UNIVERSITI PUTRA MALAYSIA

GROWTH AND BIOCHEMICAL CONTENT OF Arthrospira platensis CULTURED USING FRESH PALM OIL MILL EFFLUENT SUPPLEMENTED MEDIUM

PUGANESWARY A/P SUKUMARAN

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By

PUGANESWARY A/P SUKUMARAN

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

March 2017

DEDICATION

This thesis is dedicated to all scientists and researchers who had contributed directly and indirectly in the quest of knowledge.

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

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Chairman : Hishamuddin Bin Omar, PhD
Faculty : Science

Arthrospira, which is widely known as Spirulina, has gained growing commercial interest due to its attractive high-value biomass as an alternative and potential feedstock for food, feed, pharmaceuticals, nutraceuticals, cosmetics and so on. Numerous investigations are being conducted to optimize biomass of this microalga while reducing the production cost. Previously, studies have been done in the formulation of the cost-effective medium using wastewater. However, due to ethical reason, waste from human and animal is not suitable for microalgal cultivation intended for human consumption. Therefore, the present study was conducted to investigate the prospects of fresh palm oil mill effluent (fPOME) as an alternative and inexpensive growth medium in *Arthrospira platensis* cultivation. fPOME is non-toxic, golden-brown industrial wastewater generated as a by-product during crude palm oil extraction. The objective of this study is to determine the growth and biochemical content of *A. platensis* in fPOME supplemented medium under real environment. Accordingly, *A. platensis* was cultured in different concentrations of fPOME supplemented medium of 10 L (0%, 1%, 2%, 3% and 4% v/v) under outdoor conditions to find the optimum fPOME concentration for good growth, productivity and biochemical content of this microalga as in the control (modified Kosaric medium, MKM). Next, the biochemical content of *A. platensis* cultured using the optimum fPOME supplemented medium in scaled-up outdoor conditions (100 L) was compared with commercial Spirulina products. *A. platensis* cultured in T2 (1% v/v fPOME) reached significantly higher ($p < 0.05$) specific growth rate (0.277 $\mu\ d^{-1}$) and productivity (0.159 g L^{-1} d^{-1}) compared to control and other treatments. *A. platensis* in T2 also showed high protein (50.23% dry weight), lipid (10.37% dry weight), total chlorophyll (1.05% dry weight), carotenoids (0.56% dry weight) and phycocyanin (10.93% dry weight), which were slightly comparable with control and significantly higher ($p < 0.05$) compared to other treatments. Under scaled up outdoor conditions, *A. platensis* cultured in 1% (v/v) fPOME supplemented medium attained significantly higher ($p < 0.05$) protein (56.90% dry weight), lipid (12.63% dry weight), phycocyanin (15.95% dry weight), carotenoids (0.58% dry weight), total phenolic (5.70 mg Gallic acid equivalent per gram dry weight) content and second highest total chlorophyll content (1.20% dry weight) compared to other commercial Spirulina products. Besides, the preparation of 1,000 L of fPOME supplemented medium would cost about RM 13.52, in which, 98% and 42% of the medium cost can be saved compared to the generally used standard Kosaric medium (SKM) and MKM respectively. These findings showed the potential of fPOME as a suitable growth medium in cost-effective *Arthrospira* cultivation under natural conditions without adversely affecting the algal growth and biochemical content. Meanwhile, this study also added value to this wastewater as a cheaper and readily available fertilizer in microalgal biomass production. Moreover, it is also proven that the commercial cultivation of Spirulina at low cost is feasible in Malaysia with proper cultivation approach.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERTUMBUHAN AND KANDUNGAN BIOKIMIA *Arthrospira platensis* YANG DIKULTUR DALAM MEDIA YANG DIPERKAYA DENGAN KUMBAHAN MINYAK KELAPA SAWIT

Oleh

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Arthrospira, yang lebih dikenali sebagai Spirulina, mempunyai banyak kepentingan komersial oleh kerana biojisimnya bernilai tinggi yang menarik perhatian sebagai bahan mentah alternatif dan berpotensi dalam penghasilan makanan manusia, makanan haiwan, farmaseutikal, nutraseutikal, kosmetik dan sebagainya. Banyak kajian sedang dijalankan untuk mengoptimumkan biojisim mikroalga ini di samping mengurangkan kos pengeluaran. Sebelum ini, kajian telah dilakukan dalam merumuskan media yang murah menggunakan air sisa. Walau bagaimanapun, disebabkan alasan etika, sisa daripada manusia dan haiwan tidak sesuai untuk pengkulturan mikroalga khusus untuk kegunaan manusia. Oleh itu, kajian ini telah dijalankan untuk mengkaji prospek efluen kilang minyak sawit segar (fPOME) sebagai media pertumbuhan alternatif dan murah dalam pengkulturan *Arthrospira platensis*. fPOME adalah air sisa industri bukan toksik dan berwarna coklat keemasan yang dihasilkan sebagai produk sampingan semasa pengekstrakan minyak kelapa sawit mentah. Objektif kajian ini adalah untuk menentukan pertumbuhan dan kandungan biokimia *A. platensis* dalam media yang diperkaya dengan fPOME di bawah keadaan semulajadi. Sehubungan dengan itu, *A. platensis* telah dikulturkan dalam 10 L media yang diperkaya dengan kepekatan fPOME yang berbeza (0%, 1%, 2%, 3% dan 4% v/v) di bawah keadaan luar untuk mencari kepekatan fPOME yang optima untuk pertumbuhan, produktiviti dan kandungan biokimia mikroalga yang baik sama seperti dalam media kawalan (media Kosaric diubahsuai, MKM). Seterusnya, kandungan biokimia *A. platensis* yang telah dikulturkan dalam media yang diperkaya dengan kepekatan fPOME optima pada skala yang lebih besar (100 L) dalam keadaan luar, telah dibandingkan dengan produk Spirulina komersial. *A. platensis* yang dikultur dalam rawatan T2 (1% v/v fPOME) telah mencapai kadar pertumbuhan tertentu (0.277 $\mu\ d^{-1}$) dan produktiviti (0.159 $g\ L^{-1}\ d^{-1}$) yang tinggi ($p <$ 0.05) berbanding dengan kultur dalam kawalan dan rawatan lain. *A. platensis* dalam T2 juga telah mengandungi protin (50.23% berat kering), lipid (10.37% berat kering), jumlah klorofil (1.05% berat kering), karotenoid (0.56% berat kering) dan fikosianin (10.93% berat kering), yang lebih kurang setanding dengan media kawalan dan lebih tinggi ($p < 0.05$) berbanding dengan rawatan lain. *A. platensis*

yang dikultur di luar dalam persekitaran semulajadi pada skala besar dalam media yang diperkaya dengan 1% (v/v) fPOME telah mencapai kandungan protin (56.90% berat kering), lipid (12.63% berat kering), fikosianin (15.95% berat kering), karotenoid (0.58% berat kering) dan jumlah fenolik (5.70 mg setara dengan asid Gallic pada setiap gram berat kering) yang lebih tinggi ($p < 0.05$) dan kandungan jumlah klorofil yang kedua tertinggi (1.20% berat kering) berbanding dengan produk Spirulina komersial lain. Di samping itu, kos penyediaan 1000 L media yang diperkaya dengan fPOME adalah kira-kira RM 13.52, di mana 98% dan 42% daripada kos media boleh dijimatkan berbanding dengan media Kosaric standard (SKM) yang lazim digunakan dan MKM masing-masing. Hasil kajian ini menunjukkan potensi fPOME sebagai media pertumbuhan yang sesuai dalam pengkulturan *Arthrospira* di bawah keadaan semulajadi pada kos yang berbaloi tanpa menjejaskan pertumbuhan dan kandungan biokimia alga. Sementara itu, kajian ini juga menambah nilai kepada air sisa ini sebagai baja yang lebih murah dan mudah didapati dalam penghasilan biojisim mikroalga. Kajian ini membuktikan bahawa penanaman *A. platensis* secara komersial boleh dijalankan dengan jayanya di Malaysia melalui teknik pengkulturan yang betul.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God Almighty for giving me the strength, knowledge, ability and opportunity to undertake this research study and to persevere and complete it satisfactorily. Without His blessings, this achievement would not have been possible. My sincere gratefulness and love to my adorable parents Mr. Sukumaran and Mrs. Kuppammal for their endless love and moral support throughout my life.

Next, I would like to extend my deepest appreciation and gratitude to my supervisor, Dr. Hishamuddin Omar for his valuables ideas, concern, dedication, suggestion, guidance and enthusiasm throughout the completion of this research program. He has given me all the freedom to pursue my research while, silently and inconspicuously ensuring that I stay on course and do not deviate from the core of my research. He is not only the internal guide for this research but also a mentor, a friend, an inspiration and a living role model for me.

My acknowledgment would be incomplete without thanking my co-supervisor, Dr. Rosimah Nulit for being so kind, helpful and motivating especially during my tough phases, in which, I faced a lot of hurdles. She has always been there for me with her motherly hand whenever I needed it the most. Her constant assistance, cooperation and encouragement have always kept me going ahead. I am very much obliged to her for her every single guidance and I thank her for everything, herewith.

I am extremely grateful to my second co-supervisor, Dr. Normala Halimoon from the Faculty of Environmental Studies, University Putra Malaysia (UPM) and my external co-supervisor, Dr. Sanimah Simoh from Malaysian Agricultural Research and Development Institute (MARDI) for their constructive ideas and suggestions, besides allowing me to access to their laboratory and equipment. Furthermore, I would like to thank Dr. Suhaimi Hamzah for permitting me to conduct a part of the analysis in Malaysian Nuclear Agency, Bangi.

Not forgotten to extend gratefulness to the management of Faculty of Agriculture, UPM for providing site to conduct my outdoor cultivation study. I would like to seize this opportunity to acknowledge Mr. Bala, the manager of Palm Oil Factory, Hulu Langat who granted permission to collect fresh palm oil mill effluent (fPOME) directly from the factory outlet. Many thanks to all the technical staffs from the Biology Department, Faculty of Science and the Faculty of Environmental studies, UPM for their warmest helping hand. I owe grand thanks to Mr. Fazri from MARDI and Mr. Lim Ching Choah, Mr. Haji Abdul Halim and Mrs. Shamsiah from Malaysian Nuclear Agency for their technical support and cooperation.

My best regards to UPM for rewarding Research University Grant Scheme (RUGS) to support this research. I am very indebted indeed to The Ministry of Higher

Education for granting me the MyPhD scholarship to assist my studies financially. My special acknowledgment goes to the Head of Department of Biology Department and Dean of Faculty of Science, UPM for their continuous support and motivation towards my postgraduate affairs. A special appreciation towards all my seniors, fellow lab mates and friends for their guidance, cooperation and moral support.

Last but not least, I would like to express my heartfelt gratitude to my dearest fiancé, Mr. Thanasilan Purshothaman for his everlasting love, care, continuous encouragement, moral support, motivational phrases and constant prayers, which always cheers me up and drives me to complete this research work successfully. Throughout the fruition of my Ph.D. study, his presence means a lot to me. My beloved sisters, little brother and all my well-wishers deserve my wholehearted thanks as well.

I certify that a Thesis Examination Committee has met on 17 March 2017 to conduct the final examination of Puganeswary a/p Sukumaran on her thesis entitled "Growth and Biochemical Content of *Arthrospira platensis* Cultured using Fresh Palm Oil Mill Effluent Supplemented Medium" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

ABS	Absorbance
ANOVA	One-way independent analysis of variance
As	Arsenic
ATP	Adenosine triphosphate
B	Boron
Ba	Barium
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BOD	Biochemical oxygen demand
C:N:P	Carbon:Nitrogen:Phosphorus
Ca	Calcium
Cd	Cadmium
Co	Cobalt
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CPO	Crude palm oil
Cr	Chromium
Cu	Copper
DDW	Double distilled water
DIW	Deionized water
DO	Dissolved oxygen
DOE	Department of Environment
DOM	Dissolves organic matter

DPPH	2,2-Diphenyl-1-picrylhydrazyl
EFB	Empty fruit bunches
EQA	Environment Quality Act
FAO	Food and Agriculture Organization
Fe	Iron
FFB	Fresh fruit bunches
fPOME	Fresh palm oil mill effluent
FRAP	Ferric reducing antioxidant power
FRIM	Forest Research Institute Malaysia
GHGs	Greenhouse gases
GNI	Gross National Income
H_3PO_4	Phosphoric acid
HRT	Hydraulic retention times
HSD	Honestly Significant Difference
ICP-MS	Inductive Couple Plasma-Mass Spectrometry
IPCC	Intergovernmental Panel on Climate Change
IQ	Intelligence quotient
K	Potassium
Li	Lithium
MF	Mesocarp fibres
Mg	Magnesium
MKM	Modified Kosaric medium
Mn	Manganese
Mo	Molybdenum
MPOB	Malaysia Palm Oil Board

MPOC	Malaysian Palm Oil Council
N	Nitrogen
N:P:K	Nitrogen:Phosphorus:Potassium
Na	Sodium
Na^+	Sodium ion
NaCl	Sodium chloride
$NADPH_2$	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
$NaHCO_3$	Sodium bicarbonate
NASA	National Aeronautics and Space Administration
NBS	National Biomass Strategy
NH_4^+	Ammonium
Ni	Nickel
NO_3^-	Nitrate
NOAA	National Oceanic and Atmospheric Administration
O_2	Oxygen
OH^-	Hydroxide
P	Phosphorus
Pb	Lead
PBR	Photobioreactor
PKS	Palm kernel shells
PO_4^{3-}	Orthophosphate
POME	Palm oil mill effluent
PORIM	Palm Oil Research Institute of Malaysia
ppb	Part per billion
ppm	Part per million

PUFAs	Polyunsaturated fatty acids
PVC	Polyvinyl chloride
r^2	Pearson coefficient
RCBD	Randomized complete block design
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
S	Sulphur
Se	Selenium
SKM	Standard Kosaric medium
SPSS	Statistical Package for the Social Sciences
Sr	Strontium
TDS	Total dissolved solids
Ti	Titanium
Tl	Thallium
TN	Total nitrogen
TPC	Total phenolic content
TPTZ	2,4,6-Tripyridyl-s-Triazine
TS	Total solids
U	Uranium
UV	Ultraviolet
V	Vanadium
WFP	World Food Programme
Zn	Zinc

CHAPTER 1

INTRODUCTION

Global warming and food insecurity associated with the inevitable increasing world population are the leading international issues threatening humanity, society, economy and nature for the past several decades. As Industrial Revolution emerged about two and half centuries ago, the rapid growth of human population coupled with the development of industry sectors intensified the exploitation of natural resources such as water, land, flora and fauna and fossil fuels (McLamb, 2011; Vitousek *et al*., 1997). Meanwhile, human activities such as logging, deforestation, agricultural practices, indiscriminate land use, rapid mining and waste disposal were developed hastily in order to meet necessities of the spiraling human population. Such phenomenon led to diminishing global resources, air and water pollution, ecosystem destruction and global warming (de Sherbinin *et al*., 2007). At the same time, extensive usage of fossil fuels such as coal, oil and natural gas by the developing industries especially energy sector, transportation and agriculture sector increased the emissions of CO_2, which is the major component of greenhouse gases responsible for global warming and climate change (Omer, 2008).

Climate change is another hotly debated topic of current days that greatly interrelated with global warming and poses risk to public health, agriculture industry, socio-economy and ecosystem with soaring atmospheric temperature, alteration of precipitation patterns, glacier melting and unpredictable extreme weather events such as flood, drought, heat waves and heavy rainfall (Haines *et al*., 2006; McMichael, 2003). Anthropogenic activities simultaneously with exaggerating climate change resulted in water scarcity, depletion of cultivable land and soil degradation, which are the major bottlenecks in productive agricultural practices. On the other hand, overconsumption of energy accompanied with the increasing energy demand spiked the fuel prices and hence, made it a necessity to search towards the alternative energy sources such as biofuel that produced from energy crops, which certainly compete with food crops for resources (Pimentel *et al*., 2008). Accordingly, rapid decline in water, land and energy reserves along with escalating energy prices, directly and indirectly, affected the agricultural crops yield as well as increased the market value of food, which caused in a deleterious effect on food access, especially in third world countries (Hanjra and Qureshi, 2010; Pimentel, 2009). Failure in global food production to meet the rapidly growing world population and increasing demand for food ultimately resulted in global food crisis, hunger, malnutrition, morbidity and mortality.

With the growing concerns about the impacts of global warming and food crisis, government and non-government policies and scientific consensuses were addressed since past decades at the national and international levels to deal with these issues. Yet, there is no practical solution for these global crises to ensure long-term survivability, environmental sustainability, national security and global stability. Therefore, society started to look into sustainable, economically feasible and environmentally friendly technologies to mitigate the global warming as well as for sustainable food production. In this manner, utilization of photosynthetic organisms appears to be particularly attractive in CO_2 abatement as well as in food production (Field *et al*., 2008). From this perspective, the photoautotrophic cultivation of microalgae is starting to catch attention mainly due to its simultaneous CO_2 sequestration and profitable bio-active compounds production in a single process (Ravindran *et al*., 2016; Rosenberg *et al*., 2011).

Microalgae are microscopic organisms living basically in the aquatic environment. These photosynthetic microorganisms are capable of fixing free and soluble CO_2 into carbon-containing biochemical compounds with the aid of solar energy and release O_2 to the atmosphere as a byproduct (Rinanti, 2016). Since the early fifties, microalgal mass cultivation has been explored and developed extensively owing to the essential macro and micro nutrients content such as protein, carbohydrate, lipid, vitamins and minerals (Spolaore *et al*., 2006; Soletto *et al*., 2005). Presently, microalgae yet again emerged as a potential source of biomass for various biotechnological processes such as food and feed additives, dietary supplements, biofuel and bio-fertilizers due to its advantages over terrestrial crops (Mata *et al*., 2010). These microscopic organisms possess rapid growth rate approximately 100 times faster than terrestrial plants (Tredici, 2010) and higher photosynthetic efficiency at a rate of 11.6% compared to plants that exhibiting 1 – 2% of photosynthetic efficiency (Vasudevan and Briggs, 2008). Besides that, microalgal farming utilizes less land and water compared to land crops (Li *et al*., 2008) and does not require herbicides and pesticides. At the same time, microalgae have a remarkable ability in atmospheric CO_2 sequestration with higher bio-fixation efficiency than terrestrial plants due to their simple cell structure and fast reproduction ability (Li *et al*., 2008).

Furthermore, the arid and semi-arid land around the world, which is not suitable for food crops production, can be utilized in profitable microalgal production as this unconventional crop does not require fertile land. Moreover, these microorganisms not only grow in freshwater but, can be grown in seawater, brackish water or coastal seawater (Khan *et al*., 2009), which hold about 97% of the total Earth's water. Thus, microalgal biomass production for various industrial applications does not compete directly or indirectly with agricultural crops for resources such as land and water. Furthermore, they can grow in various ecological habitats and able to acclimatize rapidly in extreme environmental conditions such as high temperature, salinity and pH owing to their unicellular or simple multicellular structure (Mata *et al*., 2010). On the other hand, microalgal cultivation also benefited in wastewater bioremediation by consuming contaminants such as NH_4^+, NO_3^- and PO_4^{3-} as growth nutrients (Sing-Lai *et al*., 2010). Additionally, the fine molecules extracted from algal biomass including amino acids, polysaccharides, fatty acids, pigments and antioxidants have wide applications in various industrial sectors such pharmaceuticals, nutraceuticals, cosmetics, aquaculture and so on. Thus, microalgal cultivation is being suggested as a possible solution for the critical humanitarian challenges such as food and energy crises, global warming and other environmental issues.

Considering the advantages of microalgal cultivation, research and developments began with the large-scale production of *Chlorella*, *Scenedesmus* and *Dunaliella* (Pulz and Gross, 2004; Borowitzka, 1999). However, high costs of harvesting associated with the microscopic size of these algae (5 – 10 µm diameters), low digestibility due to the rigid cell wall and high risk of contamination in large-scale cultivation (in some species) became major challenges in cost-effective microalgal mass production. Hence, *Arthrospira* (previously known as *Spirulina*) has attracted public and private interest due to its peculiar properties such as large filamentous size (0.5 mm length) aiding in easy harvesting, effortlessly digestible cell membrane as well as less risk of external contamination due to its ability to grow in saline environment and alkaline condition up to pH 11, which is not suitable for other species (Pelizer *et al*., 2003). *Arthrospira* is a photosynthetic, blue-green, spiral-shaped and multicellular cyanobacterium, which is being consumed as food for thousands of years (Jensen *et al*., 2001). Currently, this microorganism is recognized as a potential dietary supplement due to its rich nutrition value such as abundant protein content (50 – 70% dry weight), low nucleic acid content, vitamins, pigments, minerals, polyunsaturated fatty acids (PUFAs) and so on (Habib *et al*., 2008).

Besides, NASA has acknowledged *Arthrospira* as ‘food of future’ and proposed this microalga as an ideal food for astronauts (Raoof *et al*., 2006). However, *Arthrospira* cultivation is not widely practiced especially in developing countries like Malaysia beyond the laboratory scales due to the high production cost with a lower yield. Thus, cost-effective microalgal cultivation techniques should be enhanced through outdoor mass production (Converti *et al*., 2006; Meseck *et al*., 2005) under natural climate using available resources. Since the temperature tolerance level of *Arthrospira* ranges from 20 °C to 40 °C (Kumar *et al*., 2011a), our Malaysian climate is suggested to be a favorable condition for the good growth of this microalga. Besides that, utilization of cost-effective cultivation medium should be considered in the development of low-cost *Arthrospira* production. Previously, studies have been done on formulating economically feasible and environmental friendly growth medium for *Arthrospira* cultivation utilizing effluent such as swine wastewater (Manikandavelu and Murugan, 2009), dry chicken manure (Ungsethaphand *et al*., 2009), piggery wastewater (Depraetere *et al*., 2013), human urine (Feng and Wu, 2006) and sago starch factory effluent (Phang *et al*., 2000) as an additional growth nutrient simultaneously benefitted in bioremediation of the wastewater. However, microalgal cultivation using human or animal waste intended for food and feed purpose is unethical and may pose health risks.

Accordingly, fresh palm oil mill effluent (fPOME) is deemed to be a suitable supplementary nutrient in *Arthrospira* cultivation. Malaysia is the second largest producer of palm oil and controls about 45% of global palm oil production (Vairappan and Yen, 2008). This huge amount of palm oil extraction, which provides a significant contribution to our economy, is also associated with the emission of abundant byproducts such as POME. fPOME is a golden brown colloidal suspension and considered non-toxic as the oil extraction process does not incorporate any hazardous chemicals (Kiyasudeen *et al*., 2016). Nevertheless, this effluent contains high COD, BOD and TS values. Besides, a huge quantity of methane gas is being released especially from the anaerobic POME treatment pond, which poses a major threat to the environment. Appropriate disposal of this highly polluted wastewater is a great challenge to the palm oil industry due to the high-cost, time-consuming and low-efficient processes involved the current treatment technologies. Hence, the idea of adding value to this wastewater as a supplementary medium in microalgal cultivation is suggested to be profitable for both palm oil and microalgal industry, aside from being environmental friendly. Microalgal cultivation using POME is not something new in Malaysia. Previously, investigations have been conducted using pretreated and digested POME to culture *Arthrospira* in laboratory (Zainal *et al*., 2012; Parkavi *et al*., 2011) and outdoor shaded conditions (Aguol, 2003). However, utilization of fPOME in *Arthrospira* cultivation never been explored before.

Therefore, the present study was conducted to determine growth and biochemical content of *A. platensis* in low-cost fPOME supplemented medium under natural conditions. In relation to the culture optimization, growth and productivity of *A. platensis* were examined under outdoor conditions in cost-effective synthetic medium formulated with industrial grade chemicals. Next, *A. platensis* was cultured in different concentrations of fPOME supplemented medium (0%, 1%, 2%, 3% and 4% v/v) under outdoor conditions using previously formulated synthetic medium as a reference medium to find the optimum fPOME concentration for growth, productivity and biochemical content of this microalga. Besides, the organic pollutants removal efficiency of *A. platensis* was studied in the different concentrations of fPOME supplemented medium. Finally, the biochemical content of *A. platensis* cultured using the optimum fPOME supplemented medium in scaled-up outdoor conditions (100 L) was compared with commercial Spirulina products to determine the prospect of this microalga in food and feed applications. The novelty of the present investigation was the utilization of fPOME in *A. platensis* cultivation under real environment focusing on the algal biomass and biochemical productivity. This study would pave the way for the development of commercial *Arthrospira* production in Malaysia using cost-effective, feasible and environmentally friendly cultivation techniques in order to increase the accessibility of this wonder food for the general public.

1.1 Study objectives

1. To examine growth and productivity of *A. platensis* cultured in outdoor conditions using cost-effective synthetic medium.
2. To study the growth, productivity and organic pollutants removal efficiency of *A. platensis* grown in different concentrations of fresh palm oil mill effluent (fPOME) supplemented medium under outdoor conditions.
3. To analyze the biochemical content of *A. platensis* cultured in different concentrations of fresh palm oil mill effluent (fPOME) supplemented medium under outdoor conditions.
4. To compare the biochemical content of *A. platensis* cultured using fresh palm oil mill effluent (fPOME) supplemented medium in scaled-up outdoor conditions (100 L) with commercial Spirulina products.

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