (0.153 ± 0.001 g L^{-1} day^{-1}). Mixed microalgae also being investigated for their proximate compositions (protein, carbohydrate, and lipid). ANOVA showed that there were significant differences ($p < 0.05$) observed on the proximate compositions of mixed microalgae grown in different weather and culture conditions. The protein content ranged from $22.72 \pm 0.33\%$ to $35.20 \pm 0.42\%$ with the highest value obtained from unsheltered fertilized mesocosm during hot weather condition. The carbohydrates were found to be the major constituent of mixed microalgae in this study. The percentage of carbohydrate ranged from 30.57 ± 0.26 to $41.38 \pm 0.33\%$ and the highest percentage was from sheltered tilapia mesocosm during rainy weather condition. The highest lipids percentage obtained was $11.28 \pm 0.30\%$

which was in sheltered tilapia mesocosm in hot weather condition. For fatty acid compositions, mixed microalgae were extracted for their lipids with methanol: chloroform mixture and after transesterification, the fatty acid methyl ester were analyzed using gas chromatography. Results showed that saturated was the major constituent fatty acids. The average percentages of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids obtained were 45.62 ± 1.37%, 20.05 ± 1.14%, and 34.33 ± 3.17% respectively. The most dominant fatty acid profiles were C18:3n3 (α-linolenic acid) and C16:0 (palmitic acid), with the overall percentages of 19.97% and 19.40% respectively. The methanolic extracts of mixed microalgae were studied for their toxicity with brine shrimp lethality assays. The LC_{50} from six samples calculated ranged from 403.98 ppm to 595.79 ppm. The extracts of mixed microalgae exhibited cytotoxic activity against the brine shrimp and considered as containing active or potent components because their LC_{50} values were less than 1000 ppm. Fertilized sheltered mesocosm has the potential for mixed microalgal production system, the mixed microalgae productivity, proximate composition and lipid acid profile is equivalent or better than terrestrial crops or other expensive single species microalgae production system. The toxicity test also revealed that mixed microalgae can be safely used as feed enhancement for animals.

Keywords: *mixed microalgae, different culture system, outdoor culture, productivity, proximate compositions, fatty acids, and toxicity*

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falasafah

PRODUKTIVITI, KOMPOSISI BIOKIMIA DAN KETOKSIKAN MIKROALGA CAMPURAN DALAM SISTEM KULTUR LUAR YANG BERBEZA

Oleh

NORHAFIZAH BINTI OSMAN

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Pengkulturan mikroalga adalah salah satu sumber biomas dunia terbesar yang masih belum dieksploitasi untuk pengeluaran sumber yang lestari dan menggantikan sumber bahan api fosil. Banyak kajian yang dilakukan bagi merungkai potensi mikroalga, tetapi masalah yang dihadapi adalah selalunya untuk menghasilkan biomas. Potensi mikroalga untuk mengatasi pelbagai masalah dunia tidak dapat direalisasikan kerana halangan dalam membekalkan mikroalga pada harga yang berpatutan. Sehubungan dengan itu, objektif kajian ini adalah untuk mengkultur mikroalga campuran dalam cuaca dan sistem kultur yang berbeza iaitu; untuk membandingkan penghasilan mikroalga campuran, komposisi proksimat, profil asid lemak dan ujian ketoksikan mikroalga campuran. Kajian ini dijalankan di Tapak Ternakan Ikan, Taman Pertanian Universiti dan Jabatan Biologi, Fakulti Sains, Universiti Putra Malaysia. Suhu dan keamatan cahaya direkodkan sepanjang tempoh pengkulturan dijalankan. Kepadatan optikal dan berat kering diukur setiap dua hari. Penghasilan dan kadar pertumbuhan spesifik dikira daripada nilai yang diperoleh daripada berat kering, Parameter pertumbuhan dipengaruhi secara signifikan ($p< 0.05$) oleh perbezaan cuaca dan sistem kultur. Semua parameter pertumbuhan menunjukkan nilai tertinggi semasa cuaca panas diikuti oleh cuaca campuran dan hujan. Produktiviti paling tinggi adalah pada cuaca campuran dalam *'mesocosm'* yang dibaja (0.153 ± 0.001 g L^{-1} $hari^{-1}$). Selain itu, kandungan komposisi proksimat (protin, karbohidrat dan lemak) di dalam mikroalga campuran juga dikaji. Analisis ANOVA menunjukkan bahawa terdapat perbezaan yang signifikan ($p<0.05$) komposisi proksimat (protin, karbohidrat dan lemak) di dalam cuaca dan sistem kultur yang berbeza. Kandungan protein adalah antara $22.72 \pm 0.33\%$ kepada $35.20 \pm 0.42\%$ dengan nilai tertinggi yang diperoleh di dalam *'mesocosm'* yang dibaja tanpa teduhan semasa keadaan cuaca panas. Karbohidrat merupakan komposisi utama mikroalga campuran dalam kajian ini. Peratusan karbohidrat adalah daripada 30.57 ± 0.26 hingga $41.38 \pm 0.33\%$ dan peratusan tertinggi adalah daripada *'mesocosm'* tilapia yang mempunyai teduhan semasa cuaca hujan. Lipid menunjukkan peratusan tertinggi di dalam *'mesocosm'* tilapia yang mempunyai teduhan iaitu $11.28 \pm 0.30\%$. Bagi analisa asid lemak, lipid mikroalga campuran diekstrak menggunakan campuran metanol:klorofom dan selepas

ditransesterifikasi, metil ester asid lemak dianalisa menggunakan kromatografi gas. Keputusan menunjukkan bahawa asid lemak tepu merupakan komposisi utama mikroalga campuran. Peratusan purata asid lemak tepu, asid lemak tak tepu mono dan asid lemak tak tepu poli masing- masing adalah 45.62 ± 1.37%, 20.05 ± 1.14%, dan 34.33 ± 3.17%. Profil asid lemak yang paling dominan ialah C18:3n3 (asid α-linolenik) dan C16:0 (asid palmitik) dengan purata masing- masing 19.97% and 19.40%. Ekstrak metanol juga digunakan bagi mengkaji ketoksikan mikroalga campuran dengan kaedah '*brine shrimp lethality assay'*. Nilai LC_{50} yang diperolehi daripada sampel mikroalga campuran yang dikaji adalah antara 403.98 ppm hingga 595.79 ppm. Ekstrak mikroalga campuran menunjukkan aktiviti sitotoksik kepada *Artemia* sp. dan dianggap mengandungi bahan aktif atau kuat kerana nilai LC_{50} yang diperoleh adalah kurang daripada 1000 ppm. *'mesocosm'* yang dibaja dan ada teduhan mempunyai potensi bagi pengeluaran mikroalga campuran, komposisi proksimat dan profil asid lemak adalah setanding atau lebih baik berbanding tanaman daratan dan mikroalga spesis tunggal. Ujian ketoksikan juga menunjukkan mikroalga campuran selamat digunakan sebagai makanan tambahan bagi haiwan.

Kata kunci: mikroalga campuran, sistem kultur berbeza, kultur luar, produktiviti, komposisi proksimat, asid lemak dan toksisiti.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ABS	Absorbance
ALA	Alpha-linolenic acid
ANOVA	One-way independent analysis of variance
ATP	Adenosine triphosphate
BSLA	Brine shrimp lethality Assay
CO_2	Carbon dioxide
COP	Conference of the parties
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EPA	Eicosapentaenoic acid
FAO	Food and Agriculture Organization
GHG	Greenhouse gas
H_3PO_4	Phosphoric acid
IPCC	Intergovernmental Panel on Climate Change
LA	Linoleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NH_4^+	Ammonia
NO_3^-	Nitrate
OD	Optical density
PBR	Photobioreactor
ppm	Part per million
ppt	Part per thousand
PUFA	Polyunsaturated fatty acid
PVC	Polyvinyl chloride
RNA	Ribonucleic acid
SPSS	Statistical Package for the Social Sciences
TPU	Taman Pertanian Universiti
UPM	Universiti Putra Malaysia
3-PGA	3- phosphorglyceric acid

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Extensive plundering of Earth resources from 19th centuries till present has caused many untold damages especially to the extinction of flora and fauna. The problem is further worsened due to global warming and climate change. The steady increases of atmospheric and oceanic temperature have caused global climate change. Steady increase in world population and the quest for steady economic development also caused steady environmental degradation, pollution and reduced quality of lives. The modern lifestyle and development of science and technology have exploited the natural resources to the maximum state which also produces waste and pollute the Earth. If the exploitation process is not controlled and waste generated has not been handled seriously, we will face severe environmental problems in near future.

Pollutions are caused by our common daily rituals from our domestic, transportation needs and energy usage. The most talk issues recently are the released of greenhouse gas in the form of carbon dioxide and methane to the atmosphere forming a blanket that preventing the heat generated by the solar radiation and through human activities from escaping, thus resulting warmer atmosphere. The warmer atmosphere in turn, alters the global air circulation and water circulation thus altering the climate. Beside climate change, the greenhouse gas also caused acid rain which leach out nutrient from topsoil, acidified the water and causing physiological stress to terrestrial and aquatic organisms.

Non-renewable oil such as petroleum has been used since the last few centuries. The usage is increasing every day. This situation also contributed to greenhouse gas emissions that are the cause of global issues such as global warming (Ribeiro *et al.,* 2007). To achieve environmental and economic sustainability, renewable sources and neutral carbon vehicle fuels are a must. There are various proposals to reduce the emission of greenhouse gas such as using greener energy generation such as hydroelectric power, solar power, wind power, wave power and geothermal power. Reduce, reuse and recycle (3R) approach is slowly adopted in many developing countries. While the intention is noble but the implementation require educating the people pertaining to the importance of 3R, logistics for waste collection and recycling technology. Political will is also very important to ensure the success of 3R.

1.2 Problem statement

In the last decade when the fuel prices were at its peak, there are many studies on the potential of using microalgae as alternative sustainable fuel of the future (Stephens *et al.,* 2013; Abishek *et al.,* 2014). The notions were based on the facts that microalgae has the highest productivity among the primary producer, has the highest oil production

compared to other plant, can be cultivated in almost any part of the world, does not compete with other plant or food production unlike maize and others, require less water than other form of agriculture, crop can be harvested at shorter period and more frequently, able to fix free carbon dioxide, produce oxygen and produce biomass that can be used as animal feed, food for human, fine chemicals for industries and renewable fuels (Ahmad *et al.,* 2011; Brennan and Owende, 2010). Most of the studies were confined to the laboratories using single species in various design photobioreactor. While the results were very promising, the technology and financial limitation prevented the implementation of mass microalgae production. There are numerous reports on pros and con of culturing microalgae in closed system aka photobioreactor and in an open system (Griffith *et al.,* 2011; Narala *et al.,* 2016). There is no right and wrong about it but depends on technology, skill, financial resources available, practicality and the prices of final product.

To date, there are no commercial production of microalgae operating at the scale that would be enough to make a significant contribution to the supply of microalgal biomass, especially for biofuel production. Most studies on microalgae cultivation focused on using a monoculture. The major challenge to grow microalgae at large-scale is the difficulty to maintain the optimal conditions for growth and lipid synthesis as established in the laboratory scale (Ugwu *et al.,* 2008). Particularly, the commercial cultivation is hard to maintain because of the issue of contamination and this issue is critical for productivity. Recent studies have shown that monocultures are difficult to maintain, but mixed cultures comprising of two or more strains may increase and stabilize productivity. According to Johnson and Admassu (2013), the microalgae grown as mixed cultures may reduce environmental risk, especially for outdoor production. A mixed microalgae culture with many varieties of strains would be less vulnerable to the fluctuation of environmental parameters compared to monoculture.

1.3 Justification of the study

This study will look into the potential of growing mixed microalgae in a sheltered open system in a fish pond. Mixed microalgae refer to all species of microalgae that exist in the fish pond throughout the culture period. In a typical fish culture, the water needs to be replaced periodically because of the buildup of nitrogenous waste from fish excrement and uneaten feed such as ammonia which is toxic and can cause fish mortality and affecting fish growth. There are two issues here: use of clean freshwater and discharging wastewater that ends up polluting the natural waterways. Now day's clean water is a precious commodity that is lacking in short supplies in any part of the world. By reducing the water use also will also reduce the waste discharge and the presence of microalgae will reduce the nutrient content in the discharge in the effluent thus reducing the pollution load to the natural water bodies.

In an extensive and semi-intensive fish culture, the presence of microalgae is a common phenomenon that is supposed to provide natural food directly or indirectly to the fish via the intricate food web in the fish pond (Brunson *et al.,* 1994). However, there is limited study on the potential of microalgae in the fish pond. To make the process of producing microalgae biomass in large-scale more economical, microalgae production has to rely on freely available sunlight, atmospheric carbon dioxide and nutrients present in

wastewater. In this regard, we have tried to explore the ability and potential of mixed microalgae cultures to be cultured in different weather conditions for a year-long production. Considering above, this present study addressing the hypothesis that mixed microalgae can be cultured in outdoor open pond system in Malaysia.

1.4 Objectives of the study

i. To compare growth and productivity of mixed microalgae cultured in outdoor under different weather conditions.
ii. To compare the proximate compositions of mixed microalgae cultured in different weather conditions.
iii. To compare the effects of different weather on the fatty acid compositions of mixed microalgae.
iv. To determine the toxicity levels of mixed microalgae using brine shrimp lethality assays.

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