

KINETIC STUDIES FOR PHYCOREMEDIATION AND NUTRIENT
REMOVAL BY *Chlorella sorokiniana* OF PALM OIL MILL EFFLUENT

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The research work is specially dedicated to my parent

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ABSTRACT

Palm Oil Mill Effluent (POME) is an untreated wastewater that is generated from palm oil industry containing high amount of nutrients such as nitrate, phosphate, ammonium and chemical oxygen demand (COD). Untreated effluent discharge into watercourse produce carcinogenic by-products in drinking water. In addition, eutrophication can cause oxygen deficiency in water thereby affecting the aquatic microorganisms. POME treatment using microalgae has been considered a more biofriendly system, however, high amount of nutrients affect the growth of microalgae. Hence, the present study investigates the effectiveness of robust *Chlorella sorokiniana* for pollutants removal in different dilutions of sterilized and raw POME. Standard and pyrosequencing methods were used in this study. The results for the former showed higher removal of nitrate, phosphate, ammonium and COD in raw POME than sterilized POME with the 80% (v/v) dilution been the best dilution accounting for higher growth and nutrients removal by *C. sorokiniana*. While results from the latter revealed the presence of *Bacillaceae*, *Paenibacillaceae*, *Enterococcaceae*, *Clostridiaceae*, *Peptostreptococcaceae*, *Caulobacteraceae*, *Enterobacteriaceae*, *Moraxellaceae* and *Pseudomonadaceae* bacterial families in raw POME. In addition, study of the biokinetic coefficients using Michaelis-Menten rate expression in optimal sterilized POME dilution showed that, the biokinetic coefficients of nitrate was; $\{k=9.2 \times 10^{-3} \text{ mg NO}_3^- \text{ mg}^{-1} \text{ dry cell weight (DCW), d}^{-1}, K_m=68.7 \text{ mg/L}, Y_N=0.1 \text{ g DCW g}^{-1} \text{ NO}_3^-\}$, for phosphate; $\{k=8 \times 10^{-3} \text{ mg PO}_4^{3-} \text{ mg}^{-1} \text{ DCW d}^{-1} \text{ and } K_m=144.6 \text{ mg/L}, Y_P=0.12 \text{ g DCW g}^{-1} \text{ PO}_4^{3-}\}$, for ammonium; $\{k=2.3 \times 10^{-2} \text{ mg NH}_4^+ \text{ mg}^{-1} \text{ DCW d}^{-1}, K_m=113 \text{ mg/L}, Y_N=0.08 \text{ g DCW g}^{-1} \text{ NH}_4^+\}$ and for COD $\{k=0.15 \text{ mg COD mg}^{-1} \text{ DCW d}^{-1} \text{ and } K_m=1662 \text{ mg/L}, Y_{\text{COD}}=0.02 \text{ g DCW g}^{-1} \text{ COD}\}$. Transesterification reaction was also carried out on the biomass for fatty acid methyl ester (FAME) production. The gas chromatography-mass spectrometer (GC-MS) conducted revealed the presence of saturated FAME in algae oil which includes tridecyclic acid, myristic acid, pentadecyclic, palmitic acid and stearic acid. Based on the effect of different nitrogen sources on biomass and lipid production, urea feed of 1 and 2 g/L was identified as the optimal concentration for maximum lipid and biomass production respectively. This study is the first report of the potential of *C. sorokiniana* for POME treatment with FAME production.

ABSTRAK

Efluen kilang kelapa Sawit (POME) adalah air kumbahan yang tidak dirawat yang dihasilkan daripada industri minyak sawit yang mengandungi jumlah nutrisi yang tinggi seperti nitrat, fosfat, ammonium dan permintaan oksigen kimia (COD). Pelepasan sisa cecair ini ke dalam saluran air tanpa rawatan menghasilkan produk sampingan yang bersifat karsinogenik dalam air minuman. Di samping itu, eutrofikasi dapat menyebabkan kekurangan oksigen dalam air sehingga mempengaruhi mikroorganisma akuatik. Rawatan POME menggunakan mikroalga telah dianggap sebagai sistem mesra-bio, namun jumlah nutrisi yang tinggi mempengaruhi pertumbuhan mikroalga. Oleh itu, kajian ini mengkaji keberkesanan *Klorela sorokiniana* yang kuat untuk menyingkirkan pencemar yang berbeza daripada POME yang steril dan mentah. Kaedah standard dan piro-penjujukan digunakan dalam kajian ini. Keputusan yang lepas menunjukkan penambahan nitrat, fosfat, ammonium dan COD yang lebih tinggi daripada POME mentah daripada POME yang disterilkan dengan pencairan 80% (v/v) merupakan yang terbaik bagi pertumbuhan yang lebih tinggi dan penyingkiran nutrien oleh *C. sorokiniana*. Selain itu, keputusan terakhir mendedahkan kehadiran *Bacillaceae*, *Paenibacillaceae*, *Enterococcaceae*, *Clostridiaceae*, *Peptostreptococcaceae*, *Caulobacteraceae*, *Enterobacteriaceae*, *Moraxellaceae* dan keluarga bakteria *Pseudomonadaceae* dalam POME mentah. Kajian koefisien biokinetik menggunakan ungkapan kadar Michaelis-Menten dalam pencairan POME yang disterilkan secara optimum menunjukkan bahawa koefisien nitrat biokinetik adalah; $\{k = 9.2 * 10^{-3} \text{ mg NO}_3^- \text{ mg}^{-1} \text{ berat sel kering (DCW), d}^{-1}, K_m = 68.7 \text{ mg/L}, Y_N = 0.1 \text{ g DCW g}^{-1} \text{ NO}_3^-\}$ untuk fosfat; $\{k = 8 * 10^{-3} \text{ mg PO}_4^{3-} \text{ mg}^{-1} \text{ DCW d}^{-1} \text{ dan } K_m = 144.6 \text{ mg/L}, Y_P = 0.12 \text{ g DCW g}^{-1} \text{ PO}_4^{3-}\}$, untuk ammonium; $\{k = 2.3 * 10^{-2} \text{ mg NH}_4^+ \text{ mg}^{-1} \text{ DCW d}^{-1}, K_m = 113 \text{ mg L}, Y_N = 0.08 \text{ g DCW g}^{-1} \text{ NH}_4^+\}$ dan $K_m = 1662 \text{ mg / L}, Y_{\text{COD}} = 0.02 \text{ g DCW g}^{-1} \text{ COD}$ untuk COD}. Reaksi transesterifikasi juga dilakukan pada biomas untuk pengeluaran metil ester asid lemak (FAME). Hasil daripada kromatografi gas-spektrometer massa (GC-MS) menunjukkan kehadiran metil ester asid lemak tepu (FAME) dalam minyak alga yang merangkumi asid tridesiklik, asid miristik, pentadesiklik, asid palmitik dan asid stearik. Berdasarkan kesan sumber nitrogen yang berbeza pada pengeluaran biomas dan lipid, penyediaan urea sebanyak 1 dan 2 g / L masing-masing telah dikenal pasti sebagai kepekatan yang optimum untuk pengeluaran lipid dan biojisim secara maksimum. Kajian ini merupakan laporan kajian pertama tentang potensi *C. sorokiniana* untuk rawatan POME dengan pengeluaran FAME.

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LIST OF ABBREVIATIONS

ACCCase	-	Acetyl-CoA carboxylase
ADP	-	Adenosine diphosphate
APHA	-	American public health association
ATR	-	Acid transesterification reaction
ASTM	-	American society for testing and materials
ATP	-	Adenosine triphosphate
BOD	-	Biological oxygen demand
BTR	-	Basic transesterification
<i>C. sorokiniana</i>	-	<i>Chlorella sorokiniana</i>
COD	-	Chemical oxygen demand
DCW	-	Dry cell weight
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediaminetetraacetic Acid
ETR	-	Enzyme catalysed transesterification
FAEE	-	Fatty acid ethyl ester
FAME	-	Fatty acid methyl ester
FAP	-	Facultative anaerobic pond
FFA	-	Free fatty acid
GC-MS	-	Gas chromatography-mass spectrometer
ICP-OES	-	Inductive coupled plasma optical emission spectroscopy
ISO	-	International standards organization
kW	-	Kilowatts
MLSS	-	Mixed liquor suspended solid
MLVSS	-	Mixed liquor volatile suspended solid
NADPH	-	Nicotinamide adenine dinucleotide

NTU	-	Nephelometric units
OD	-	Optical density
PCR	-	Polymerase chain reaction
pH	-	Potential of hydrogen
POME	-	Palm oil mill effluent
RF	-	Radiative forcing
RPM	-	Rotation per time
SEM	-	Scanning electron microscope
SLP	-	Solvent lipid complex
ssDNA	-	Single-stranded DNA
sp.	-	Species
TAG	-	Triacylglyceride
UV	-	Ultraviolet

LIST OF SYMBOLS

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	-	Copper Sulphate
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	-	Cobalt (II) Nitrate Hexahydrate
CO_2	-	Carbon dioxide
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	-	Iron (II) Sulfate Heptahydrate
g/L	-	Gram per Litre
g	-	Gram
H_3BO_3	-	Boric acid
H_2SO_4	-	Sulphuric acid
HNO_3	-	Nitric acid
KOH	-	Potassium hydroxide
K_m	-	Saturation rate constant
mL	-	Millilitre
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	-	Manganese (II) Chloride Tetrahydrate
MoO_3	-	Molybdenum (VI) oxide
NH_4^+	-	Ammonium
NaOH	-	Sodium Hydroxide
NO_3^-	-	Nitrate
NO_2^-	-	Nitrite
PO_4^{3-}	-	Phosphate
%	-	Percentage
k	-	Reaction/Removal rate constant
Y	-	Yield coefficient
Y_P	-	Yield coefficient of phosphate removal
Y_N	-	Yield coefficient of nitrate removal
Y_{COD}	-	Yield coefficient of COD removal
R_x	-	Specific rate of substrate removal

v/v	-	Volume by volume
°C	-	Degree centigrade
ρ	-	Density
μm	-	Micrometre
μmol	-	Micromole
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	-	Zinc Sulfate Heptahydrate

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CHAPTER 1

INTRODUCTION

1.1 Background of the Research

Due to rapid industrialisation which in effect had caused high levels of pollution especially of water systems, there has been an increased interest in the discovery of microphytes to mitigate pollution concentrations in contaminated waters. Most water pollution arose from the discharge of wastewater from a piggery, dairy, refinery, textile and palm oil industry (Abdullah *et al.*, 2013). Palm oil industries in Malaysia have grown rapidly over the years (Neoh *et al.*, 2013). The various processes of palm oil mill production (sterilization, stripping, digestion, pressing and clarification) lead to the generation of wastewater known as palm oil mill effluent (POME) (Lam and Lee, 2011). A great deal of concern on the quality and quantity of POME generated and discharge into natural water bodies have recently indicated the need for better treatment strategy to handle water pollution. POME contained high amount of nitrogen, phosphorus, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), Total Solid (TS) and Suspended Solid (SS).

The release of such high chemical effluent poses serious environmental challenges such as eutrophication to the receiving water bodies (De-Bashan and Bashan, 2010; de Godos *et al.*, 2009; Munoz and Guieysse, 2006). Conventional treatment systems such as ponding system, anaerobic digestion, mechanical aeration, activated sludge system and chemical method was currently under practice, however,

these are methods that suffer many disadvantages such as poor pollutants removal, generation of secondary pollutants and expensive to practice. One promising approach to achieve effective bioremediation and energy reduction during the treatment is to integrate algal facility within the existing industrial wastewater treatment system. This implies that the wastewater was used as feed for microalgae growth. The advantage of this system is that while the microalgae remove excess nutrients in the wastewater, there will be a concomitant accumulation of biomass for downstream processing (Chinnasamy *et al.*, 2010b; Olguí, 2003; Rawat *et al.*, 2011). The major nutrients required for the cultivation of algal cell is basically nitrogen and phosphorus and they are contained in wastewater. This kind of industrial symbiosis can critically decline the economic and environmental cost of wastewater treatment plant. Thus, phycoremediation which uses naturally occurring microorganism to treat the industrial wastewater and this treatment process can prove less expensive with no secondary production of pollution and easier to handle than other technologies employed to clean up hazardous waste (de Godos *et al.*, 2009; Kshirsagar, 2013).

Microalgae are microscopic plant belonging to both prokaryotic and eukaryotic microorganism. The prokaryotic algae are the cyanobacteria that resemble typical bacteria. The three most important classes of eukaryotic algae are basically diatoms (Bacillariophyceae), green algae (Chlorophyceae) and the golden algae (Chrysophyceae) (Saidu *et al.*, 2017). Their unicellular feature gives them competitive advantages of converting about 50% of global carbon present in the atmosphere. Microalgae exist in chain or individually with no root, stem and leave (Mondal *et al.*, 2016). The presence of green pigment allows them to conduct photosynthesis using available sunlight and nutrients and therefore grow rapidly. Although most of the microalgae species live in freshwater and marine environment, there are few other microalgae species that can grow under harsh and unfavourable environmental conditions. Some species of microalgae contained a robust cell wall which enables them to withstand unfavourable condition (Sahay and Braganza, 2016). Depending on the species, some microalgae can acclimatize to different environmental changes which are due to their ability to produce chemical substances in order to neutralize the effect of this environmental changes. From a practical point of view, many of their species are easy to cultivate because they are not too

demanding of constant monitoring especially in water that is rich in nutrients and unsuitable for human use. In addition, an increase in the concentration of the nutrients was also reported to increase their growth cycle (Choudhary *et al.*, 2016). As a result of that, microalgae has recently been fully incorporated as an important bioremediation agent and are already been used by many wastewater facilities.

Although the main focus of this research is to evaluate the viability of using microalgae for nutrients stripping in POME, it is of high crucial to put into consideration the utilization of the cultivated biomass post-wastewater treatment. Many species of microalgae had evolved to produce biomass which contains high amount of lipid with the potential of converting it to biodiesel (an alternative to petroleum-based diesel) (Ahmad *et al.*, 2016). Fatty acid methyl ester (FAME) is a renewable form of bio-energy that fulfilled the promise of providing an alternative source of energy and future reduction of environmental pollution. This is why the continuous use of petroleum-based fuels is now widely recognized as unsustainable because of depleting supplies and the contribution of these fuels to the accumulation of carbon dioxide in the environment (Li *et al.*, 2011b; Luthfi *et al.*, 2017). Literature has stated that high productivity of lipid is strongly correlated with the growth of algae in a particular growth medium (Bertoldi *et al.*, 2006). It is therefore suggested that the use of wastewater as an algae growth substrate will go a long way in reducing the operational cost of algae cultivation. For instance, *C. vulgaris* was demonstrated to grow on artificial medium resulted in 20-42% (dry biomass) lipid while reported to have nutrients removal efficiency for ammonium and phosphorus to be 97% and 96% respectively (Feng *et al.*, 2011). Nevertheless, studies have reported the occurrence of some algal species with the ability to removed nutrients and have high lipid content when grown in wastewater (Kamyab *et al.* 2015; Lekshmi *et al.*, 2015; Wang *et al.*, 2010b). Specifically, on POME, a very limited research on its treatment using microalgae exist (Ding *et al.*, 2016a; Hadiyanto and Nur, 2012). Yet, none of this literatures reported the use of mixotrophic microalgae species belonging to particular genera identified to have robust cell wall and lipid-rich such as *Chlorella sorokiniana* for simultaneous treatment of POME under sterilized and raw conditions, coupled with biodiesel production. This species grow mixotrophically, giving it the ability to grow well in a strange environment. The aim of this research is

to assess the potential of utilizing *C. sorokiniana* for the treatment of POME. By conducting a biokinetic study for pollutants removal from POME, a comprehensive understanding of the biological processes taking place during the treatment can be obtained. As microalgae wastewater treatment, hold a promise of proving zero waste generation, the biomass of wastewater-grown algae will be investigated for lipid and fatty acid methyl ester (FAME). This is a one-step strategy of achieving effective phycoremediation and developing the sustainability of biofuel production.

1.2 Statement of Problem

Malaysia is the second producers of palm oil across the globe (Lam and Lee, 2011). During the processing activities of palm oil in the palm oil industry, about 5-7.5 tonne of water is required for the production of 1 tonne of crude palm oil, and more than 50% of the used water produce POME (Ahmad *et al.*, 2016). The presence of high amount of nitrates, phosphate, chemical oxygen demand and ammonium in POME affects both human, aquatic biota and the surrounding environment. It causes eutrophication which promotes excessive growth of algae and causes oxygen depletion in water body thereby threatening the life of the aquatic organisms (Cai *et al.*, 2013). The conventional treatment methods of POME include vermicomposting, mechanical aeration, anaerobic digestion and ponding system. However, these are methods that suffer disadvantages such as inefficiency for pollutants removal, expensive to practice and introduction of secondary pollutant into the environment. Therefore the ultimate solution of finding an efficient method that can remove pollutants from POME with no production of secondary pollutant is to utilize the biological method, and the use of microalgae in this case can provide dual role of efficient POME treatment and production of valuable biomass.

The major challenges of using microalgae for POME treatment are slow rate of pollutant removal, which therefore prolongs the complete removal of these pollutants in POME. Therefore microalgae that have the ability to remove organic pollutant by both assimilatory and dissimilatory process are adequately required in

wastewater treatment plant. During the process of assimilatory and dissimilatory activity, nitrate and phosphate are been converted into ammonium and orthophosphate respectively in the algal cell before they are utilized for growth (Cai *et al.*, 2013). These processes are considered useful more especially to a situation where there is need to utilize the captured nitrate and phosphate in the algal biomass for the production of value-added substances.

The large volumes of the biomass that are left after POME treatment are mostly left to decay with the aim of improving the soil fertility (Kamyab *et al.*, 2014). This is considered unacceptable by the local community as it causes aesthetic pollution to the environment. A quite remarkable opportunity is the utilization of wastewater grown algal biomass as a promising feedstock for biodiesel production. This will add more value to the economy of biofuel production in terms of reducing the over-dependence on food crop as a feedstock for bioenergy production. Another key problem hindering the up-scaling of algae-based biofuel production is high cost of production due to unusual cultivating system (Doma *et al.*, 2016). The use of POME as medium will completely eradicate the need for cultivating medium (synthetic) which will reduce the cost of biodiesel production in the biodiesel industry.

1.3 Objectives of the Research

The objectives of the research are;

- i. To analyze the performance of *Chlorella sorokiniana* for nutrients removal in POME under sterilized and raw condition.
- ii. To study the biokinetic coefficients of nitrate, phosphate, ammonium and COD removal in the best-diluted POME by *Chlorella sorokiniana*.
- iii. To investigate the potential of wastewater-grown microalgae for the production lipid and fatty acid methyl ester (FAME).

1.4 Scope of the Research

The project gave more priority on the cultivation of *C. sorokiniana* in different dilutions of POME for bio-treatment and biokinetic coefficients study. The specific POME dilution that supported the maximum growth of algae was selected and subjected to the subsequent experiments. POME was first characterized according to the standard method of APHA before the commencement of phycoremediation (Apha, 2005). Various kinetic coefficients for removal of nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+) and COD were studied for over 15 days. Next, the potential of utilizing biomass of wastewater-grown algae for lipid and fatty acid methyl ester (FAME) production was determined using solvent extraction and transesterification method. Gas chromatography-mass spectrometer (GC-MS) was used for the identification of FAME. The obtained FAME was characterized based on physical features and its quality was compared with world standard quality criteria for diesel. Next-generation sequencing (pyrosequencing) was used to identify the indigenous microbial communities that interact with microalgae during the treatment of POME under raw condition.

1.5 Summary of Chapter 1

The increase in wastewater discharge from various industries into the water bodies has been considered as the major causes of water pollution across the globe. In Malaysia, the rapid growth of the palm oil industries has made it the second producers of palm oil in the world. POME is a wastewater that is generated during the processing of palm oil in palm oil milling industry. POME contain high amount of chemical compounds and nutrients such as BOD, COD, nitrate, phosphate and heavy metals. The release of these chemical compounds into the water bodies affects both human and aquatic biota and therefore it is imperative to find an efficient method that will remove these pollutants before it is discharged out to the environment.

The conventional methods of POME treatment were unable to meet the regulatory discharge limit set by the Malaysian Department of Environmental (DOE) that is why using microalgae to treat POME is considered highly efficient. Microalgae are suitable for the treatment of POME because the necessary and fundamental components of their growth are mainly nitrogen and phosphorus and are adequately contained in POME wastewater. This gave microalgae the ability to remove various pollutants such as nitrogen and phosphorus from water. POME treatment using microalgae results into the production of large volume of biomass which has a potential to be used for the production of value-added production. This means that POME grown algae can be used for biofuel production.

In spite of the fact that few researches were done on the use of microalgae for POME treatment, no research reported any species of microalgae that can treat POME efficiently. Hence, this research reported the potential of utilizing *C. sorokiniana* for the treating of POME. The mechanism employed for the pollutant removal from POME was studied using Michaelis-Menten kinetic equation. The application of the biomass obtained after treatment of POME was examined for its potential for biodiesel production.

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