LIPID PRODUCTION FROM PALM OIL MILL EFFLUENT BY MICROALGAE

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To my beloved family

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ABSTRACT

In tropical countries, the palm oil industry discharges a large amount of wastewater. The wastewater can serve as an economical nutrient source or substrate that can support the cultivation of microalgae. This study aimed to identify the local species of microalgae potentially existing in the industrial wastewater of palm oil mill effluent (POME). POME was selected as the key source of waste due to its higher potential in producing lipids from microalgae as biofuel substrate. A novel green microalgal strain was isolated from POME of Kulai-Johor west Palm Oil Mill in Malaysia and was identified as *Chlamydomonas sp*. and subsequently named *Chlamydomonas sp*. UTM 98 with Catalogue No. of KR349061. This strain was cultivated in media with different volume ratios of POME and Basal Bald Medium (BBM). Lipid is generally a group of organic compound that serves as the primary raw material for biofuel. Therefore, this study emphasizes the effectiveness of POME as the main carbon source to maintain the growth of microalgae and simultaneously to increase the lipid content. In addition, glucose ($C_6H_{12}O_6$) was also used to compare the effectiveness of their cultivations against POME. Furthermore, four selected strains of green microalgae are applied namely *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Chlorella sorokiniana*, *Tetraselmis sp* and isolated microalgae from POME. All cultivation of microalgae were initially carried out in 250mL Erlenmeyer flask containing 100 mL medium at ± 30°C with continuous illumination (± 14 μmol^{-1} m^{-2} s^{-1}) and up to 20 days of cultivations. The study demonstrated that *Chlamydomona incerta* (*C. incerta*) is the predominant species for specific growth rate (μ), biomass productivity and lipid content in the diluted POME with the value of 0.099/d, 8.0 mg L^{-1}.d, 2.68 mg lipid mg^{-1} Cell Dry Weight (CDW), respectively. However, *C. incerta* showed that there was about one and the half times more lipid productivity when the biomass cells utilized glucose as carbon source, compared to POME. The best condition was determined with various carbon-to-total nitrogen (C:TN) ratio and light/dark (L:D) cycles, respectively. As a result, the highest lipid content was achieved when the condition was controlled at C:TN (100:7) and with continuous light (24 hr) which recorded a value of 17 mg lipid mg^{-1} CDW. These results concluded that *C. incerta* had the highest growth rates and lipid production in the diluted POME compared to other strains of microalgae. Finally, the study suggested several improvement of the experiment to achieve higher lipid production at steady state condition by manipulating the ratio of carbon-to-total nitrogen and the light intensity on the bio-substrate. The Nile Red method was used to measure the lipid content in the culture. Fatty Acid Methyl Esters (FAMEs) and samples were analyzed via gas chromatography. POME with COD 250mg L^{-1} concentration showed the greater lipid content with absorbance 3.138a.u. The result showed that C*hlamydomonas sp* UTM 98 grown in the media of diluted POME exhibited a high potential of microalgae for biomass production and POME nutrients removal.

ABSTRAK

Di negara tropika, industri minyak kelapa sawit melepaskan sejumlah besar air sisa. Air sisa boleh menjadi sumber nutrien ekonomi atau substrat yang boleh menyokong penanaman mikroalga. Kajian ini bertujuan untuk mengenal pasti spesies tempatan mikroalga yang berpontensi dan sedia ada dalam air sisa perindustrian kilang minyak sawit (POME). POME telah dipilih sebagai sumber utama sisa kerana potensinya yang lebih tinggi dalam menghasilkan lipid dari mikroalga yang boleh dijadikan bahan api bio-substrat. Sejenis mikroalga hijau yang baru ditemui telah diasingkan daripada POME Kilang Minyak Sawit barat Kulai-Johor di Malaysia dan telah dikenal pasti sebagai *Chlamydomonas sp*. dan dinamakan *Chlamydomonas sp*. UTM 98 dengan No. Catalogue KR349061. Strain ini telah dikultur dalam media POME dan Basal Bald Medium (BBM) dengan nisbah isipadu yang berbeza. Lipid merupakan sebatian organik yang penting sebagai bahan mentah utama banhan api. Oleh itu, kajian ini memberi penekanan kepada keberkesanan POME sebagai sumber karbon utama untuk mengekalkan pertumbuhan mikroalga dan juga untuk meningkatkan kandungan lipid. Di samping itu, glukosa ($C_6H_{12}O_6$) juga digunakan untuk membandingkan keberkesanan pengkulturan mikroalga berbanding POME. Tambahan pula, empat jenis mikroalga iaitu *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Chlorella sorokiniana*, T*etraselmis sp* dan microalga yang diasing dari POME. Semua pengkulturan mikroalga telah dilakukan di 250 mL kelalang Erlenmeyer yang mengandungi 100 mL media pada ± 30^{o}C suhu dengan pencahayaan yang berterusan (± 14 µmol^{-1} m^{-2} s^{-1}) untuk selama 20 hari. Kajian ini menunjukkan bahawa *Chlamydomonas incerta* (*C. inserta*) adalah spesies utama untuk kadar pertumbuhan spesifik (µ), produktiviti biojisin dan kandungan lipid pada kadar pencairan POME adalah masing-masing pada 0.099 / d, 8.0 mg L^{-1}.d, 2.68 mg lipid mg^{-1} Berat Sel Kering (CDW). Walau bagaimanapun, *Chlamydomona incerta* menunjukkan bahawa terdapat kira-kira satu setengah kali lebih produktiviti lipid apabila glukos digunakan sebagai sumber karbon, berbanding POME. Keadaan terbaik ditentukan pada pelbagai nisbah karbon / nitrogen (C: TN) dan kitaran cahaya / gelap (L: D). Hasilnya, kandungan lipid yang paling tinggi dicapai apabila keadaan dikawal pada C: TN (100: 7) dan cahaya yang berterusan (24 jam), di mana nilai yang direkodkan sebanyak 17 mg lipid mg^{-1} CDW. Keputusan ini menyimpulkan bahawa *C. incerta* mempunyai kadar pertumbuhan yang paling tinggi dan pengeluaran lipid dalam POME yang dicairkan berbanding mikroalga yang lain. Akhir sekali, kajian ini mencadangkan beberapa penambahbaikan eksperimen untuk mencapai pengeluaran lipid yang tinggi pada keadaan mantap dengan memanipulasi nisbah karbon/ nitrogen dan keamatan cahaya ke atas bio-substrat. Kaedah Merah Nile telah digunakan untuk mengukur kandungan lipid dalam media. Fatty Acid Methyl Esters (FAMEs) dan sampel dianalisis melalui kromatografi gas. POME pada kepekatan COD 250mg L^{-1} menunjukkan kandungan lipid yang lebih besar dengan nilai *absorbance* 3.138a.u. Hasilnya menunjukkan Chlamydomonas sp. UTM 98 yang dikultur dalam media POME yang dicairkan menunjukkan potensi yang tinggi untuk pengeluaran biojisim dan penyingkiran POME nutrien.

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LIST OF SYMBOLS

α	-	alpha
$C_6H_{12}O_6$	-	glucose
CH_4	-	methane gas
CI	-	concentration of lipids at the end of batch run
CO_2	-	carbon dioxide
d	-	day
et.al.	-	and others
g	-	gram
g/L.d	-	gram per liter per day
H_2O	-	water
H_2S	-	hydrogen sulphide
μ	-	specific growth rate
X	-	biomass
X_m	-	concentration of biomass at the end of batch run
X_0	-	concentration of biomass at the beginning of a batch run
Km	-	Michaelis-Menten constant, expressed in the same units as X
Ki	-	dissociation constant for substrate binding in such a way that two substrates can bind to an enzyme
LN	-	natural Log
m^3	-	cubic meter
$P_{Biomass}$	-	biomass Productivity (mg/L.d)
P_{Lipid}	-	lipid Productivity (mg/L.d)
t	-	time, duration (hour,day)
v/v	-	volume per volume
Vmax	-	maximum enzyme velocity

LIST OF ABBREVIATIONS

AN	-	Ammonical nitrogen
ATP	-	Adenosine TriPhosphate
BBM	-	Bald's Basal Medium
BOD	-	Biological Oxygen Demand
C	-	Carbon
CDW	-	Cell Dry Weight
CPH	-	Corn Powder Hydrolysate
COD	-	Chemical Oxygen Demand
DNA	-	Deoxyribonucleic acid
EQA	-	Environmental Quality Acts
FFB	-	Fresh fruit brunches
LD	-	Light/ dark cycle
MLSS	-	Mixed Liquor Suspended Solid
MLVSS	-	Mixed Liquor Volatile Suspended Solid
N	-	Nitrogen
NADPH	-	Nicotinamide Adenine Dinucleotide Phosphate-oxidase
nm	-	nanometer
POME	-	Palm Oil Mill Effluent
OLR	-	Organic Loading Rate
OMW	-	Olive Mill Waste
O&G	-	Oil & Gas
PAR	-	Photosynthetically active radiance
RPM	-	*Revolutions per minute* (r/min)
S	-	Substrate
SS	-	Suspended solid
TCA	-	Tricarboxylic acid cycle
TS	-	Total Solid

TSS	-	Total Suspended Solid
TN	-	Total Nitrogen
VFA	-	Volatile fatty acids

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Oil palm is one of the world's most rapidly expanding equatorial crops. In Malaysia, palm plantation currently occupies the largest acre of farmed land and the palm oil industry is growing rapidly. Malaysia is one of the major palm oil producers in the world (Lam *et al*., 2009; MPOB, 2012). It was estimated that for each tonnes of crude palm oil produced, 5-7.5 tonnes of water are required, and more than 50% of the water will end up as palm oil mill effluent (POME) (Wu *et al*., 2007 and Singh *et al*., 2010).While the palm oil industry has been recognized strongly for its contribution towards economic growth and rapid development, it has also contributed to environmental pollution due to the production of large quantities of by products during the process of oil extraction (Singh *et al*., 2010; Parthasarathy *et al*., 2016).

Freshwater microalgae are globally ubiquitous and highly deserve, with tens or perhaps hundreds of thousands of species, in a myriad of forms and sizes (Guiry and Guiry, 2014). Current classifications consider most microalgae to be protists with chloroplast, but there also photosynthetic prokaryotes (cyanobacteria) and a subset of land plants (Wehr *et al*., 2015).

Microalgal culture has received much attention, given its prospects as a source of bioenergy and its potential for wastewater treatment. In this respect, simple and easily cultivated biomass has a number of applications, ranging from its direct use such as biodiesel and various pigments (Fulton, 2004). The demand for these products is increasing due to their adverse properties, which are economically and environmentally viable options. The complication of cultivation methods and the high cost of growth medium have become a major drawback for the algal industry; nevertheless, the integration along with wastewater treatment has provided a feasible solution due to the fact that exploitation of wastewater as the source of growth medium simultaneously eliminates the requirement for expensive medium and at the same time remediates the wastewater. Recently, much attention has been given to the production of biodiesel from all over the world. The world production of biodiesel was appraised to be around 1.8 billion liters annually (Fulton, 2004; Gnansounou and Raman, 2016). It is also found that biodiesel can be produced using vegetable oils of terraneous oil plants, such as soybean, sunflower and oil palm. However a recent development in the production of biodiesel from microalgae is highly needed to be competitive in the fuel industry.

The current study, investigated the potential, benefits, strategies, and challenges of microalgae to be integrated with wastewater treatment, POME treatment in Malaysia due to the hazardous properties of POME, which may lead to severe environmental pollution. The integration of POME treatment using microalgal culture will potentially reduce the wastewater treatment retention time and eliminate toxic elements, which serve as nutrients for the growth of microalgae. Moreover, microalgae are gaining considerable attention as a feedstock for biodiesel production as they can be grown away from the croplands and hence do not compromise food crop supplies (Liu *et al*., 2008). The exploration of new microalgae integration methods for the development and formation of valuable products is also being discussed in this study.

1.2 Background of Study

Palm oil mill Effluent (POME) is the wastewater generated by processing oil palm and consists of various suspended materials. POME has a very high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), which is 100 times more than the municipal sewage. The effluent also contains higher concentration of organic nitrogen, phosphorus, and other nutrient contents (Hadiyanto, 2013). POME is a non- toxic waste, but will pose environmental issue due to large oxygen depleting capability in aquatic system due to organic and nutrient contents. It is also known to be a good source of nutrients (Kamyab *et al*., 2014a).

The waste products generated during palm oil processing consists of oil palm trunks (OPT), oil palm fronds (OPF), empty fruit bunches (EFB), palm pressed fibers (PPF) and palm kernel shells, less fibrous material such as palm kernel cake and liquid discharge palm oil mill effluent (POME) (Singh *et al*., 2010).The wastes are in the form of high organic matters concentration, such as cellulosic wastes with a mixture of carbohydrates and oils. The discharge of untreated POME creates adverse impact to the environment (Abdul Aziz, 2007).

Nowadays biological process is the most common practice for the treatment of POME based on anaerobic and aerobic ponding system (Singh *et al*., 2010).While the emerging technologies for the treatment of POME, the notion of nurturing POME and its derivatives as valuable resources should not be dismissed. Furthermore, it is necessary to properly address the POME treatment so as not to contribute to human health hazards and environmental pollution. When compared to the conventional wastewater treatment process which introduces activated sludge and biological floc to degrade organic carbonaceous matter to CO_2, microalgae can assimilate organic pollutants into cellular constituents such as lipid and carbohydrate, thus achieving pollutant reduction in a more environmental friendly way (de Andrade *et al*., 2016). In fact, microalgae have become the focus of attention for both wastewater treatment and biomass production as early as 1950s (Oswald and Gotaas, 1957).

Microalgae can assist the treatment and purification of wastewater such as municipal, animal and industrial runoff, while benefiting from using the nutrients. For several years, it has been studied that microalgae have the potential to remove organic and inorganic matters present in the polluted water. It is also concluded that this method is an economic method for removing inorganic and organic materials from the wastewater, resulting in better quality water discharge and obtaining valuable algal biomass which could be useful for different purposes such as the production of biofuel, fertilizer, animal feedstock, biogas etc., (Becker, 2007; Gonçalves *et al*., 2016).

Several studies have shown that microalgae are able to remove nitrogen and phosphorus from the wastewater (Aslan and Kapdan, 2006; Gonçalves *et al*., 2016; Lu *et al*., 2016). Microalgae grow rapidly; able to divide once every 3-4 h, but mostly would divide every 1-2 days under favorable growth conditions (Griffiths and Harrison, 2009; Huang *et al*, 2010). Moreover, parameters such as temperature, irradiance and, most markedly, nutrient availability have been shown to affect both lipids composition and content in many microalgae (Rodolfi *et al*., 2009; Karpagam *et al*., 2015; Suganya *et al*., 2016). The average lipid content of algal cells varies between 1% and 70%, but can reach 90% of dry weight under certain conditions (Xin *et al*., 2010; Lordan and Stanton 2011). The total content of lipids in microalgae may vary from about 1- 85% of the dry weight, with values higher than 40% being typically achieved under nutrient limitation. The interest in microalgae for biodiesel production is due to the presence of high amount of lipid content in some species, and also due to the fact that lipid synthesis, especially of non-polar TAGs (triacylglycerols), which are considered to be the best substrate for producing biodiesel, can be modulated by varying the growth conditions (Monari *et al*., 2016).

1.3 Problem statement

In 2011, Malaysia was the second largest palm oil producer in the world, with a total of 16.6 million tonnes, an amount lesser than 1% from the total world's supply behind Indonesia. Since the palm oil industry is huge, with 67% of agricultural land covered with oil palm tree, biomass from oil palm contributes the most. Currently, 85.5% of the biomass residues are coming from the palm oil industry. Palm oil has a very good potential in producing alternative energy due to its calorific content (Ahmad *et al*., 2011 and MPOB, 2012). More than 85% of palm oil mills in Malaysia have adopted ponding system for treating POME (Ma *et al*., 1993;Chin *et al*.,2013) while, the rest have opted for open digesting tank (Yacob *et al*., 2005). These methods are regarded as conventional POME treatment method, whereby longer retention time and large treatment areas are required (Poh and Chong 2009). The effluent that comes out from palm oil mill is hazardous to the ecosystem. The discharge can lead to land and aquatic pollution if it is left untreated (Salihu, 2012).

Based on the statistic value of total crude palm oil production in May 2001, the production of 985,063 tonnes of crude palm oil means a total of 1,477,595 m^3 of water is being used, and 738,797 m^3 released as POME, in a month. Without proper treatment, this wastewater will pollute the nearby watercourses. The current treatment technology of POME typically consists of biological aerobic and anaerobic digestion. Biologically treated effluent is disposed of via land application system, thus providing essential nutrients for growing plants. This method may be a good choice for the disposal of treated effluent. However, considering the rate of daily wastewater production, for example, approximately 26 m^3/d for an average palm oil mill with an operating capacity of 35 t/d FFB, it is doubtful that the surrounding plantations receiving it could efficiently absorb all the treated effluent (Wah *et al*., 2002).

The waste water treatment technologies are expensive, dependent on skilled personnel and hard to carry out (Darajeh *et al*., 2014). Furthermore, the common conventional treatment is unable to meet the regulations set by the Department of Environment (DOE) with the level of BOD at 100 mg/L. According to Ahmad *et al*., 2003, large quantities of water are used during the extraction of crude palm oil from the fresh fruit bunch, and about 50% of the water results in POME. POME is a thick brownish liquid that contains high amount of total solids (40,500 mg/L), oil and grease (4000 mg/L), COD (50,000 mg/L) and BOD (25,000 mg/L). The disposal of this highly polluting effluent is becoming a major problem if it is not being treated properly besides a stringent standard limit imposed by the Malaysian Department of Environment for the discharge of effluent. A POME treatment system based on membrane technology shows high potential for eliminating the environmental problem, and in addition, this alternative treatment system offers water recycling (Ahmad *et al*., 2003).

POME contains high content of degradable organic matter, which could become one of the promising sources for renewable energy in Malaysia (Ahmad *et al*., 2011; Chin *et al*., 2013). The discharge of improperly treated POME creates adverse impact to the environment. However, the substances in POME are able to support the growth of microalgae. Microalgae naturally exist in many palm oil mill processes, phenomenon known as "algae bloom", hence declining the water quality. Because POME consists of large amount of organic compounds and inorganics which is hazardous to environmental health, therefore microalgae have been suggested as a potential candidate to remove these pollutants and able to breakdown the organic compounds present in it (Munoz and Guieysse, 2006; Kamyab *et al*., 2015a).

On the other hand, culturing microalgae in wastewater offers an inexpensive alternative to the conventional forms of wastewater treatment (Hoh *et al*., 2016; Ge *et al*., 2016). At the same time microalgae can utilize the nitrogen and phosphorus compound in wastewater to generate microalgae biomass for different types of lipid production, which can serve as a substrate for biofuel production (Huang *et al*., 2010; Kamyab *et al*., 2014a).

In addition, still there is a need to investigate an efficient microalgae candidate to apply in wastewater treatment method for remediation and simultaneously produce lipid. Utilizing microalgae into the treatment system cause to several advantageous comprise enhancing treatment method, microalgae growth, decreasing nutrients, reducing cost and time saving.

1.4 Objectives of study

The main objectives of this study are as follows:

1. To isolate, identify and screen the algal species that can grow in different concentrations of POME.
2. To investigate the growth and the physio-chemical parameters in achieving higher lipid from the selected micro algal species.
3. To determine the lipid content from the selected microalgae based on different conditions (carbon to total nitrogen ratio, photo periods, and organic loading rate).
4. To quantify and characterize the fatty acid content of the selected microalgae to serve as substrate for the production of biodiesel.

1.5 Scope of study

This study aimed to isolate and identify the potential microalgae isolated from the palm oil mill located in Johor, Malaysia (geographical location, latitude N 3^{o} 57' 20.01and longitude E 101^{o} 11' 55.69) which has the existing POME that are capable of producing high lipid using microalgae. The scope of this study is to assess the main component of POME to be used as organic carbon for microalgae. The research is mainly focused in developing a lab scale prototype to produce high lipid content from the microalgae. The applicable parameters such as optical density (OD), Light intensity, Chlorophyll content, Mixed Liquor Suspended Solid (MLSS), Mixed Liquor Volatile Suspended Solid (MLVSS), Cell Dry Weight (CDW), Scanning Electronic Microscope (SEM) and FTIR, were also investigated in this study to enhance the lipid production. The study is more focused on lipid production of POME using different species of microalgae at laboratory scale.

1.6 Significance of the study

The use of wastewater for the growth of microalgal cultures is considered beneficial for minimizing the use of freshwater, reducing the cost of nutrient addition, removing nitrogen and phosphorus from wastewater and producing microalgal biomass as bio resources for biofuel or value added by products. There are three main sources of wastewater (municipal (domestic), agricultural and industrial wastewater) which contains a variety of ingredients. Some components in the wastewater, such as nitrogen and phosphorus, are useful ingredients for microalgal cultures (Chiu *et al*., 2015).

There are several important aspects to be considered during the current study. POME is the major source of water pollutant in Malaysia (Kamarudin *et al*., 2015). For example, in a conventional palm oil mill, 600-700 kg of POME is generated for every 1000 kg of processed FFB (fresh fruit bunches) (Aini *et al*., 1999; MPOB, 2014). By utilizing the ingredients present in POME, this study will play a major role to solve the pollution problem resulting from the POME as it will pollute the environment if it is improperly discharged into the environment.

Furthermore, culturing microalgae in wastewater offers an inexpensive alternative in comparison to conventional forms of biological wastewater treatments in reducing the main contents such as nitrogen and phosphorus compounds present in the wastewater. Therefore, this study using POME has the potential to offer carbon source for the microalgal growth in promoting high lipid content (Kamyab *et al*., 20154b; Chiu *et al*., 2015). Microalgae are able to produce the highest potential outputs of oil. Furthermore, the characteristics of microalgae include all year long growth and a short life cycle that makes it the fastest growing plant on Earth; microalgae growth is 100 times faster than trees. Additionally, microalgae cultivation is inexpensive with its necessities only being readily available raw materials such as sunlight, water, carbon dioxide and nutrients. Besides that, the information and knowledge gained through this research will provide a strong foundation for further research in biochemistry, genetic engineering, and technology enhancement of oil producing microalgae (Clarens *et al*., 2010).

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