ASSESSMENT OF MICROALGAE FOR SUSTAINABLE PROCESSING OF PALM OIL WASTE.



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ABSTRACT

Industrial production of palm oil adversely affects the environment since it contains substantial harmful quantities of solids as well as viscous and oily liquid. Due to the high capital expenditure and operationally intensive nature of current treatments, phycoremediation, which employs microalgae species, provides an alternative and sustainable method of biological wastewater treatment. Six microalgae species, *Stichoccocus bacillaris*, *Brachiomonas submarina pulsifera*, *Tetraselmis sp*., *Dunaliella salina*, *Euglena mutabilis*, and *Oscillatoria sp.* were initially investigated, due to the range of biochemical and physical characteristics, and to analyse the potential of each strain, given their less well-characterised information on the growth in industrial effluent and under different light regimes. Among the selected species, novel chlorophyta *S.bacillaris* and cyanophyta *Oscillatoria sp.* were found to grow successfully in palm oil mill effluent under 16:8 and 8:16 light regimes. In comparison to the growth observed in the control medium, chlorophyta *S.bacillaris* exhibited a highly significant increase in cell numbers when it was cultivated in palm oil mill effluent: 89.95% (p-value = 0.0004) and 87.72% (p-value = 0.0009) under the 16:8 light and 8:16 light regimes respectively. Chlorophyll and protein contents were also found to be significantly higher when cultivated in palm oil mill effluent than grown in the control medium. In parallel to the cells growth in palm oil mill effluent, the novel species was also able to remediate the industrial wastewater as there was a simultaneous decrease in palm oil loads after 10 days of cultivation and treatment. It was indicated by a significant decrease in Chemical Oxygen Demand (COD) by *S.bacillaris*; by 85.31% (p-value = 0.000027) and 80.88% (p-value = 0.000023) under 16:8 light and 8:16

light regimes respectively. Further assessment revealed the significant reduction of total phosphate by 93.07% (*p*-value = 0.00015) under 16:8 light and 88.25% (*p*-value = 0.000039) under 8:16 light, and nitrate by 78.14% (*p*-value = 0.00037) under 16:8 light and 72.9% (*p*-value = 0.00056) under 8:16 light. Although the cultivated cyanophyta *Oscillatoria sp.* in palm oil mill effluent showed an increase in cell counts, chlorophyll, and protein content under both light regimes, assessments of these factors showed them to be non-significant when compared to their growth in the control medium. Despite these results, pollution loads in palm oil mill effluent were significantly reduced by both species. COD was decreased by 78.22% (*p*-value = 0.00001), and 82.9% (*p*-value = 0.0000015) under the 16:8 and 8:16 light regimes respectively, whereas total phosphate and nitrate were both demonstrably reduced by between 78.69% and 85.85% under both light regimes. Turbidity level was also demonstrated to have reduced significantly in palm oil mill effluent after the cultivation of *S.bacillaris*, by 44.04 % (*p*-value = 0.013), and 19.08% (*p*-value = 0.0047) under 16:8 and 8:16 light regimes respectively, after 10 days of treatment. *Oscillatoria sp*. was consistently efficient at reducing turbidity, by 34.69% (*p*-value = 0.00435) under 16:8 light and 36.64% (*p*-value = 0.00006) under 8:16 light.

Besides the importance of applied photoperiods for the study of algal biomass growth, the findings established also provide an insight into phycoremediation, an environmentally desirable and sustainable solution for pollutant removal. The optimisation of this technology requires further biochemical assessments in order to determine possible technoeconomic advantages of phycoremediation and the key parameters dictating pollution load reduction.

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LIST OF ABBREVIATIONS

ANOVA	Analysis Of Variance
ASW	Artificial Seawater
BAT	Best Available Technology
BEP	Best Environmental Practice
BG11	Blue-Green medium
BOD	Biological Oxygen Demand
BSA	Bovine Serum Albumin
B. submarina puls.	*Branchiomonas submarina pulsifera*
Chl	Chlorophyll
CCAP	Culture Collection Algae & Protozoa Scotland
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic Acid
DO	Dissolve Oxygen
DOC	Dissolved Organic Carbon
D.salina	*Dunaliella salina*

EG	Euglena Gracilis medium
EMS	Environmental Management System
EPA	Environmental Protection Agency
E.mutabilis	*Euglena mutabilis*
f/2	Half strength f medium
GHG	Greenhouse Gases
GDP	Gross Domestic Product
HRT	Hydraulic retention time
LCA	Life Cycle Assessment
MBA	The Marine Biological Association UK
MBBM	Modified Bold Basal Medium
mRNA	Messenger Ribonucleic Acid
nm	nanometer
NTU	Nephelometric Turbidity Units
OGM	Optimal Growth Medium
Oscillatoria sp.	*Oscillatoria* species

PBR	Photobioreactor
POME	Palm Oil Mill Effluent
S.bacillaris	*Stichoccocus bacillaris*
Tetraselmis sp.	*Tetraselmis* species
TKN	Total Kjedahl Nitrogen
TN	Total Nitrogen
TSS	Total Suspended Solids
UV	Ultraviolet
VSS	Volatile Suspended Solids

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Palm oil is among the most rapidly expanding equatorial crops, but the generation of excessive palm oil waste has become a major problem in the world's producers and exporters of palm oil due to the substantial harmful quantities of solids as well as viscous and oily liquid it contains. Liquid palm oil waste, commonly known as Palm Oil Mill Effluent (POME) is a polluting organic residue. POME is generated from palm oil processing and contains a high nutrient load costing of mainly carbohydrate; crude protein and lipid (such as amino), fatty acids, moisture and carotene, and ash. When left untreated, POME affects aquatic life and ground water supplies by depleting dissolved oxygen (Khalid and Wan Mustafa, 1992). Current palm oil wastewater management using pond systems and physicochemical treatments such as membrane technology, flocculation, and coagulation provide possible treatments, but have several unavoidable flaws including emission of irrepressible methane and carbon dioxide, requirement of a long hydraulic retention time (HRT), entailment of a vast amount of land mass and high capital expenditure, not to mention intensive operational demands (Rupani *et al.,* 2010; Putri *et al.,* 2011).

A holistic view of community based water supply, wastewater and resources recovery requires a paradigmatic shift in the water industry to meet the challenges of providing a more sustainable and cost effective operational wastewater treatment. Sustainable bioremediation using microalgae species, or phycoremediation, provides the basis for

designing and operating new advanced biological wastewater treatment systems that may remove pollutants and hazardous or toxic constituents and additionally recover important and increasingly valuable resources. Biotic and abiotic factors which include cell size/age, light and diurnal cycle, pH, salinity, and dissolved organic carbon (DOC) exercise huge influences over the growth of microalgae species and their tolerance of organic pollutants (Levy *et al*., 2007). Hence, the ability of microalgae species to remove and recover pollutants provides a strong potentially robust system of cost-effective and environmentally friendly treatment for commercial wastewater treatment.

Palmer (1969) and Pittman (2010) reported that several microalgae species are tolerant to highly organically-enriched wastewater. Removal of dissolved organic and inorganic compounds using *Chlorella* and *Scenedesmus* genus (>80%) from municipal sewage wastewater also showed high growth rates of the microalgae species over the batch growth within light-emitted photobioreactors (Lau *et al*., 1995, Martinez *et al*., 2000, Shi *et al*., 2007, Zhang *et al*., 2008, Bhatnagar *et al*., 2010, Wang *et al*., 2010). Semi-continuous culture within photobioreactors was also found for initial growth of *S. obliquus* (Ruiz-Marin *et al*., 2010). Further studies by Wilkie and Mulbry (2002), An *et al*. (2003), and Mulbury *et al*. (2008) using agricultural wastewater demonstrated efficient growth of microalgae species and high nutrient uptake rates among benthic freshwater microalgae species such as *Microspora wileana*, *Ulothrix sp*., and *Rhizoclonium hierglyphicum*. There has been significant interest in phycoremediation using industrially-derived wastewater, primarily to remove heavy metal pollutants such as cadmium, chromium, copper, and zinc, and organic chemicals such as hydrocarbons was also investigated. Despite the lower concentrations of Nitrogen (N) and Phosphorus (P), industrial wastewater was able to

support the growth of *B. braunii* and *Chlorella saccharophila* in carpet mill effluent (Chinnasamy *et al*., 2010). Therefore, Palm Oil Mill Effluent (POME), which is generated from both agricultural and industrial sites, could provide a suitable substrate for microalgae biomass growth that could potentially prove to be a good food source for fish and thus a protein source which is in increasing demand globally.

The increasing freshwater scarcity and the critical stress of water supply (UN-Water, 2007) has had a major impact on the current bioenergy industry. Phycoremediation - exploits microalgae species not only to reclaim water, but also to biotransform pollutants, and generate biomass for sustainable bioenergy production. The studies of Clarens *et al.* (2010) confirmed that phycoremediation would deliver an eco-friendly performance as they can be cultivated in surroundings that require renewable light sources, minimal total land usage, and less fresh water supply, and can reduce the tendency towards eutrophication. Further research and development in phycoremediation may enable the maximization of economy, health and social benefits, and help the sustainability of global ecosystems.

1.2 LITERATURE REVIEW

1.2.1 PALM OIL

The palm oil industry has grown rapidly over the years. For example, Malaysia produces 18.79 million tonnes of crude palm oil on roughly 5,000,000 hectares or 19,000 sq mi of land (USA Foreign Agricultural Service, 2012). The expansion of the palm oil industry contributes significantly to gross domestic product (GDP) and provides employment. Although it significantly contributes towards economic growth, the palm oil industry adversely effects the environment due to the generation of polluted wastewater. In 2009, 43.8 million m^3 of POME was generated from Malaysia's palm oil mill industry (Malaysian Palm Oil Board, 2010) and this amount is predicted to increase in future years due to continuous demand.

The process water generated by the palm oil industry is mainly produced from wash water during production or at the end of a cycle when cleaning equipment. Furthermore, leaks and spills might also contribute to the toxic waste generated, and thereby maximise the negative impact on the environment. The typical characteristics of industrial wastewater are tested for their Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (COD), Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), Total Kjeldahl Nitrogen (TKN), Ammonia (NH_3-N), Nitrites and Nitrates (NO_2-N and NO_3-N), Phosphorus (P), pH, temperature, alkalinity, conductivity, as well as the possibly for several heavy metals such as Iron (Fe), Aluminium (Al), Magnesium (Mg), Lead (Pb), inc (Zn), and Copper (Cu), and also possible inorganic content like silica, bicarbonate, and organic compounds. Turbidity,

colour, taste and odour tests provide the physical aggregate characteristics of water quality (Tchobanoglous and Schroeder, 1985).

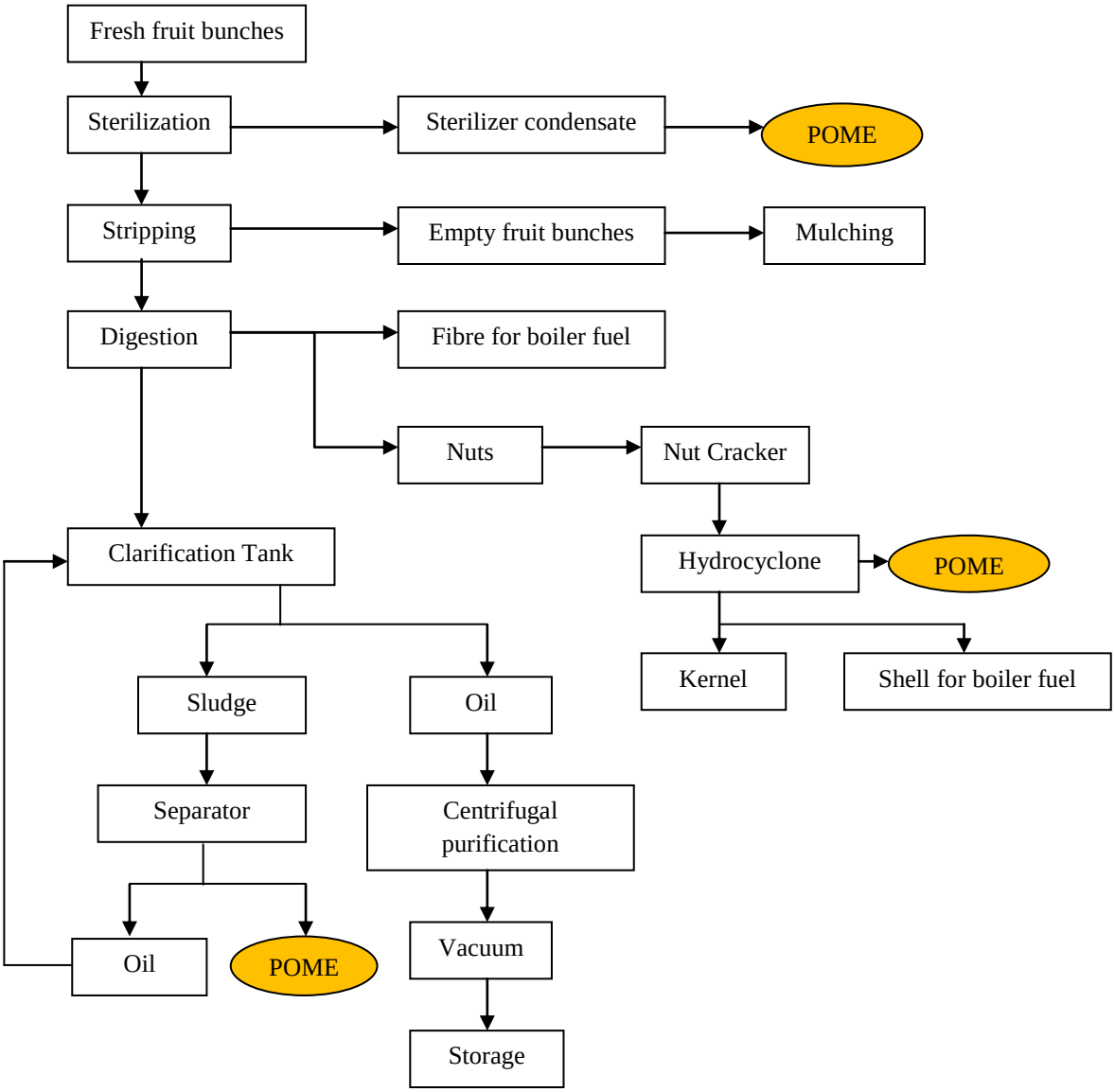


Figure 1.1 Flow diagram of a palm oil mill and the production of Palm Oil Mill Effluent (Lam and Lee, 2011).

1.2.1.1 CHARACTERISITCS OF PALM OIL MILL EFFLUENT

Palm Oil Mill Effluent (POME) contains substantial quantities of solids and high values of COD, BOD, oil and grease, total solids and suspended solids, which are harmful to aquatic organisms (Ma, 1995). Raw POME is a brown coloured suspension, which is slightly acidic and consists mainly of water (94 – 96%), 0.6 – 0.7% oil, and 4 – 5 % colloidal suspension (Wu *et al*., 2009).

Table 1.1

Characteristics of POME. All parameters are in mg/l except pH (Lam and Lee, 2011)

Parameters	Range	Mean
pH	3.4-5.2	4.2
Oil and Grease	130 – 18,000	6000
COD	15,000 – 100,000	51,000
BOD3	10,250 – 43,750	25,000
Total solid	11,500 – 79,000	40,000
Suspended solid	5000 – 54,000	18,000
Total volatile solid	9000 – 72,000	34,000

POME is the most polluted organic residue generated from palm oil processing and is composed of high nutrient content: carbohydrate (29.55%); free extract nitrogen (26.39%); ash (14.88%), crude protein and lipid (27.63%) such as amino (glutamic acid, aspartic acid, glycine, alanine, methionine, serine etc.) and fatty acids (e.g. palmitic acid, oleic acid, myristic acid, stearic acid, linoleic acid and several others), moisture and carotene (Habib *et al.*, 1997).

Table 1.2

The composition of mineral in POME (Adapted from Habib *et al*., 1997)

Minerals	Composition (µg/g dry weight)
P	14377.38
K	8951.55
Ca	1650.09
Mg	911.95
Na	94.57
Mn	38.81
Zn	17.58
Al	16.60
S	13.32
Fe	11.08
Cu	10.76
Si	10.50
As	9.09
B	7.60
Pb	5.15
Cr	4.02
Co	2.40
Sn	2.30
Ni	1.31
Cd	0.44

1.2.2 PHYCOREMEDIATION

The wastewater technology sector offers opportunities to rectify several unanswered issues relating to energy efficiency and pollution prevention. Best Environmental Practices (BEPs) such as Environmental Management Systems (EMSs), Environmental Audits and Best Available Technology (BAT) offer management systems to identify subsequent commitment in the wastewater technology sector.

1.2.2.1 ANAEROBIC TREATMENT

For palm oil waste, the most conventional wastewater treatment is the anaerobic ponding system, in which the high values COD and BOD, as well as TSS are reduced by the degradation of complex organic matter. This occurs in the absence of oxygen to produce methane, carbon dioxide, and water via hydrolysis, acidogenesis (including acetogenesis) and methanogenesis. However, ponding systems have several unavoidable flaws, such as the irrepressible emission of methane and carbon dioxide from the treatment process, which might contribute to the effect of global warming (Putri *et al*., 2011), that it also requires long hydraulic retention time (HRT) and that it entails a vast amount of land mass (Rupani *et al*., 2010). While the method is widely accepted as an effective one, anaerobic treatment alone has difficulty meeting discharge limits due to process inhibition by the high organic strength of POME.

1.2.2.2 PHYSICOCHEMICAL TREATMENT

Physicochemical treatments using membrane technology, flocculation, or coagulation provide possible alternative treatments. For example, chitosan, a natural organic polyelectrolyte derived from chitin is used as a coagulant or flocculant and shows

tremendous reduction in turbidity, TSS, and COD levels by 99.9%, 99.15%, and 60.73% respectively when compared to Alum, a typical flocculant, which shows a reduction of 99.45% in turbidity, 98.60% in TSS, and 4.24% in COD level (A. Hassan *et al.*, 2009). The capital expenditure as well as the operative expenditure of such treatments is high. Wastewater management based on phycoremediation hence offers a sustainable energy process with far less operating area and equipment.

1.2.2.3 PREVIOUS WORK

During the 1940s, the Stanford Research Institute and Arthur D. Little at the Carnegie Institute translated phycology studies into engineering practice. Researchers at both institutes worked specifically with a eukaryotic microalga, *Chlorella pyrenoidosa* and from this they designed a large scale pilot plant for mass cultivation of the microalgae. This breakthrough became a major stepping-stone for later researchers working on microalgae.

Phycoremediation exploits microalgae species not only to biotransform pollutants, but also to generate biomass for sustainable bioenergy production. Palmer (1969) and Pittman (2010) reported that several microalgae species are tolerant to wastewater with a high organic content. For example, *C. vulgaris* was demonstrated to remove more than 90% of nitrogen and 80% of phosphorus content (Lau *et al*., 1995). This suggests the effectiveness suitability for wastewater in microalgae growth and nutrient/metal ion removal.

Further it was reported that *C. minutissima* grew in high concentrations of raw sewage and dominated inside the oxidation pond system. This species was found to grow by utilising a variety of organic carbon substrates over a wide pH range and in the presence of high salt content. Agricultural wastewater from animal husbandries has demonstrated growth of

microalgae species and efficiency in removing nutrients and metal ions (Gonzalez *et al*., 1997; Wilkie and Mulbury, 2002; An *et al*., 2003).

Due to lower levels of nitrogen and phosphorus in many industrial wastewaters, there is less potential for utilising it for a large-scale generation of microalgae biomass. However, a study suggested that certain noteworthy species of microalgae were viable for growth in industrial wastewater, if provided with sufficient nutrient resources (Chinnasamy *et al*., 2010).

Artificial wastewater could also be utilised for microalgae growth. Due to the lack of solid organic material and other potential toxins, exploitation of artificial wastewater provides benefits in laboratory-based experiments, as it allows for a basic analysis of the major biological and chemical mechanisms and removes unidentified variables.

Table 1.3

Comparison of biomass and lipid of microalgae growth in varied wastewater media (Pittman *et al*., 2010)

Wastewater Type	Microalgae species	Biomass DW Productivity [mg/L/d]	Lipid Content [% DW]	Lipid Productivity [mg/L/d]	References
Municipal	*C. reinhardtii*	2000	25.25	505	Kong *et al.* (2010)
Municipal	*B. braunii*	345.6 (i)	17.85	62	Orpez *et al.* (2009)
Municipal	Mix of *Chlorella, Micractinium, Actinastrum*	270.7	9	24.4	Woertz *et al.* (2009)
Agricultural (piggery manure) (a)	*B. braunii*	700 (ii)	nd	69	An et.al (2003)
Agricultural (dairy manure)	*Chlorella*	$2.6\frac{g}{m^2}/d$	9 (iii)	$230\,\frac{mg}{m^2}/d$	Johnson and Wen (2010)
Agricultural (swine effluent) (b)	*R. hieroglyphicum*	10.7	0.7 (iii)	$72\,\frac{mg}{m^2}/d$	Mulbery et.al (2008)
Agricultural (dairy effluent) (c)	*R. hieroglyphicum*	17.9	1.2 (iii)	$210\,\frac{mg}{m^2}/d$	Mulbery et.al (2008)
Industrial (carpet mill)	*B. braunii*	34	132.20	4.5	Chinnasamy *et al*. (2010)
Artificial	*Scenedesmus*	126.54	12.8	16.2	Voltolina *et al.* (1999)

nd – not determine; DW –dry weight

(i) Estimated from biomass value of 14.4mgLh
(ii) Estimated from biomass value of 812 mgLh after 3 days
(iii) Fatty acid content and productivity determined rather than total lipid
(a) with high NO_3-N
(b) maximum manure loading rate
(c) +CO_2, maximum manure loading rate

1.2.2.4 TECHNOLOGICAL DEVELOPMENT

The requirements for phycoremediation include a light source, which initiates a biochemical reaction of photosynthesis, and a container of simple mechanical design for optimal mixing (Cooper and Herr, 1987). Sunlight is the most abundant source of energy for microalgae growth, and has the full spectrum of light energy. The light spectrum between 400 and 700 nm can be used by microalgae and a sufficient amount of light energy can be utilised for biomass production. Approximately 12-14% of the light source is utilised by microalgae via the photosynthesis process. For indoor purposes, homogenous illumination is preferable for the growth of microalgae species. The typical temperature required for the majority of microalgae species to grow successfully is between 16°C and 35°C. Besides the lighting system, Drapcho *et al.* (2000) mentioned that an incremental change in the mixing rate in microalgae cultivation also increases biomass production. Therefore, the hydrodynamic mixing and mass transfer must also be carefully taken in account as it might result in shear-sensitive cells and disrupt the growth rate of the microalgae (Wu and Merchuk, 2004). A pump is necessary to allow a mixing mechanism during microalgae cultivation.

Traditional open pond systems are believed to be a cheaper method for large-scale phycoremediation as they require lower energy input, but they also have drawbacks due to fluctuations in local environmental conditions. The diurnal variation of light intensity from sunlight leads to uneven light distribution, and can cause a negative impact on productivity. Furthermore, the monocultures are susceptible to contamination from other microalgae species or bacteria and open systems cannot be kept axenic and require intensive labour to control the conditions such as temperature, pH, salinity and other environmental factors,

including the light regime. Photobioreactors (PBR) are therefore designed to overcome the problem by eliminating factors that could prevent or reduce the growth of microalgae.

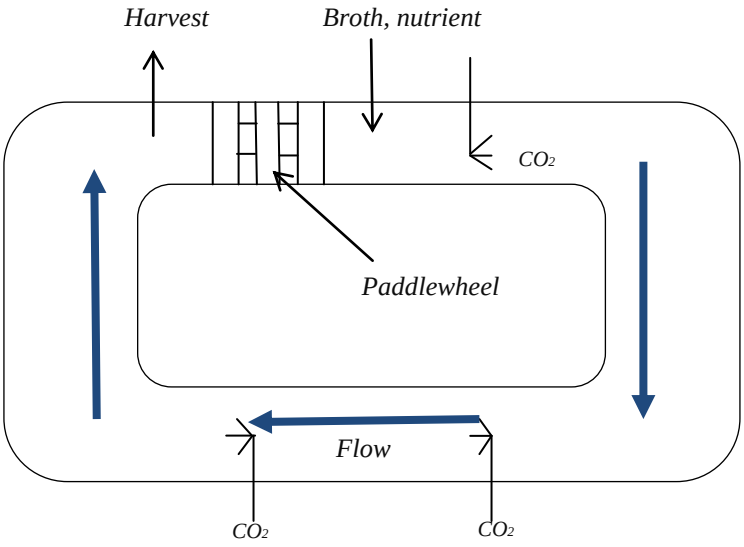


Figure 1.2 Plan view of a raceway pond (Adapted from Christi, 2007).

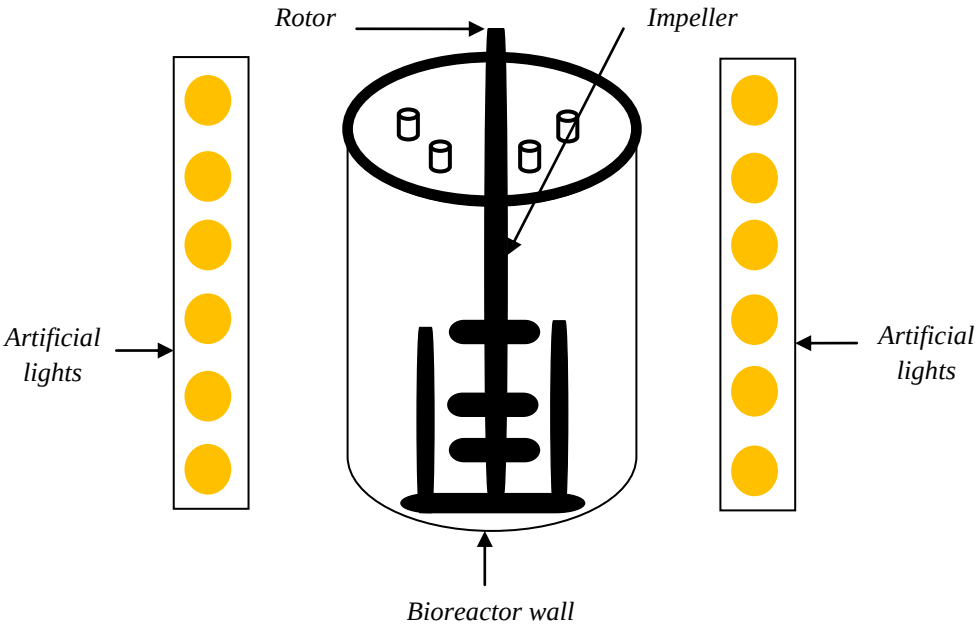


Figure 1.3 Example of a vertical-column photobioreactor. The diagram shows a standalone wall connected to a rotor, as well as an attached artificial light source (Adapted from Ugwu *et al*., 2002).

1.2.2.5 ARTIFICIAL LIGHT

The installation of an artificial light plays a key role in photobioreactor (PBR) design as a source that improves the light supply by means of the diurnal cycles and also helps to overcome an uneven light distribution. PBRs are designed with different illumination strategies in order to enhance the production rate of biomass and oil/lipid content (Ma and Hanna, 1999). The oil yield which could be reached by using artificial light sources on a laboratory scale of microalgae cultivation is up to 170m^3/ha in comparison to 100-130 m^3/ha using sunlight, due to the stable and continuous light energy which enhances the growth and oil accumulation. Nevertheless, the operation of the installed artificial light sources also provides an operational drawback due mainly to the power consumption of a high duration of light intensity for the large-scale cultivation process. An overdose of light energy could damage the photosynthetic metabolism in a process called photoinhibition (Janssen *et al*., 2000). Such photodamage provides strong evidence that a controlled light/dark light cycle can be very important with respect to the photosynthetic efficiency of microalgae growth in photobioreactors. Therefore, the efficiency of photoperiod, with the aim of increasing the biomass yield, is associated with basic parameters which include two main characteristics; the light fraction i.e. the ratio between the light period and the cycle time; and the length of the light/dark cycle. An increase in light fraction leads to an increase in the biomass yield (Janssen *et al*., 2000).

In spite of this, PBRs have high production costs compared to the open pond systems. Such constraints on efficient large-scale cultivation demand the development of innovative engineering solutions that need to be worked out in order to develop a cost-effective cultivation system in photobioreactors. Studies have shown that in the laboratory,

microalgae grow better under mixed light cycles (Garcia *et al*., 2010). Due to what is termed photo zone (arbitrarily defined as the depth at which 90% of the incoming photon flux is absorbed) – species that are stratified outside the zone receive no light. Part of the light energy absorbed in the photo zone also dissipates as heat due to the limitation of microalgae species to fix the light energy. Therefore, mixing mechanism provides an important role for cell exposure to the light. If light energy could be optimised in both photic and non-photic zones, a higher biomass yield would be predicted.

Table 1.4

Comparison of open pond and vertical-column photobioreactor (Christi, 2007; Ugwu *et al*., 2008)

	Open pond		Vertical-column PBR	
Advantages	(i)	Economical for mass cultivation	(i)	High-mass transfer
			(ii)	Mixing with low shear stress
			(iii)	Low energy and power consumption
			(iv)	Potential for scalability
			(v)	Easy for process to sterilise
			(vi)	Efficient in immobilization of microalgae species
			(vii)	Lower photoinhibition and photooxidation
			(viii)	Compact and low cost
Disadvantages	(i)	Low control of culture conditions and low mass transfer rate	(i)	Small illumination surface area
			(ii)	Cell sedimentation (if airlift or mixing mechanism are not used)
	(ii)	Ineffective for low period of cultivation		
	(iii)	Poor light utilization		
	(iv)	Probability of contamination		
	(v)	Not suitable for all microalgae species		

1.3 AIM AND OBJECTIVES

Phycoremediation provides the basis for designing and operating new advanced biological wastewater treatment systems and conveys potential new avenues in removing pollutants and hazardous or toxic constituents, and recovering important and increasingly valuable resources.

Effective growth of microalgae through the utilization of abundant organic and inorganic chemical constituents in wastewater simultaneously permits the removal of nitrogen and phosphorus which helps to reduce the risks of eutrophication; allowing the absorption or bioaccumulation of heavy metal pollutions (Zhou *et al*., 2012). Phycoremediation also generates far less sludge than chemical treatment, and offers an opportunity for efficient generation of useful by-products, for example low-cost fertilisers or animal feed (Munoz and Guieysee, 2006; Wilike and Mulbry, 2002). Pittman *et al*. (2010) has calculated that if 50% or 405 billion m^3 of wastewater is made available for microalgae cultivation, it could generate about 247 million tons of microalgae biomass (or 37 million tons of oil).

The aims of this study are to investigate the growth of microalgae species in industrial palm oil mill effluent under different light/dark cycle conditions and also to evaluate the species which have the potential to phycoremediate industrial effluent and recover potentially exploitable chemical constituents from wastewater.

To this end, the research objectives were as follows:

i. To compare the growth of selected microalgae species within industrial palm oil mill effluent under different light/dark cycle regimes. This includes optical density for cell number, chlorophyll extraction analysis and protein determination using the Bradford Assay.

ii. To verify the reduction of the pollution load, specifically Chemical Oxygen Demand (COD) of Palm Oil Mill Effluent (POME) in correlation to the biomass growth.

iii. To investigate the turbidity changes of industrial palm oil mill effluent after the cultivation of the dominant microalgae species; this is to test whether phycoremediation is able to 'bleach' the dirty industrial effluent for aesthetic reasons.

iv. To gain insight into phycoremediation as an alternative and economical biological treatment for palm oil mill effluent.

CHAPTER 2

MATERIALS AND METHODS

Phycoremediation provides the basis for an alternative and advanced biological wastewater treatment system; a new way to remove pollutants and hazardous or toxic constituents, and to recover important and increasingly valuable resources. At the core of this whole system is the utilization of microalgae cultures. Experiments were designed to measure the growth of microalgae both in a control medium and in palm oil waste under different light regimes, and simultaneously to show a reduction in pollution load. This was also to demonstrate whether microalgae species can remediate palm oil waste.

2.1 SELECTION OF MICROALGAE SPECIES

Microalgae are microscopic organisms, which are typically found in freshwater and marine systems. They exist either individually, or in chains or groups of unicellular species. Depending on the species, their size can range from a few micrometers to a few hundred micrometers (μm) across. The biodiversity of microalgae is enormous; it has been estimated that about 200,000-800,000 species exist, of which about 35,000 species have been described in scientific literature (John *et al*., 2002). For my research, 6 species have been chosen from a range of biochemical and physical varieties in order to obtain information and to analyse the potential of each strain to grow under selected conditions, given that the data on the growth in industrial effluent and under different light regimes has not been previously documented. Table 2.1 summarises the strains chosen for this research.

Stichococcus bacillaris

These freshwater green microalgae readily fragment into solitary cells as they reproduce by fragmentation of filaments and multiplication of cells through vegetative cell division. Their typical width is between 2 – 3.8 µm, and the length is normally 2 to 6 times greater than their width. Their cylindrical cell walls are thin and grow best in an acidic medium (John *et al.*, 2002; Lee, 2008).

Brachiomonas submarina pulsifera

Brachiomonas submarina pulsifera falls within the order of Volvocales. It is actively motile with 2 or more contractile vacuoles that define the thin walls which are ridged (pear /wedge-shaped), has 2 or 4 anterior flagellas, and is colourless (John *et al.*, 2002). This marine chlorophyceae microalgae species contain chloroplasts that are usually single cell and have 'visible' eyespots (Lee, 2008); it also osmoregulates over a wide range of external salinities and exhibits maximum water content in 100% artificial seawater (ASW). Accumulation of glycerol, amino acids and other inorganic ions (e.g. sodium, potassium and chloride) are found to be responsible for the osmolarity characteristic (Ahmad, Hellebust, 1985).

Tetraselmis sp.

Tetraselmis sp. is green, motile, and usually grows to be 10 µm long x 14 µm wide. The species contains very high lipid concentration which stimulates feeding in marine organisms by means of natural amino acids and has 4 flagellas emerging from an anterior pit. The anterior pit is a cell-heart shape or somewhat cherry-shaped, ellipsoidal or almost spherical (John *et al.*, 2002; Lee, 2008).

Dunaliella salina

This phototrophic green microalga is a halophile species with the richest natural source of the carotenoid β-carotene in concentrations that are responsible for its orange-red pigmentations which protect against the intense light, and high concentrations of glycerol which provide protection against osmotic pressure. Their optimal temperature is between 20 - 40°C and their known ability to consume energy mixotrophically makes *D. salina* easy to handle. This species is motile, unicellular, and rod to ovoid shaped (9 - 11 µm). *Dunaliella sp.* also has a very broad pH tolerance ranging from pH 1 to pH 11 (John *et al.*, 2002; Lee, 2008). In fact, *D. salina* is one of the most environmentally tolerant eukaryotic organisms known to man and can also cope with a broad salinity range.

Euglena mutabilis

Euglena mutabilis falls within the order of Euglenales of Euglenophyta. They are normally within 60 to 95 µm in length and 4 – 9 µm in width, long and narrowly cylindrical in shape, and contain yellowish chloroplasts. They are reported to be denser in water in the early morning or early evening as their brilliant eyespots help aid cellular movement according to the light intensity (John *et al.*, 2002; Lee, 2008). Their swimming rate depends directly upon the temperature and vitamin B12 starvation is reported to increase cell volume and generate more than usual chloroplasts in the cell (Carell, 1969; Bertaux and Valencia, 1971). Euglenoids are also the genus most documented to have a high-tolerance resistance, thriving where there is an abundance of rich organic waste and able to grow in an acidic medium (John et al., 2002; Lee, 2008). The uptake of the energy is a phototrophic process and they are also readily heterotrophically viable.

Oscillatoria sp.

This filamentous phototrophic cyanophyta is a filament containing a chain of cells, trichome, and enveloping sheath (John *et al.*, 2002; Lee, 2008). They have sheaths which vary in thickness and consistency as the environmental conditions change (Baker and Bold, 1970) – these are responsible for protecting the cells from drying out (Nobles *et al*., 2001). Typical blue green algae contain chlorophyll *a* that are able to free oxygen (they have the same characteristics as bacteria though bacteria do not release oxygen) but are also able to form the blue pigment (phycocyanin) if light intensity decreases. Under microscopic investigation the cells visually appear to be blue-green with slight purplish shades or sometimes brownish and orange due to the presence of scytonemin pigments which protect against UV-lights, or even yellowish due to the an unhealthy sheath resulting from overly high light intensity. The size of cyanophyta ranges from 0.6 µm to 45 µm but some can extend to 100 µm in length. Blue green algae are prokaryotic, meaning that they lack membrane-bounded nuclei. The foundation of the blue-green algae cell wall is a complex multilayered structure. It lies between the plasma membrane and the sheath (slime layer). The presence of gas vacuoles provides the buoyancy characteristic of the cell; therefore allowing them to exist in a free-floating state. They are ubiquitous in water of a great range of salinity and temperature, but more abundant in neutral or slightly alkaline habitats.

Table 2.1

Summary of the selected microalgae species

Microalgae species	Division	Environment	Motility	pH tolerance	Temp. tolerance	Salinity tolerance	Growth medium
Stichococcus bacillaris	Chlorophyta	Brackish but acid-tolerant	Motile at pH 7.0 (1)	Acidic	16-27°C	Up to 30 g/L	MBBM (2)
Brachiomonas submarina puls.	Chlorophyta	Marine	Motile	Both acidic and alkaline	16-27°C	Up to 50 g/L	f/2 (3)
Tetraselmis sp.	Chlorophyta	Marine	Motile	Alkaline	16-27°C	Up to 50 g/L	f/2 (3)
Dunaliella salina	Chlorophyta	Hyper-saline	Motile	Alkaline	Up to 40°C	Up to 100g/L	f/2 +NaCl (1:1) (4)
Euglena mutabilis	Euglenophyta	Freshwater	Motile	Neutral, but survives in a slightly acidic environment	Up to 40°C	Up to 0.5 g/L	EG (5)
Oscillatoria sp.	Cyanophyta	Brackish	Motile (glides)	Alkaline	16-27°C	Up to 30 g/L	BG11 (6)

(1) adapted from Olivieri *et al*., 2012
(2) modified Bold Basal medium with 3-fold Nitrogen and vitamins
(3) half-strength f medium
(4) half-strength f medium with additional of 35 g/L sodium chloride solution to produce double-strength saline medium
(5) Euglena Gracilis medium
(6) Blue-green medium

Six microalgae species were selected and purchased from The Plymouth Algal Culture Collection of The Marine Biological Association (MBA), U.K., and Culture Collection Algae and Protozoa (CCAP), Scotland. Stock cultures remained in non-aerated isolation in lit conditions and under low temperature before being inoculated into the specified growth medium as a starter culture. Besides the optimal growth medium for each species, three main physical parameters were taken into consideration for inoculation: light source, temperature, and aeration (air/CO_2). The cultures were maintained for between 1 and 2 months, in order to ensure longevity. Subculturing was carried out to make sure that the

cells were continuously receiving fresh and sufficient nutrients, so that the cultures could be maintained for a longer time period.

The optimally tolerable temperature for microalgae cultures is generally between 16 and 27°C, although this may vary with the composition of the culture medium and the species. In this research, the surrounding temperature for cell growth was maintained by means of a lightproof and waterproof catchment tent called BudBox™ Grow Tent (Growell Hydroponics & Plant Lighting). In order to prevent sedimentation of the cells, an aeration technique was applied using Air Infusion Pumps Blagdon Koi Air 25. This allowed the cells to be exposed to light and improved gas exchange between the cultured cell and the growth medium. The pump used 230V/18W and ran at a frequency of 50 Hz, with a maximum air flow of 25 litre/min and a maximum pressure of 0.028 mpa (Blagdon the Pond Master). Two 125-Watt Compact Eco-Fluorescent lamps which emit both a blue and a red light spectrum were used to provide the most active light range for plant growth. A reflector was attached to each lamp at a precise angle for an optimum light distribution, and was hung up to 60 cm above the cultures.

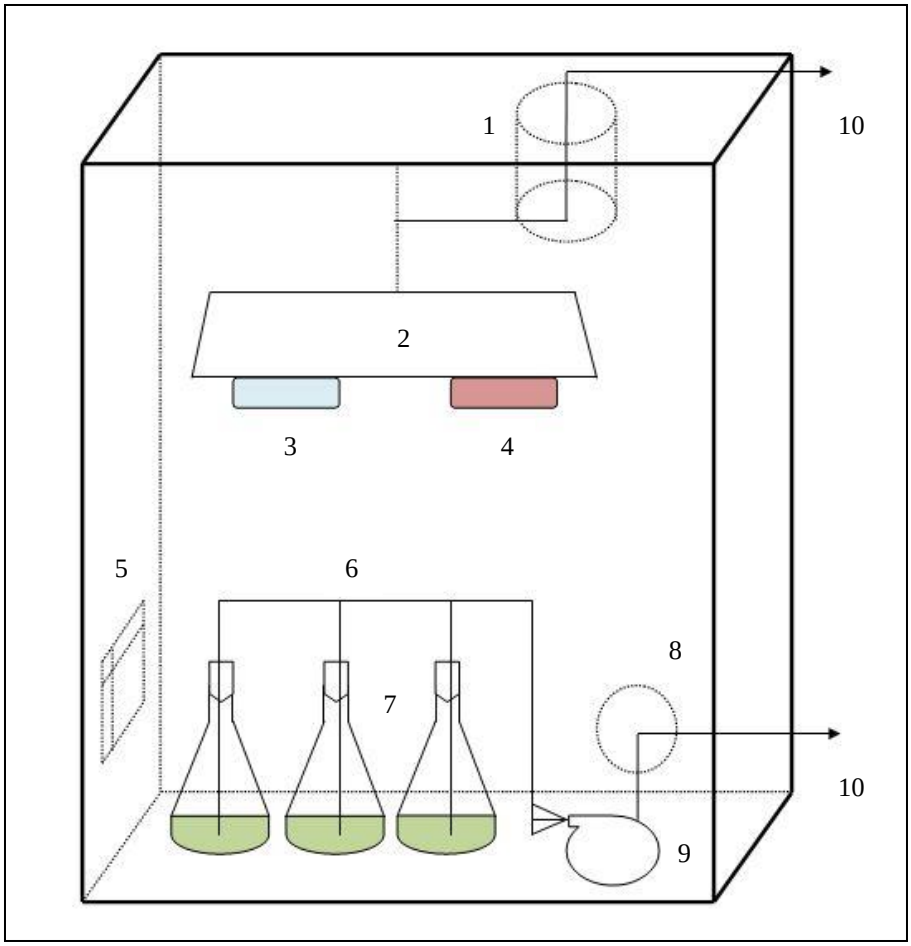


Figure 2.1 Sketch of BudBox™ Grow Tent and experimental installation

1. Vents for installing lamps, or extractor fan if required.
2. Fluorescent grow lamp reflector.
3. 125 Watt-Compact Eco Fluorescent growth lamp (blue light).
4. 125 Watt-Compact Eco fluorescent growth lamp (red light).
5. Lightproof air intake vents.
6. Tubes connecting rlenmeyer flasks and Air Infusion pump for aeration purposes.
7. Series of Erlenmeyer flasks containing microalgae samples.
8. Cabling access port.
9. Air Infusion Pump.
10. Cables connected to power supply

2.2 OPTIMAL GROWTH MEDIUM AND SALINITY TEST

Cultures are maintained in order to preserve healthy populations, which are physiologically, morphologically, and genetically representative (Harrison *et al.*, 2010). The growth medium provides a nutrient-rich environment for the microalgae. Nutrient enrichment is vital to ensure the species has enough food supply to grow. Macronutrients (e.g. nitrogen, phosphate, and silicon) and trace amounts of microelements (e.g. iron, manganese, and selenium) are essential for cell growth. Three vitamins are generally used: B_1, B_{12} and H (Watanabe, 2005). Antibiotics and other antimicrobial agents prevent the growth of contaminants without harming the microalgae species. Seawater can also be used as the nutrient provider for microalgae, but for the cultivation of microalgae in the laboratory, Artificial Sea Water (ASW) can be utilised to simulate seawater. This recipe contains trace solutions of vitamins or organic compounds which enhance its suitability for microalgae growth (Goldman & McCarthy, 1978). Furthermore, the salinity of the prepared medium can be measured in the laboratory for control purposes using several techniques. These methods include evaporation tests, electrical conductivity, using refraction of light through the medium, or using titration of the chloride ion against Silver Nitrate ($AgNO_3$).

Four standardised recipes for the optimal growth medium were as followed: f/2 medium for marine microalgae species, with an additional 35 g/L NaCl solution for the species that tolerate high salinity; Modified Bold Basal medium (MBBM) for acidic freshwater species; Blue-Green medium (BG-11) for freshwater Cyanobacteria; Euglena Gracillis medium (EG) for euglenophyta species. The media were prepared under carefully controlled

conditions to minimise risk of contamination and an autoclave was used to sterilise both the glassware and the media.

Modified Bold Basal medium

MBBM stock solution was prepared by first weighing 25.0 g of $NaNO_3$, 2.5 g of $CaCl_2.2H_2O$, 7.5 g of $MgSO_4.7H_2O$, 7.5 g of $K_2HPO_4.3H_2O$, 17.5 g of KH_2PO_4, and 2.5 g of NaCl, then each of these salts were mixed in 1000 ml of distilled water. Trace element solution was prepared by weighing 0.75 g Na_2EDTA which was added to a 100 ml fresh distilled water before the following minerals were added in this exact sequence: 97 mg of $FeCl_3.6H_2O$, 41 mg of $MnCl_2.4H_2O$, 2 mg of $ZnCl_2$, 2 mg of $CoCl_2.6H_2O$, and 4 mg of $Na_2MoO_4.2H_2O$. 0.12 g of Thiaminhydrochloride was added to 100 ml distilled water. 0.1 g of Cyanocobalamin was also added to 100 ml distilled water, before 1 ml of this solution was pipetted and 99 ml of distilled water was added to it to make up a 100 ml solution. 30 ml of $NaNO_3$ stock solution and 10 ml of the rest of the stock solution were measured and mixed in a flask with 6 ml of trace element solution, 1 ml of filter-sterile Thiaminhydrochloride, and 1 ml of filter-sterile Cyanocobalamin. The mixture was made up to 1 l in volume with distilled water. 1 g of Bactopeptone and 5 g of sucrose were finally added to this mixture.

f/2 medium

For the marine microalgae, f/2 medium was used. Trace metal solution was prepared by weighing 4.16 g of Na_2 EDTA, 3.15 g of $FeCl_3.6H_2O$, 0.01 g of $CuSO_4.5H_2O$, 0.022 g of $ZnSO_4.7H_2O$, 0.01 g of $CoCl_2.6H_2O$, 0.18 g of $MnCl_2.4H_2O$, and 0.006 g of $Na_2MoO_4.2H_2O$, which were added to 1000 ml of distilled water and mixed. Vitamin mix

was prepared by weighing 0.0005 g of Cyanocobalamin (vitamin B12), 0.1 g of Thiamine HCl (vitamin B1), and 0.005 g of Biotin, then added to 1000 ml of distilled water. 0.075 g of $NaNO_3$ 0 and 0.00565 g of $NaH_2PO_4.2H_2O$ were weighed, and 1 ml of the trace elements stock solution and 1 ml of vitamin mix stock solution were added, before the solution was made up with 1 liter of filtered seawater. For microalgae species *D.salina*, an additional solution was prepared by adding 1000 ml of 35 g/l NaCl solution in 1000 ml of f/2 in order to provide a high saline solution or double strength artificial seawater.

Euglena Gracilis medium

EG Medium, the specified optimal growth medium for *E. mutabilis* was prepared by weighing 1 g of $CaCl_2$ salt and dissolving it in 1000 ml of distilled water. 10 ml of $CaCl_2$ stock solution was then added with 1 g Sodium acetate trihydrate, 1 g "LabLemco" Powder (Oxoid L29), 2.0 g Tryptone (Oxoid L42), and 2.0 g yeast extract (Oxoid L21), and topped up with 1000 ml distilled water.

Blue-green medium

Oscillatoria sp. grows best in BG11 medium. A solution of 15 g of $NaNO_3$ was mixed with 1000 ml of distilled water. 2.86 g of H_3BO_3, 1.81 g of $MnCl_2.4H_2O$, 0.22 g of $ZnSO_4.7H_2O$, 0.39 g of $Na2MoO_4.2H_2O$, 0.08 g of $CuSO_4.5H_2O$, and 0.05 g of $Co(NO_3)_2.6H_2O$ were weighed and each of these trace metals was mixed in separate flasks of 500 ml of distilled water. Stock solution was then prepared by weighing 2.00 g of K_2HPO_4, 3.75 g of $MgSO_4.7H_2O$, 1.8 g of $CaCl_2.2H_2O$, 0.6 g of citric acid, 0.15 g of Ammonium Iron Sulphate, 0.05 g of $EDTANa_2$, and 1 g of Na_2CO_3, which were diluted in 1000 ml of

distilled water. 100 ml of $NaNO_3$ Solution, 10 ml of each trace metal solution, and 1 ml of each stock solution were pipetted into a flask and topped up with 1000 ml of distilled water.

Salinity Test

A simple analytical method of mass evaporation was used to evaluate the salinity of each growth medium. Firstly, the pH value of each solution was recorded using a Jenway 3345 Ion Meter, then 10 ml of each medium solution was transferred into a fresh beaker and weighed to determine the difference in mass. The beakers were left on a Stuart CB-162 Heat-Stirrer at a temperature of 300°C for 20 - 30 minutes, or until they were completely dried up. The samples were allowed to cool, and were once again weighed to determine the amount of salt. The procedure was repeated 3 times to get an average salinity value. The salinity of each sample was determined using the following equation:

$$Salinity = \frac{weight\ of\ salt}{weight\ of\ solution}\ x\ 1000\ ‰$$

2.3 PALM OIL MILL EFFLUENT

Raw palm oil mill effluent is a brown coloured suspension which is slightly acidic and made up of 94–96% water (Idris *et al*., 2010). Freshly discharged POME is viscous and oily with a very distinctive odour, and contains a huge amount of oil and grease from the extraction plant. Five litres of palm oil mill effluent was shipped from FELDA Agricultural Services Sdn Bhd, Malaysia, in conjunction with Universiti Putra Malaysia. For safety and health purposes a test certificate was released by the supplier (Certificate Nr: 509/2010) and is tabulated in Table 2.2.

Table 2.2

Characteristics of the working POME

Parameter	Value
pH	6.3
Suspended solid, SS	141 mg/l
Oil and grease	2 mg/l
Ammonia nitrogen	2 mg/l

Table 2.3 summarises the methods used to evaluate changes of parameters in POME in order to investigate the reduction of pollution load within POME.

Table 2.3

Methods for tests on POME

Parameter	Method
pH	Jenway 3345 Ion Meter
COD	LCK 014 COD Cuvette Test + Hach Lange DR 2800 Spectrophotometer
Total phosphate	LCK 350 Phosphate Cuvette Test + Hach Lange DR 2800 Spectrophotometer
Nitrate	LCK 339 Nitrate Cuvette Test + Hach Lange DR 2800 Spectrophotometer

2.4 OPTICAL DENSITY AND CELL NUMBER

The phases in the growth of microalgae species can be illustrated by a typical growth curve (S-curve). Growth curves are an important way to determine and express the relative ecological success of a species in adapting to the experimental environment that has been imposed upon it. In this research, optical density was shown to effectively monitor cell growth i.e. cell number. The growth curves for each species within the standard growth medium were generated before those for the species when applied under different parameters.

2.4.1 CORRELATION BETWEEN ABSORBANCE AND CELL NUMBER IN OPTIMAL GROWTH MEDIUM

This experiment was designed to establish the correlation between absorbance and cell number for the different microalgae species. Aliquots from the cultures were transferred to Corning 96 transparent bottomed microtiter plates on the initial day of the cultivation (Day 0). The plates were placed in a TECAN Infinite F200 Microplate Reader and the absorbance measurement at 595 nm was then analyzed using a Magellan 6 v6.4. Ten microlitres of each aliquot was then brought under a microscope to count the number of cells using haemocytometers. These were done three times and a mean was calculated. Results were computed to generate standard equations. From Day 1 to 10, aliquots of cultivated species were transferred to Corning 96 transparent bottomed microtiter plates and the absorbance at 595 nm was analyzed. Readings were again done in triplicate. The growth curves of *S. bacillaris, B. submarina pulsifera, Tetraselmis sp., D. salina, E.*

mutabilis, and *Oscillatoria sp*. cultivated under light regimes of 16:8, 8:16, and 0:24 for 10 days in the optimal growth medium were generated on MS Excel.

2.4.2 CORRELATION BETWEEN ABSORBANCE AND CELL NUMBERS IN INDUSTRIAL PALM OIL MILL EFFLUENT

The growth of *S. bacillaris*, *B. submarina pulsifera*, *Tetraselmis sp*., *D. salina*, *E. mutabilis*, and *Oscillatoria sp*. in POME was determined using the same methods referred to in Chapter 2.4.1. The results in POME were then compared to those in the optimal growth medium.

2.5 OPTICAL DENSITY AND CHLOROPHYLL ANALYSIS

Determination of chlorophyll content provides further information on biomass growth under selected light regimes. The photosynthetic pigments, Chlorophyll *a* (Chl *a*), Chlorophyll *b*, *c*1, *c*2, *c*3, and *d* pigments, can be used as a standard basis to represent the productivity of microalgae species.

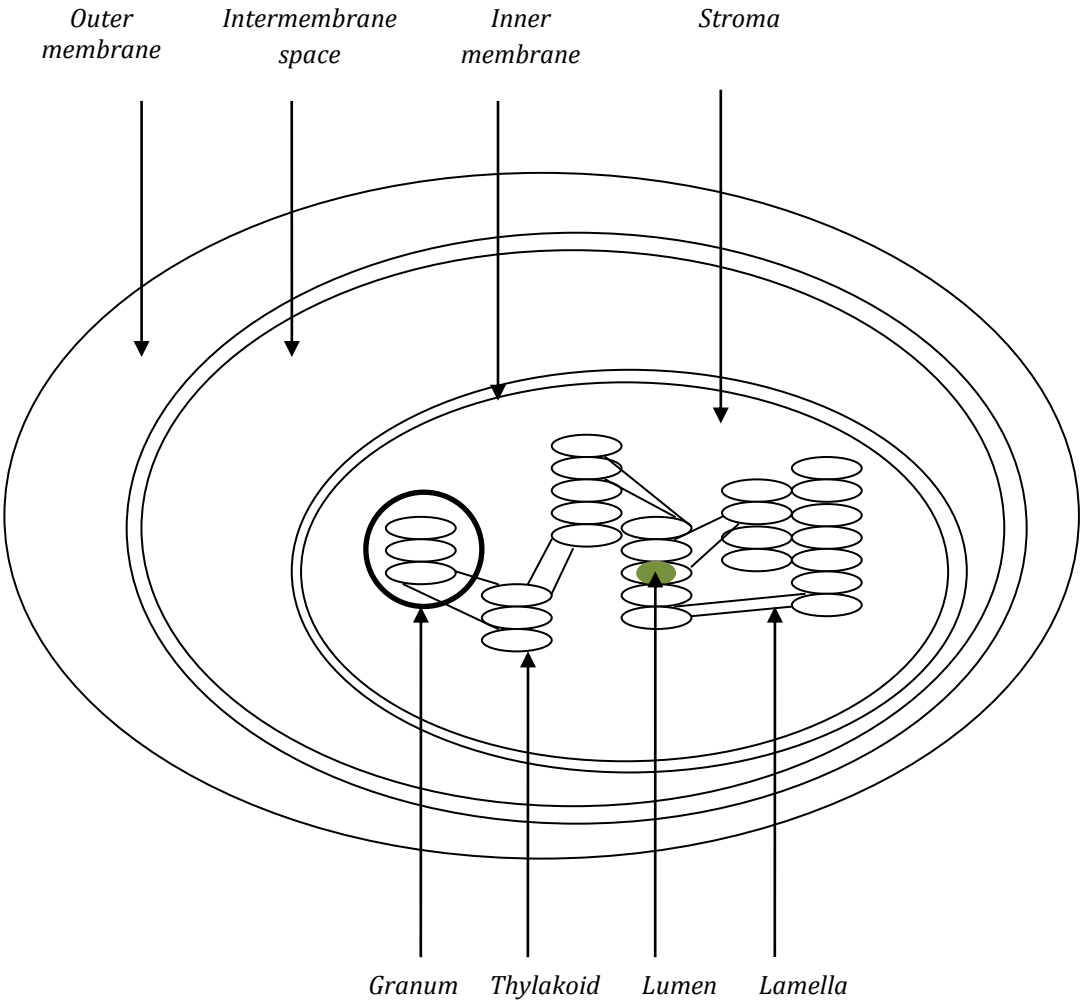


Figure 2.2 Chloroplast

Several methods can be used to quantify chlorophyll content, including the extraction and spectrophotometric methods. A standardise protocol is used to minimise errors and inaccuracy. A common protocol based on the spectrophotometry method includes the separation of microalgae cells from the supernatant and the extraction of pigments with an organic solvent. A complete extraction demonstrates maximal chemical composition of chlorophyll. Therefore, a good solvent plays an important role in the lysis process. Acetone is usually used (Jeffrey and Humphrey, 1975; Humphrey and Jeffrey, 1997), but due to its volatility, high flammability, and properties as a skin irritant chemical, as well as the fact that it is narcotic when highly concentrated, methanol and ethanol are preferably used (Scheer, 1991; Ritchie, 2006). Ultrasound is used for cell wall disruption.

2.5.1 CHLOROPHYLL EXTRACTION

The primary photosynthetic pigments, Chlorophyll *a* (Chl *a*) and/or Chlorophyll *b*, *c*1, *c*2, or *c*3 pigments, can be used as a standard basis to represent the productivity of microalgal biomass. In this experiment, the two most dominant microalgae species from previous studies - *Stichococcus bacillaris* and *Oscillatoria sp*. - were selected for the quantification of their chlorophyll content. *S. bacillaris*, a freshwater green microalgae species, grows best in an acidic medium (John *et al.*, 2002; Lee, 2008). Chlorophyta *S. bacillaris* contains chlorophylls *a* and *b* and stores food as starch in its plastids. *Oscillatoria sp*., on the other hand, is a filamentous prokaryotic cyanophyta microalgae species and contains mainly chlorophyll *a*.

A 50 ml volume of each species' starter culture was cultivated in 200 ml of growth medium, and simultaneously in 200 ml of sterilised POME. The samples were kept inside BudBox™ growth tent (Growell Hydroponics & Plant Lighting) under the 125-Watt Compact Eco-Fluorescent lamps, which were set to two different light regimes i.e. 16:8 and 8:16; the temperature was kept between 16 and 27°C. The experiments were performed in triplicate. For chlorophyll analysis, 2 ml of each culture sample was withdrawn daily, and centrifuged at 3,000 rpm for 10 minutes. When cells were pelletted, the supernatant, which had become fairly clear, was pipetted off, avoiding disturbance of the pellet. Cells were then re-suspended in 2 ml of distilled water to remove any salts that could have been retained by the biomass, before being submitted again to centrifugation at 3,000 rpm for 10 minutes. This washing process was repeated twice. Two millilitres of organic solvent and 0.1 g $MgCO_3$ were suspended with the cell pellets at room temperature and mixed using a vortex mixer for 15 s. The suspension of cells was kept placed in the ultrasonic water bath, which was set at 250 Hz, for 30 minutes, and the homogenate was then centrifuged at 13000 rpm for 2 minutes, or until the supernatant was clear. Using a Pasteur pipette, the sonicated liquid was transferred into a clean centrifuge tube. The pigment extracts were kept on ice and in the dark. The remaining pellets contained damaged cells, including fragmented chloroplast membranes that still contained pigments. The protocol was repeated for a complete extraction, or until the sonicated liquid was colourless. The working extract was analysed using a spectrophotometer at wavelengths of 630, 645, 665, and 750 nm to determine its optical density. The absorbance values at these wavelengths were manipulated using the standard of total chlorophyll analysis of the Australian National Algae Culture Collection Methodologies. The absorbance at 750 nm was subtracted from the absorbance

of each of the other collected wavelengths and the values were substituted in the following equations:

$$Chlorophyll\ a \text{ extract} = (11.85 * \text{A664} - 1.54 * \text{A647} - 0.08 * \text{A630}) * l$$

$$Chlorophyll\ b \text{ extract} = (21.03 * \text{A647} - 5.43 * \text{A664} - 2.66 * \text{A630}) * l$$

$$Chlorophyll\ c \text{ extract} = (24.52 * \text{A630} - 1.67 * \text{A664} - 7.6 * \text{A647}) * l$$

The concentration of chlorophyll in the sample (μg chlorophyll/ml) was obtained by the following equation:

$$Chlorophyll\ a/b/c \text{ sample} = Chlorophyll\ a/b/c \text{ extract} * (v/V)$$

The total concentration of chlorophyll in the sample (μg/ml) was obtained by adding up the chlorophyll *a*, *b* and c samples:

$$Chl \text{ total} = Chl\ a \text{ sample} + Chl\ b \text{ sample} + Chl\ c \text{ sample}$$

Note
l : The spectrometric length; v : Volume of solvent; V : Volume of sample

2.6 OPTICAL DENSITY AND PROTEIN DETERMINATION

Protein determination can also be used as a coupled method in order to estimate the algal biomass growth (Harrison and Thomas, 1988). There are several methods to determine the concentration of protein in microalgae cells (Bergmeyer and Grabl, 1983): the most common method is the spectrophotometric measurements described by Bradford (1976).

The Bradford Assay involves the binding of Coomassie Brilliant Blue dye (which exists in three forms - cationic (red), neutral (green), and anionic (blue)) to proteins through the van der Waals forces and hydrophobic interactions (Compton and Jones, 1985), which are then converted to a stable unprotonated blue form (Sedmark and Grossberg, 1977). Protein analysis in algae is relatively new; therefore using the Bradford Assay is an innovative approach in the study of microalgae (Barbarino, Lourenço, 2005).

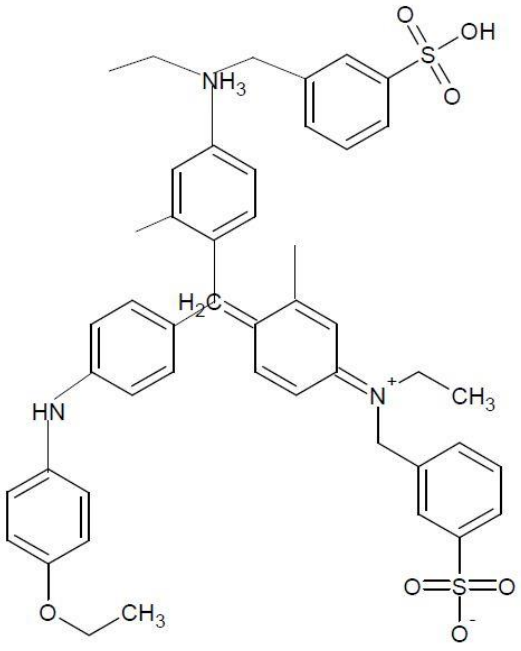


Figure 2.3 Structure of Coomassie Brilliant Blue G-250 dye, which binds to basic (arginine) and aromatic amino acid (Sketch using ACD/Chemsketch).

2.6.1 STANDARD CURVE CALIBRATION

A standard curve for protein determination was generated using 10 µl, 50 µl, and 100 µl of 1 mg/ml Bovine Serum Albumin (BSA) with the Coomasie Blue G250. A series of dilutions were made in triplicate using the wells of a microplate. The absorbance measured at 595 nm using a TECAN Infinite F200 Microplate Reader was then analyzed using a Magellan 6 v6.4. The results obtained were plotted using MS Excel.

2.6.2 BRADFORD ASSAY

Determination of the protein content of *S. bacillaris* and *Oscillatoria sp*. constituted a further investigation of the microalgae growth under the selected light regimes.

A 50 ml volume of each species' starter culture was cultivated in 200 ml of growth medium, and simultaneously in 200 ml of sterilised POME. The samples were kept inside BudBox™ Grow Tent (Growell Hydroponics & Plant Lighting) under the 125-Watt Compact Eco-Fluorescent lamps, which were set to two different light regimes i.e. 16:8 and 8:16; the temperature was kept between 16 and 27°C. The samples were kept in triplicate.

One millilitre of each culture was transferred into an Eppendorf tube and spun down at 4,000 rpm for 5 minutes. The supernatant was deposited, and another 1 ml of fresh strains was added to accumulate more pellets. The pellets were brought onto a weighing scale. They were then stored at -80ºC for 2 to 3 hours before being ground using a micropestle with clean sterilised sand (0.5 g of sand in every 1 g of microalgae biomass). A 0.25 ml volume of 0.3M Phosphate buffer was then added for every 1 g of microalgae biomass, and the mixture was ground further. This solution was spuned down at 10,000 rpm for 2 minutes. Amounts of 10µl, 50 µl, and 100 µl of the supernatants were each mixed with 1 ml

of Coomassie Blue G250-Reagent. The same procedure was repeated in triplicate. Sample processing was carried out in minimal light exposure to avoid protein degradation.

The daily spectrophotometric absorbance was converted to protein concentration using the calibration curve established. The protein content of each microalgae species was calculated using the following equation (Lopez *et al.*, 2010):

$$\text{Protein } \left(\%, \frac{\text{w}}{\text{w}}\right) = \frac{C.V.D}{m} \text{ x } 100\%$$

C is the protein concentration (mg/l) obtained from the calibration curve, *V* is the volume of the phosphate buffer used to re-suspend the biomass, *D* is the dilution factor, and *m* is the amount of dry microalgae biomass (mg).

2.7 TURBIDITY OF PALM OIL MILL EFFLUENT

Turbidity can be used to monitor water quality due to the physical characteristics of scattered light, which is absorbed by the molecules presented in the sample. In this study, the turbidity of palm oil mill effluent was tested using a Nephelometer, which works according to the following principles: the more particles are present, the more light is scattered; subsequently more light reaches the detector, and the value of turbidity is higher. This can be presented by a calibrated Nephelometer in a unit of Nephelometric Turbidity Units (NTU).

2.7.1 TURBIDITY TEST

This experiment elaborates the effect on cultivated *S. bacillaris* and *Oscillatoria sp.* in highly turbid palm waste, comparing it to what it could be if, simply stated, the turbidity of POME can be reduced during the 10 day cultivation period.

Two-hundred millilitres of each species' starter culture was cultivated in 800 ml of sterilised and totally suspended-solid free POME. The samples were kept inside the BudBox™ Grow Tent (Growell Hydroponics & Plant Lighting) under the 125-Watt Compact Eco-Fluorescent lamps, which were set to two different light regimes i.e. 16:8 and 8:16; the temperature was kept between 16 and 27°C. The samples were kept in triplicate. A 10 ml volume of the culture was brought to the Nephelometric Instrument on the 2^{nd}, 4^{th}, 6^{th}, $8^{th,}$ and 10^{th} days of cultivation, to evaluate the turbidity level in Nephelometric Turbidity Units (NTU). The samples were centrifuged and spun down at 13,000 rpm for 2 minutes and the aliquots were again brought for turbidity level readings. Each reading was performed in triplicate.

CHAPTER 3

RESULTS

Phycoremediation offers new possibilities for the biotransformation of pollutant loads and hazardous or toxic constituents, as well as the recovery of important and increasingly valuable resources. Several microalgae species are tolerant to highly organically-enriched wastewater (Oswald *et al*., 1957) and if 50% or 405 billion m^3 of all wastewater is made available for microalgae cultivation it could generate about 247 million tons of microalgae biomass or 37 million tons of oil (Pittman *et al*., 2010). The following results of the experiments executed prove that the selected microalgae species have the potential to phycoremediate industrial palm oil mill effluent when cultivated in it under different light/dark cycle conditions.

3.1 SALINITY TEST

Salinity is a measure of the total salts dissolved in a medium and plays an important role regarding the biochemical composition of microalgae species since different species are tolerant to different level of salinity. It is extremely important to guarantee optimal growth for each species, so a salinity test for each growth medium was carried out.

Highly saline water, also known as brine water, is classified as hyperhaline or metahaline; whereas euhaline water, or typical marine water, can be divided into two types, which are mixoeuhaline or saline, and (poly-, meso-, oligo) haline, or brackish. The salinity of freshwater is usually classified as below 0.5‰.

Table 3.1

Summary of the comparisons of the freshly prepared growth media

Growth medium	Environment	pH	Salinity, ‰	Classification
MBBM	Acidic	6.57	3.13	Oligohaline
f/2	Alkaline	8.52	25.19	Polyhaline
f/2 + 35g/L NaCl	Alkaline	9.62	37.16	Mixoeuhaline
EG	Neutral	7.54	4.24	Oligohaline
BG11	Alkaline	7.40	2.61	Oligohaline
Artificial Seawater	Alkaline	8.37	32.73	Mixoeuhaline

The salinity test was done in triplicate and the percentage is the mean value of the results obtained. Classification is in reference to the Venice System for the Classification of Brackish Waters, sponsored by the International Association of Limnology and the International Union of Biological Sciences in Venice 1958.

3.2 POLLUTION LOAD IN PALM OIL MILL EFFLUENT

Raw palm oil mill effluent is a brown coloured suspension which is slightly acidic and made up of 94–96% water (Idrisa *et al*., 2010). Freshly discharged POME is viscous, oily and acidic. Five litre of palm waste was shipped from FELDA AGRICULTURAL SERVICES Sdn Bhd, Malaysia, in conjunction with Universiti Putra Malaysia. Prior to the arrival of the POME sample several preliminary analytical tests were conducted for preliminary experiments in order to determine the basic characters of the palm waste sample. The results of the tests are tabulated in Table 3.2.

Table 3.2

Results of the preliminary tests done on the POME sample

Parameter	Method	Result
pH	Jenway 3345 Ion Meter	6.34
COD (1)	LCK 014 COD Cuvette Test + Hach Lange DR 2800 Spectrophotometer	1713 mg/l
Total phosphate	LCK 350 Phosphate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	12.1 mg/l
Nitrate	LCK 339 Nitrate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	13.7 mg/l

Each test is done in triplicate and the results are the mean average.
(1) Chemical Oxygen Demand

The significance of the results was to determine the presence of Chemical Oxygen Demand (COD), total phosphate and nitrate. The same assessment was done on the palm waste before and after cultivation with the selected microalgae species in order to determine the efficiency of each strain in removing the pollutant loads.

3.3 STANDARD CALIBRATION CURVES

The measurement of absorbance and cell number in a serial dilution of microalgae samples in both the specified optimal growth medium (control medium) and the palm oil waste was used to determine the growth of the microalgae species as there is a direct correlation between these two parameters when a linear regression is performed (see Chapter 2.4.1.). The absorbance of each species was assessed in both their optimal growth medium and the palm oil waste. Table 3.2 tabulates the standard calibration equations that were used to evaluate the growth for the 10 days of cultivation.

Table 3.3

Standard calibration curves for each microalgae strain under different parameters

Condition	Species	Equation	$\mathbf{R^2}$	End point (cell /ml)
MBBM (1); I	*S. bacillaris*	$y = 1 \times 10^{-8} x + 0.0791$	0.9564	8.64×10^{7}
POME; I	*S. bacillaris*	$y = 8 \times 10^{-9} x + 0.482$	0.9709	1.57×10^{8}
MBBM (1); II	*S. bacillaris*	$y = 3 \times 10^{-9} x + 0.0369$	0.9687	1.31×10^{8}
POME; II	*S. bacillaris*	$y = 1 \times 10^{-9} x + 0.3043$	0.9699	1.07×10^{8}
MBBM (1); III	*S. bacillaris*	$y = 3 \times 10^{-9} x + 0.0369$	0.9601	1.25×10^{8}
POME; III	*S. bacillaris*	$y = 1 \times 10^{-9} x + 0.3043$	0.9669	1.05×10^{8}
f/2 (2); I	*B. submarina puls.*	$y = 5 \times 10^{-8} x - 0.0224$	0.8379	3.53×10^{7}
POME; I	*B. submarina puls.*	$y = 1 \times 10^{-7} x - 0.0815$	0.8385	6.79×10^{7}
f/2 (2); II	*B. submarina puls.*	$y = 5 \times 10^{-8} x - 0.0224$	0.8309	3.49×10^{7}
POME; II	*B. submarina puls.*	$y = 1 \times 10^{-7} x - 0.0815$	0.8306	6.81×10^{7}
f/2 (2); III	*B. submarina puls.*	$y = 5 \times 10^{-8} x - 0.0224$	0.8318	3.50×10^{7}
POME; III	*B. submarina puls.*	$y = 1 \times 10^{-7} x - 0.0815$	0.8347	6.80×10^{7}

Table 3.3 (continued)

Standard calibration curves for each microalgae strain under different parameters

Condition	Species	Equation	R^2	End point (cell/ml)
f/2 (2); I	*Tetraselmis sp.*	$y = 1 \times 10^{-6} x + 0.0359$	0.9665	2.01×10^6
POME; I	*Tetraselmis sp.*	$y = 5 \times 10^{-7} x + 0.5622$	0.9698	2.56×10^6
f/2 (2); II	*Tetraselmis sp.*	$y = 7 \times 10^{-7} x + 0.0646$	0.9719	2.89×10^6
POME; II	*Tetraselmis sp.*	$y = 9 \times 10^{-7} x + 0.353$	0.9703	2.97×10^6
f/2(2) ; III	*Tetraselmis sp.*	$y = 7 \times 10^{-7} x + 0.0646$	0.9723	2.87×10^6
POME; III	*Tetraselmis sp.*	$y = 6 \times 10^{-7} x + 0.2315$	0.9701	2.73×10^6
f/2+NaCl (3); I	*D. salina*	$y = 3 \times 10^{-9} x + 0.0528$	0.9638	1.26×10^8
POME; I	*D. salina*	$y = 2 \times 10^{-9} x + 0.4694$	0.9591	1.09×10^8
f/2+NaCl (3); II	*D. salina*	$y = 2 \times 10^{-9} x + 0.0775$	0.9607	1.12×10^8
POME; II	*D. salina*	$y = 5 \times 10^{-9} x + 0.3462$	0.9701	1.71×10^8
f/2+NaCl (3); III	*D. salina*	$y = 2 \times 10^{-9} x + 0.0775$	0.9619	1.14×10^8
POME; III	*D. salina*	$y = 4 \times 10^{-9} x + 0.5162$	0.9719	1.68×10^8
EM (4); I	*E. mutabilis*	$y = 1 \times 10^{-8} x + 0.0791$	0.9406	4.67×10^6
POME; I	*E. mutabilis*	$y = 2 \times 10^{-8} x - 0.034$	0.9389	3.91×10^6
EM (4); II	*E. mutabilis*	$y = 2 \times 10^{-8} x + 0.00005$	0.9478	5.03×10^6
POME; II	*E. mutabilis*	$y = 4 \times 10^{-8} x - 0.1025$	0.9401	4.56×10^6
EM (4); III	*E. mutabilis*	$y = 2 \times 10^{-8} x + 0.00005$	0.9451	5.11×10^6
POME; III	*E. mutabilis*	$y = 3 \times 10^{-8} x - 0.1177$	0.9391	4.21×10^6

Table 3.3 (continued)

Standard calibration curves for each microalgae strain under different parameters

Condition	Species	Equation	$\mathbf{R^2}$	End point (cell/ml)
BG11 (5); I	*Oscillatoria sp.*	$y = 8 \times 10^{-9} x + 0.0928$	0.9877	2.68×10^8
POME; I	*Oscillatoria sp.*	$y = 5 \times 10^{-9} x + 0.4947$	0.9831	2.57×10^8
BG11 (5); II	*Oscillatoria sp.*	$y = 3 \times 10^{-9} x + 0.041$	0.9809	2.35×10^8
POME; II	*Oscillatoria sp.*	$y = 4 \times 10^{-9} x + 0.2864$	0.9819	2.45×10^8
BG11 (5); III	*Oscillatoria sp.*	$y = 3 \times 10^{-9} x + 0.041$	0.9811	2.39×10^8
POME; III	*Oscillatoria sp.*	$y = 4 \times 10^{-9} x + 0.2835$	0.9815	2.42×10^8

The equations are for the slope of the regression line for cell number over time. The absorbance values were read using a TECAN Infinite F200 Microplate Reader with the absorbance measurement at 595 nm. I is the light regime of 16:8; II is the light regime of 8:16; III is the light regime of 0:24.

(1) modified Bold Basal medium with 3-fold nitrogen and vitamins
(2) half-strength f medium
(3) half-strength f medium with an additional 35 g/l sodium chloride solution to produce double-strength saline medium
(4) Euglena Gracilis medium
(5) Blue-green medium

Standard calibration curves in the optimal growth medium and POME in all 6 species showed good correlation coefficients and would be used in further assessing the cell growth during the cultivation period. This was done by replacing the daily absorbance (y-values) in the generated equations to quantify the number of cell (x-values). Cell number was then plotted against the day.

3.4 GROWTH

3.4.1 CELL NUMBER

Monitoring microalgae growth using the light scattering properties of the medium solutions may be difficult owing to several physical anomalies, such as backscattering, therefore standard calibration curves are important to normalise the data regarding cell number. The following results demonstrate the cultivated cell growth for 10 days using daily optical density based on the standard calibration equations of cell number in the respective optimal growth medium.

Table 3.4

The end-of-point of cell number after 10 days of cultivation in the respective optimal growth medium under different light regimes

Species	16:8 light	8:16 light	0:24 light
S. bacillaris	2.13×10^7 cell number	1.89×10^7 cell number	1.036×10^8 cell number
B. submarina pulsf.	2.83×10^7 cell number	1.74×10^7 cell number	N.A.
Tetraselmis sp.	8.04×10^5 cell number	2.90×10^5 cell number	N.A.
D. salina	5.01×10^7 cell number	4.41×10^7 cell number	N.A.
E. mutabilis	6.70×10^5 cell number	N.A.	N.A.
Oscillatoria sp.	1.14×10^8 cell number	1.58×10^8 cell number	2.18×10^8 cell number

N.A. not available – no growth was detected

Growth of *B. submarina pulsifera, Tetraselmis sp.* and *D.salina* was reduced relative to controls shortly after the period of 2 to 3 days under the total dark regime. Although *E. mutabilis* showed growth under 16:8 light, no growth was detected under 8:16 and 0:24 light regimes. This shows that these species grow under high light regimes only in their optimal growth medium. The photoheterotrophic-like characteristics were shown to be more practicable for *S. bacillaris* and *Oscillatoria sp.* Further experiments include a screening test to investigate the ability of 6 microalgae species to grow in palm oil waste. Only two species were found to grow under 16:8 and 8:16 light regimes only, and these results are tabulated as follows.

Table 3.5

Summary of growth ability of microalgae species within optimal growth medium and palm oil mill effluent under different light regimes

Species	Optimal growth medium			Palm oil mill effluent		
	16:8	8:16	0:24	16:8	8:16	0:24
S. bacillaris	√	√	√	√	√	*x*
B. submarina pulsifera	√	√	*x*	*x*	*x*	*x*
Tetraselmis sp.	√	√	*x*	*x*	*x*	*x*
D. salina	√	√	*x*	*x*	*x*	*x*
E. mutabilis	√	*x*	*x*	*x*	*x*	*x*
Oscillatoria sp.	√	√	√	√	√	*x*

√ represents the growth availability of microalgae cells under the specified parameters
x represents the insignificant growth of microalgae cells under the specified parameters

Results showed that only *S. bacillaris* and *Oscillatoria sp.* were able to grow successfully under artificial light in palm waste, although no growth was detected under 0:24 light for all six species. The filamentous structures of *S.bacillaris* and *Oscillatoria sp*. were found to

fragment and also provided evidence that physiological adaptation of the cells to different concentrations of industrial palm oil mill effluent under different light regimes results in different metabolic modifications of the cell reproduction. Therefore only chlorophyta *S. bacillaris* and *Oscillatoria sp.* were selected for further studies due to their growth ability to grow under these selected parameters.

3.4.1.1 GROWTH IN PALM OIL MILL EFFLUENT

Phycoremediation exploits high-tolerance microalgae species to biotransform pollutants from organically-enriched wastewater. The following results show the growth of two novel microalgae species, *S. bacillaris* and *Oscillatoria sp.*, in palm oil waste under different light regimes i.e. 16:8 and 8:16.

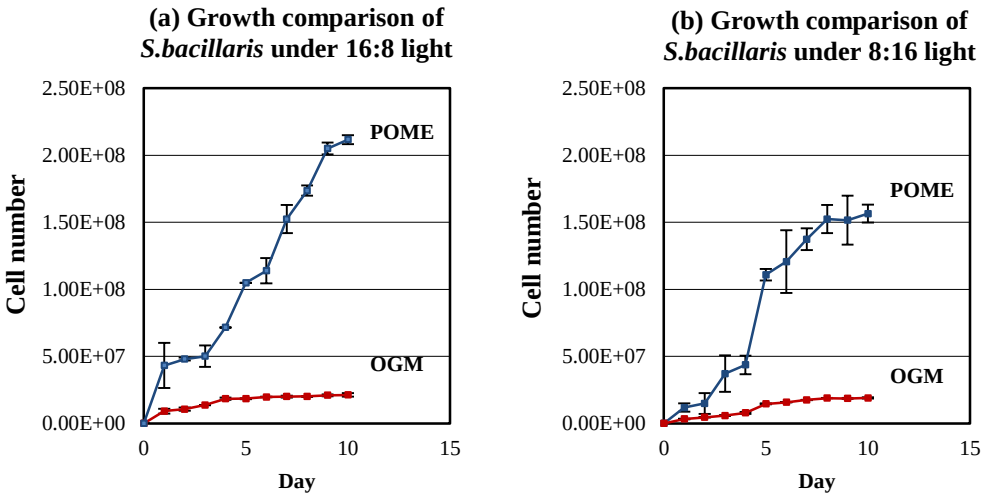


Figure 3.1 Growth curves of *S. bacillaris* in control (OGM) and Palm Oil Mill Effluent (POME) under **(a)** 16:8 light and **(b)** 8:16 light

Figure 3.1 (a) and (b) dictate the efficiency of chlorophyta *S. bacillaris* in palm oil mill effluent under different light regimes. The ANOVA test was used to determine the

significance of difference in growth under the selected conditions. An ANOVA analysis of $\alpha = 0.05$ for *S. bacillaris* was calculated using MS EXCEL. The *p* value from the ANOVA analysis indicated that the cell number under 16:8 light in palm oil mill effluent was significantly higher than the other exploited parameters.

Figure 3.2 (a) and (b) dictate the efficiency of cyanophtya *Oscillatoria sp*. in palm oil mill effluent under different light regimes. The ANOVA test was also demonstrated to evaluate the significant growth under the selected parameters. As for *Oscillatoria sp*., cell number was not significantly different.

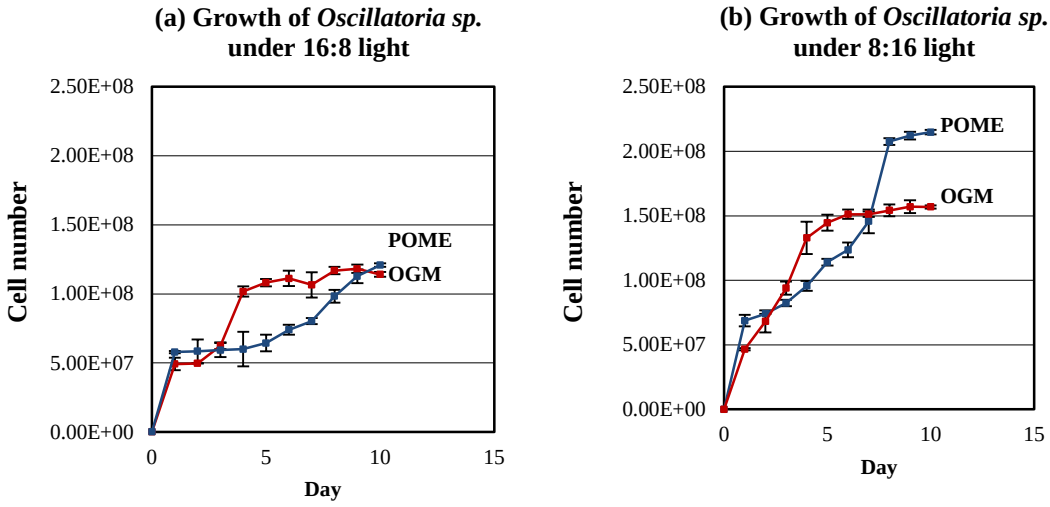


Figure 3.2 Growth curves of *Oscillatoria sp.* in control (OGM) and Palm Oil Mill Effluent (POME) under **(a)** 16:8 light and **(b)** 8:16 light

The student *t*-test was used to make pair-wise comparison of the differences in growth between *S. bacillaris* and *Oscillatoria sp.* in palm oil mill effluent and the control medium under each light regime. Chlorophyta *S.bacillaris* showed a highly significant value under the high light regime when employed within palm oil waste. On other hand, despite the

percentage increment under each light regime, the cell number cyanophyta *Oscillatoria sp*. was found to be non-significant (see Table 3.6).

Table 3.6

t-test analysis of cell number

Species	Light regime 16:8	Light regime 8:16
S. bacillaris		
(1) Difference (end-point in POME to control)	+89.95%	+87.72%
(2) *p*-value	0.0004	0.0009
Oscillatoria sp.		
(1) Difference (end-point in POME to control)	+5.56%	+26.98%
(2) *p*-value	0.11	0.32

(1) Difference : percentage in increment or decrement of cell number at the end-of-point between POME and control
(2) *p*-value : generated from the *t*-test of cell number in optimal growth medium and palm oil mill effluent

3.4.2 CHLOROPHYLL ANALYSIS

Determination of the chlorophyll content of selected microalgae species provides further information on biomass growth and formation under the selected light regime. The primary photosynthetic pigments, Chlorophyll *a* (Chl *a*) and/or with the presence of Chlorophyll *b*, *c*1, *c*2, *c*3 pigments, can be used as a standard basis to calculate the growth. The two most dominant microalgae species, which are *S. bacillaris* and *Oscillatoria sp*., were selected for the extraction and quantification of chlorophyll content. The growths in the optimal growth medium and in palm oil mill effluent were plotted as follows:

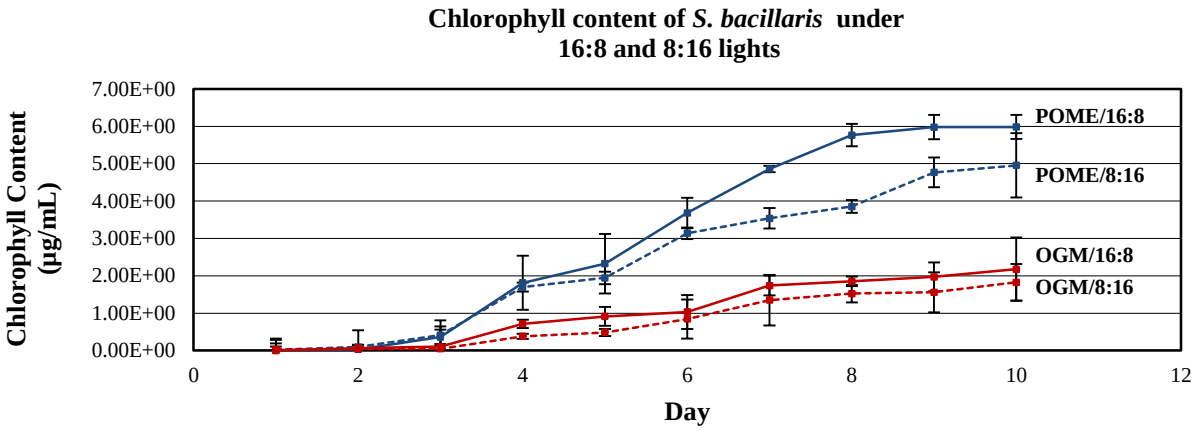


Figure 3.3 Chlorophyll content of *S. bacillaris* in control (OGM) and Palm Oil Mill Effluent (POME) under 16:8 and 8:16 light regimes

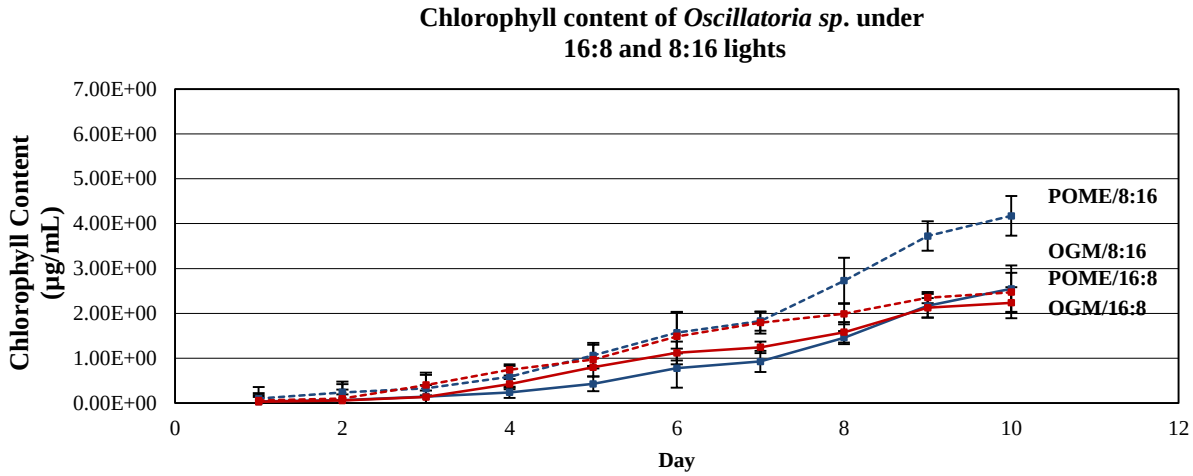


Figure 3.4 Chlorophyll content of *Oscillatoria sp.* in control (OGM) and Palm Oil Mill Effluent (POME) under 16:8 and 8:16 light regimes

In order to compare the chlorophyll content in *S. bacillaris and Oscillatoria sp.*, the ANOVA test was carried out to evaluate the significance of the difference in growth observed under the selected parameters. The ANOVA analysis of $\alpha = 0.05$ for *S. bacillaris* and *Oscillatoria sp.* was calculated using MS EXCEL. For *S. bacillaris* the *p* value from ANOVA analysis was 0.010088. It can be concluded that the strains under 16:8 light in

palm oil mill effluent contained a significantly higher value of chlorophyll content in comparison to the other exploited parameters. As for *Oscillatoria sp*., the analysis showed differences to be non-significant under the designated parameters. Further analysis was performed using the *t*-test to evaluate distribution within the groups.

Table 3.7

t-test analysis of chlorophyll content

Species	Light regime 16:8	Light regime 8:16
S. bacillaris		
(1) Difference (end-point of chlorophyll in POME to control)	+ 63.62 %	+ 63.17 %
(2) *p*-value	0.0165	0.0125
Oscillatoria sp.		
(1) Difference (end-point of chlorophyll in POME to control)	+ 12.21 %	+ 40.8 %
(2) *p*-value	0.404	0.239

(1) Difference : percentage in increment or decrement of chlorophyll content at the end-of-point between POME and control
(2) *p*-value : generated from the *t*-test of chlorophyll content between optimal growth medium and palm oil mill effluent

Student *t*-test analysis of the chlorophyll contents of the *S. bacillaris* within the groups (light regimes) revealed that they were significantly higher when it was cultivated in palm oil mill effluent rather than in the control medium under both light regimes. In spite of the increment in the percentage differences of chlorophyll content from the cultivation within palm oil mill effluent compared to those in the control medium, the *t*- test statistical analysis of *Oscillatoria sp*. indicates non-significant values under both light regimes.

3.4.3 PROTEIN DETERMINATION

Determination of the protein content of *S. bacillaris* and *Oscillatoria sp*. was investigated to determine the microalgae growth formation under selected physical parameters i.e. the 16:8 and 8:16 light regimes. Due to physiological processes that are dependent on the activity of free protein in the cell (Harrison and Thomas, 1988), protein determination in microalgae species represents biochemical measure of algal biomass. Daily absorbance of protein extraction using the Bradford Assay was converted into protein concentration using the experimental calibration curves, and protein percentage weight-to-weight (%, w/w) of each growth medium and light regime were plotted as follows.

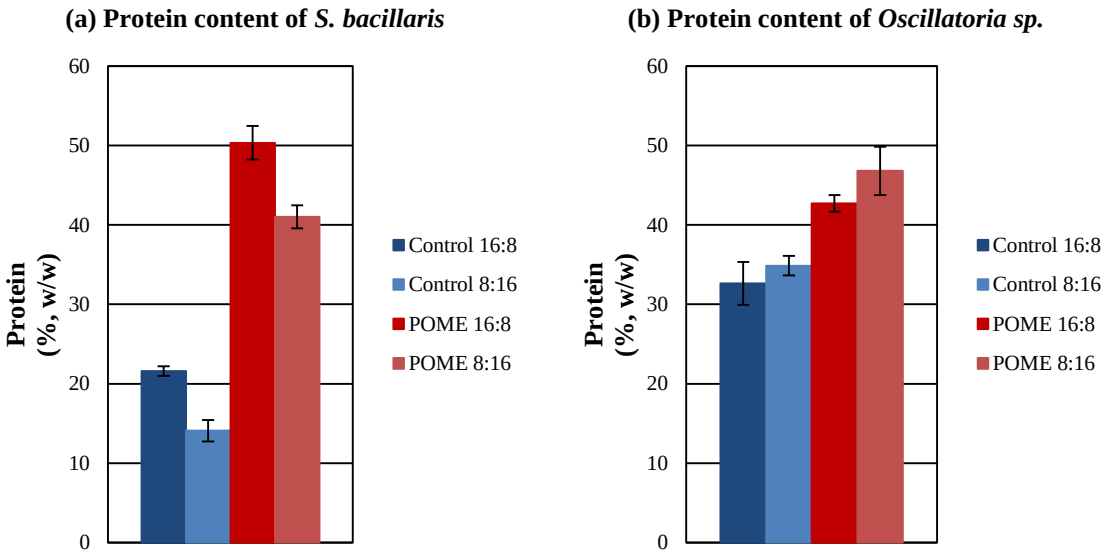


Figure 3.5 Protein content of **(a)** *S. bacillaris* and **(b)** *Oscillatoria sp.* in the control medium and palm oil mill effluent (POME) under 16:8 light and 8:16 light

To compare the protein content in each microalgae species under different light regimes and in different growth media, ANOVA test was carried out to evaluate the significant growth under the selected parameters. The ANOVA analysis of $\alpha = 0.05$ for *S. bacillaris* and *Oscillatoria sp.* was calculated using MS EXCEL. As per the ANOVA analysis for

chlorophyll content, protein of *S. bacillaris* under cultivated 16:8 light regime in palm waste was significantly higher than those of the other parameters. As for *Oscillatoria sp*., the analysis was shown to be non-significant. For further analysis, the *t*-test was performed to evaluate distribution within the groups (Table 3.8).

Table 3.8

t-test analysis of protein content

Species	Light regime 16:8	Light regime 8:16
S. bacillaris		
(1) Difference (end-point of protein content in POME to control)	+ 57.1%	+ 65.66 %
(2) *p*-value	0.091	0.0333
Oscillatoria sp.		
(1) Difference (end-point of protein content in POME to control)	+ 23.57 %	+ 25.53%
(2) *p*-value	0.1003	0.1068

(1) Difference : percentage in increment or decrement of protein content at the end-of-point between POME and control
(2) *p*-value : generated from the *t*-test of protein content between optimal growth medium and palm oil mill effluent

Student *t*-test analysis of the protein contents of both microalgae species within the groups (light regimes) protein contents of both microalgae species revealed that they were significantly higher when cultivated in palm oil mill effluent than in the control medium when cultivated under both light regimes for *S. bacillaris* and non-significant for *Oscillatoria sp*.

3.5 REMOVAL OF POLLUTION LOAD

POME is the most polluted organic residue generated from palm oil processing and is composed of high nutrient content. Phycoremediation removes pollutants, hazardous or toxic constituents, and recovers important and increasingly valuable resources.

The experiments in this chapter were designed to investigate the abilities of *S. bacillaris* and *Oscillatoria sp.* to reduce chemical oxygen demand, total phosphate and nitrate levels. Changes in pH were also monitored. Results are contained in the following tables and plotted on the graphs as shown on Figures 3.6 – 3.9.

Table 3.9

Results of the POME sample before and after the cultivation of *S.bacillaris* under 16:8 light regime

Parameter	Method	Before	After	Reduction
pH	Jenway 3345 Ion Meter	6.54	6.07	-7.19%
COD (1)	LCK 014 COD Cuvette Test + Hach Lange DR 2800 Spectrophotometer	1389 mg/l	204 mg/l	-85.31%
Total phosphate	LCK 350 Phosphate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	17.47 mg/l	1.21 mg/l	-93.07%
Nitrate	LCK 339 Nitrate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	12.03 mg/l	2.63 mg/l	-78.14%

Table 3.10

Results of the POME sample before and after the cultivation of *S.bacillaris* under 8:16 light regime

Parameter	Method	Before	After	Reduction
pH	Jenway 3345 Ion Meter	6.48	6.09	-6.02%
COD (1)	LCK 014 COD Cuvette Test + Hach Lange DR 2800 Spectrophotometer	1313 mg/l	251 mg/l	-80.88%
Total phosphate	LCK 350 Phosphate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	15.44 mg/l	1.85 mg/l	-88.25%
Nitrate	LCK 339 Nitrate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	11.33 mg/l	3.07 mg/l	-72.90%

Table 3.11

Results of the POME sample (control)

Parameter	Method	Before	After	Reduction
pH	Jenway 3345 Ion Meter	6.39	6.36	-0.47%
COD (1)	LCK 014 COD Cuvette Test + Hach Lange DR 2800 Spectrophotometer	1319 mg/l	1298 mg/l	-1.59%
Total phosphate	LCK 350 Phosphate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	11.49 mg/l	11.51 mg/l	+ 0.17%
Nitrate	LCK 339 Nitrate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	9.71 mg/l	9.64 mg/l	-0.72%

Table 3.12

Results of the POME sample before and after the cultivation of *Oscillatoria sp.* under 16:8 light regime

Parameter	Method	Before	After	Reduction
pH	Jenway 3345 Ion Meter	6.98	6.52	-6.59%
COD (1)	LCK 014 COD Cuvette Test + Hach Lange DR 2800 Spectrophotometer	1419 mg/l	309 mg/l	-78.22%
Total phosphate	LCK 350 Phosphate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	18.85 mg/l	3.4 mg/l	-81.96%
Nitrate	LCK 339 Nitrate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	10.98 mg/l	2.34 mg/l	-78.69%

Table 3.13

Results of the POME sample before and after the cultivation of *Oscillatoria sp.*under 8:16 light regime

Parameter	Method	Before	After	Reduction
pH	Jenway 3345 Ion Meter	6.89	6.48	-5.95%
COD (1)	LCK 014 COD Cuvette Test + Hach Lange DR 2800 Spectrophotometer	1398 mg/l	239 mg/l	-82.9%
Total phosphate	LCK 350 Phosphate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	19.08 mg/l	2.7 mg/l	-85.85%
Nitrate	LCK 339 Nitrate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	10.08 mg/l	1.49 mg/l	-85.22%

Table 3.14

Results of the POME sample (control)

Parameter	Method	Before	After	Reduction
pH	Jenway 3345 Ion Meter	6.37	6.35	-0.31%
COD (1)	LCK 014 COD Cuvette Test + Hach Lange DR 2800 Spectrophotometer	1387 mg/l	1358 mg/l	-2.09%
Total phosphate	LCK 350 Phosphate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	10.99 mg/l	11.09 mg/l	+0.91%
Nitrate	LCK 339 Nitrate Cuvette Test + Hach Lange DR 2800 Spectrophotometer	9.3 mg/l	9.46 mg/l	+1.72%

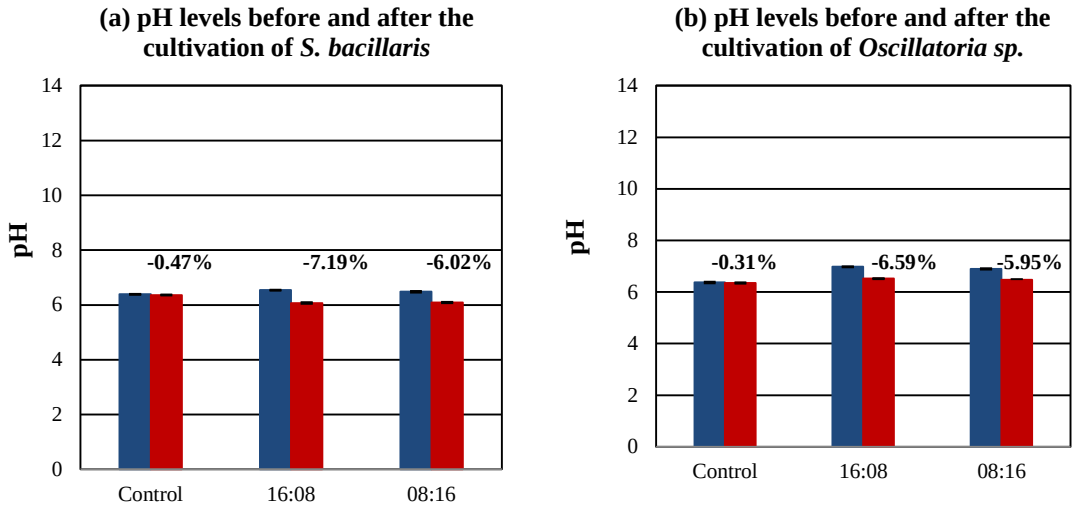


Figure 3.6 Comparison of pH level in POME before (blue bars) and after (red bars) phycoremediation of (**a)** *S. bacillaris* and **(b)** *Oscillatoria sp.* under different light regimes.

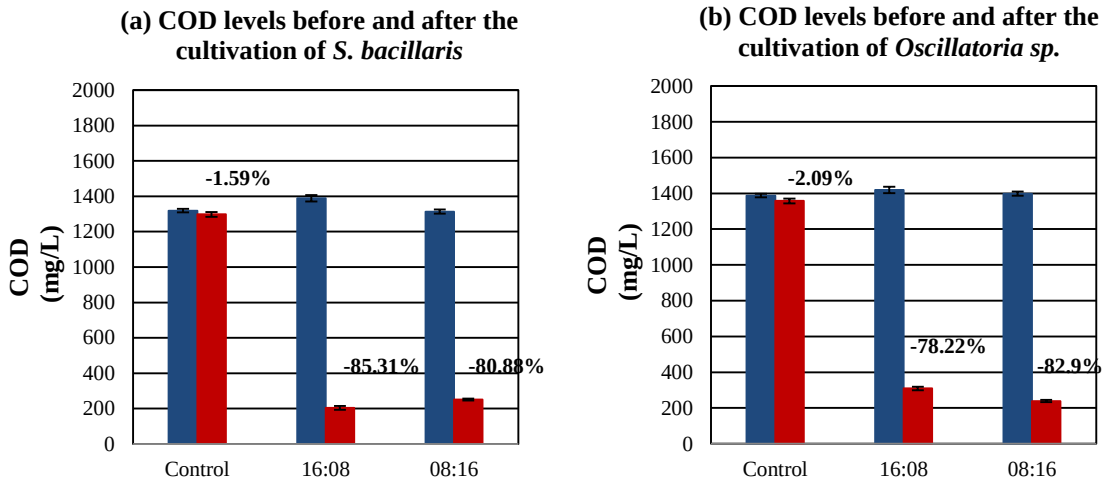


Figure 3.7 Comparison of Chemical Oxygen Demand (COD) level in POME before (blue bars) and after (red bars) phycoremediation of **(a)** *S. bacillaris* and **(b)** *Oscillatoria sp*. under different light regimes.

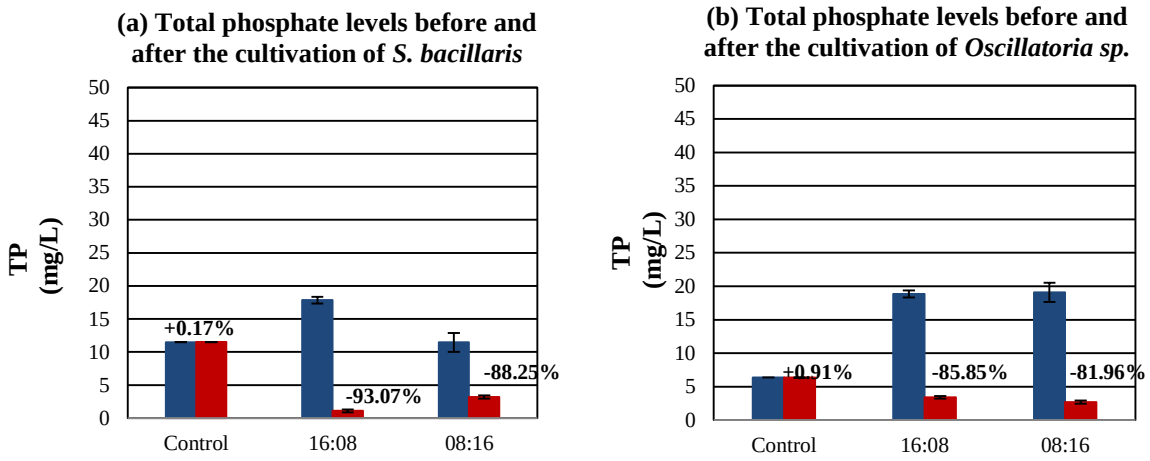


Figure 3.8 Comparison of total phosphate level in POME before (blue bars) and after (red bars) phycoremediation of (**a)** *S. bacillaris* and **(b)** *Oscillatoria sp.*under different light regimes.

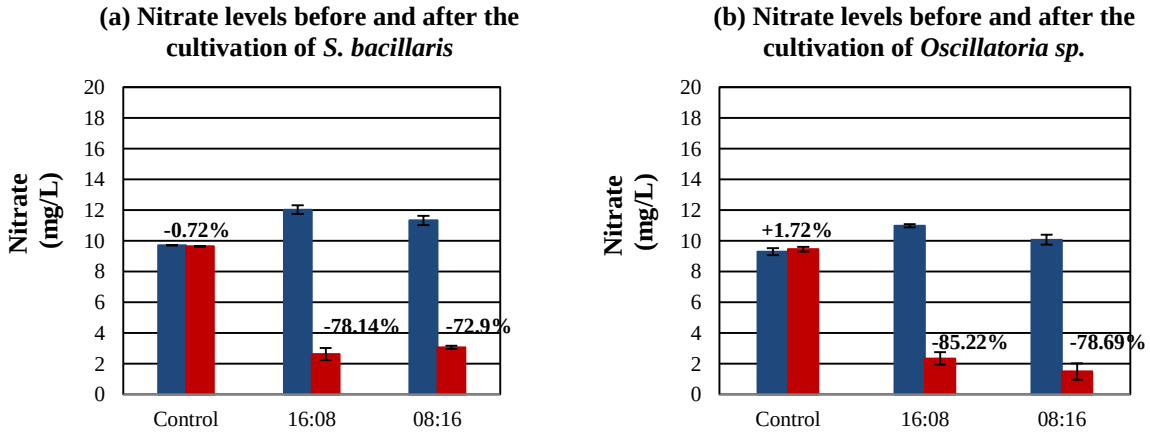


Figure 3.9 Comparison of nitrate level in POME before (blue bars) and after (red bars) phycoremediation of (**a)** *S.bacillaris* and **(b)** *Oscillatoria sp.* under different light regimes.

To compare the pollution load removal by each microalgae species within the groups, the *t*-test was performed using MS EXCEL and the results are tabulated in Tables 3.15 – 3.17.

Table 3.15

t-test analysis of Chemical Oxygen Demand (COD) in POME under different light regimes

Species	Light regime 16:8	Light regime 8:16
S. bacillaris		
(1) Difference	- 85.31%	- 80.88%
(2) *p*-value	0.000027	0.000023
Oscillatoria sp.		
(1) Difference	- 78.22%	- 82.9%
(2) *p*-value	0.00001	0.0000015

(1) Difference : percentage in increment or decrement of Chemical Oxygen Demand (COD) before and after treatment
(2) *p*-value : generated from the *t*-test of Chemical Oxygen Demand (COD) in palm oil mill effluent under specified light regime

Table 3.16

t-test analysis of total phosphate in POME under different light regimes

Species	Light regime 16:8	Light regime 8:16
S. bacillaris		
(1) Difference	- 93.07 %	- 88.25 %
(2) *p*-value	0.00015	0.000039
Oscillatoria sp.		
(1) Difference	- 85.85 %	- 81.96 %
(2) *p*-value	0.0025	0.00017

(1) Difference : percentage in increment or decrement of total phosphate before and after treatment
(2) *p*-value : generated from the *t*-test of total phosphate in palm oil mill effluent under specified light regime

Table 3.17

t-test analysis of nitrate in POME under different light regimes

Species	Light regime 16:8	Light regime 8:16
S. bacillaris		
(1) Difference	- 78.14 %	- 72.90 %
(2) *p*-value	0.00037	0.00056
Oscillatoria sp.		
(1) Difference	- 85.22 %	- 78.69 %
(2) *p*-value	0.00029	0.00077

(1) Difference : percentage in increment or decrement of nitrate before and after treatment
(2) *p*-value : generated from the *t*-test of nitrate in palm oil mill effluent under specified light regime

Student *t*-test analysis within the groups (light regimes) of the removal of all three pollution components by both microalgae species revealed that they were significantly high when cultivated under both light regimes, with preference towards 16:8 light by both *S. bacillaris* and *Oscillatoria sp.* for phosphorus and nitrate removal; and higher percentage reduction of Chemical Oxygen Demand (COD) under 16:8 light for *S. bacillaris* and 8:16 light for *Oscillatoria sp*. (see Tables 3.15 - 3.17)

Chemical oxygen demand (COD) represents the ultimate oxygen demand to oxidise organic carbon. Carbon consumption could be calculated as follows (Edwards *et al*., 1980):

$$\text{Carbon (mg litre}^{-1}) = \text{Chemical Oxygen Demand (COD)} \times \frac{12}{32}$$

Furthermore, the value of nitrate is also an important tool to assess water quality. Fully nitrified wastewater also has little or no organic nitrogen. In theory, total nitrogen (TN) is the sum of nitrate-nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N), ammonia-nitrogen (NH_3N) and organically bonded nitrogen. As for this research, TN is simplified as the summation of

nitrate-nitrogen solely based on the above-mentioned assumptions. To achieve this, the conversion of nitrate-N (mg/L) to nitrate-NO_3 (mg/l) can be executed as follows:

$$\text{Nitrate} - \ NO_3 \ \left(\frac{\text{mg}}{\text{l}}\right) = \text{Nitrate} - \text{N} \ \left(\frac{\text{mg}}{\text{l}}\right) \text{x } 4.4268$$

C/N ratio determines the limitations of microalgal growth (Donaghay *et al.*, 1978). Muhrizal *et al.* (2006) reported that POME is characterised by a low C:N ratio (6-15:1) in comparison to the C:N ratio of sawdust (186:1). The C:N ratio derived from the chemical oxygen demand and nitrate-nitrogen level in the palm waste sample before the treatment in this research demonstrated a consistency to the literature, and the consumption of C:N ratio showed that higher amounts of carbon were consumed under the lower light regime i.e. 8:16 light, which also means that more nitrogen was immobilised during heterotrophic conditions.

Table 3.18

Carbon to nitrogen ratio

Species	Light Regime	C before	C after	N-NO_3 before	N-NO_3 after	C:N before	C:N after
S. bacillaris	16:8	520.75	76.5	53.3	11.6	9.8:1	6.6:1
	8:16	492.5	94.1	50.2	13.6	9.8:1	9.6:1
Oscillatoria sp.	16:8	532.3	115.9	48.6	10.8	11:1	10.7:1
	8:16	524.3	89.6	44.6	6.6	11.8:1	13.6:1

C Carbon
N-NO_3 Nitrate-nitrogen
C:N Carbon-to-nitrogen ratio

3.6 TURBIDITY

Turbidity is considered a mostly aesthetic characteristic of water. Testing the turbidity of palm oil waste after phycoremediation provides further information to discover the ability of microalgae species in removing organic pollutants or in improving the quality of the effluent. The effect of cultivated *S. bacillaris* and *Oscillatoria sp.* in palm oil mill effluent were tabulated in the following figures.

Figures 3.10 (a) – (d) show the correlation of microalgae biomass growth to the reduction of turbidity in palm oil mill effluent (POME) over the 10 days of cultivation. In comparison to the control, turbidity was reduced when *S. bacillaris* and *Oscillatoria sp*. grew over the time period. The following figures show the comparison of each microalgae species in improving the turbidity level of POME under different light regimes. The results are tabulated as mean averages.

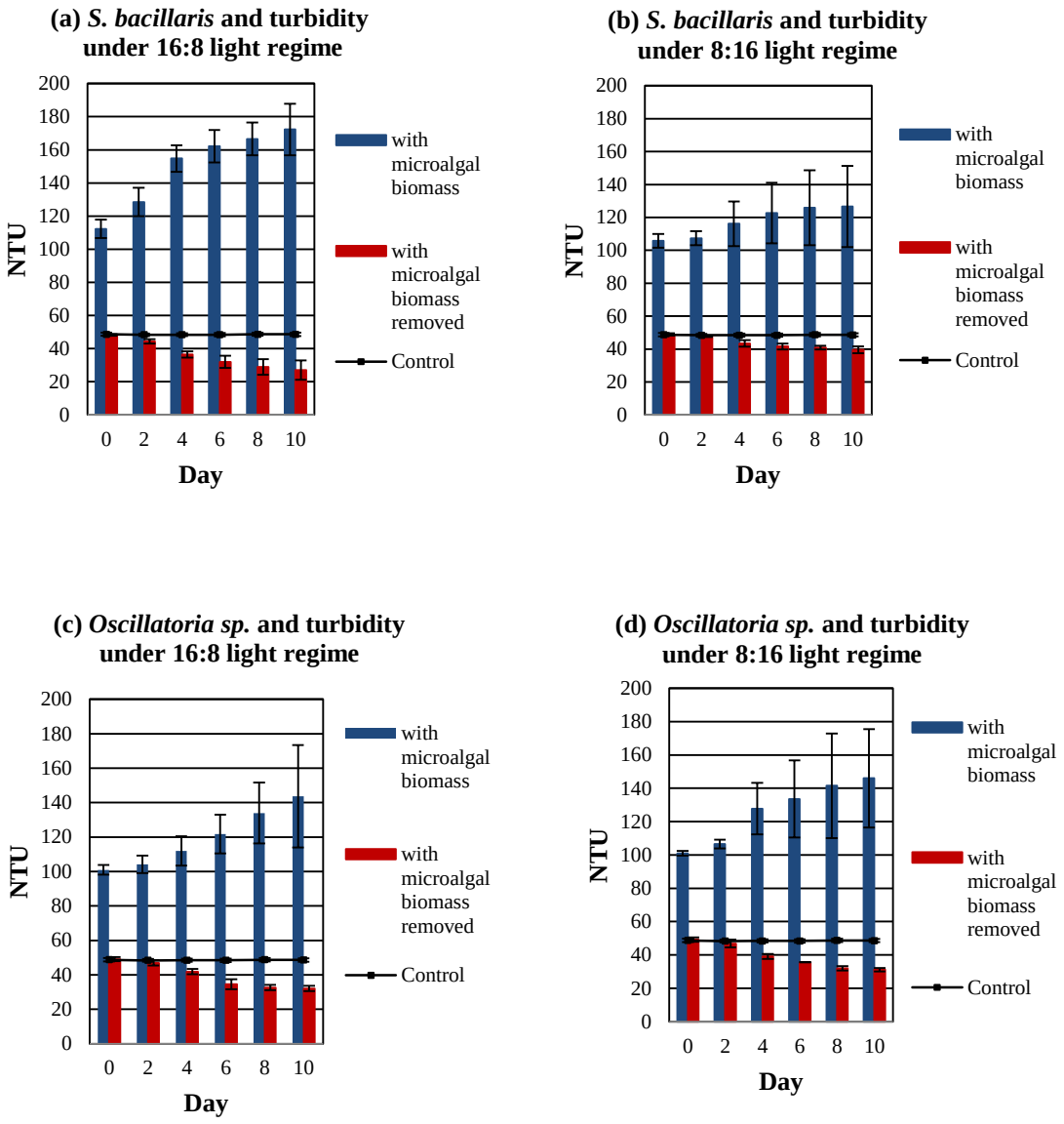


Figure 3.10 Turbidity levels in palm oil mill effluent during 10 days of cultivation of **(a)** *S.bacillaris* under 16:8 light; **(b)** *S.bacillaris* under 8:16 light; **(c)** *Oscillatoria sp.* under 16:8 light; and **(d)** *Oscillatoria sp.* under 8:16 light.

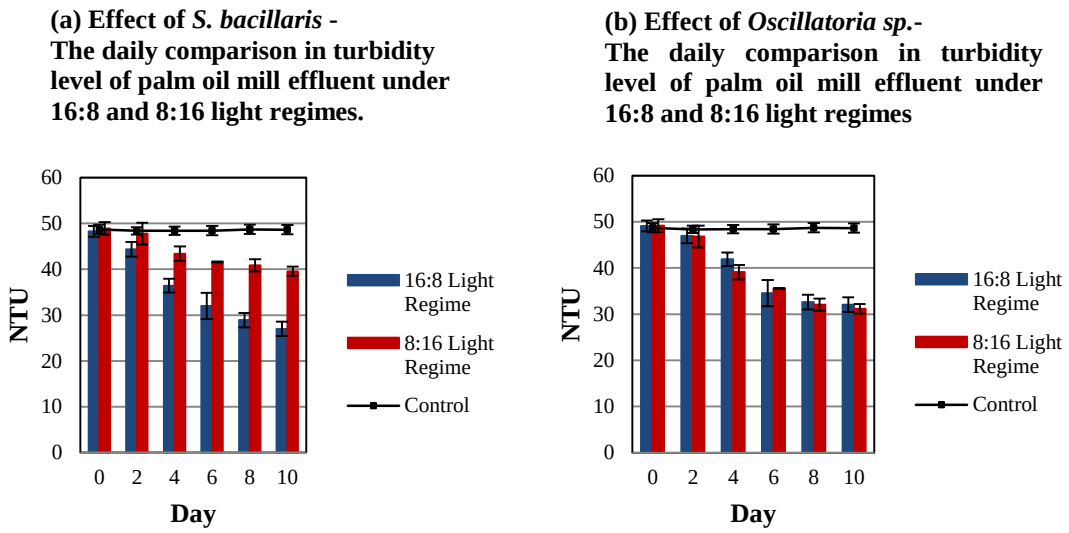


Figure 3.11 Comparison of daily turbidity level under 16:8 and 8:16 light regimes during the 10-day-cultivation of **(a)** *S. bacillaris* and **(b)** *Oscillatoria sp.*

Student *t*-tests were performed and showed that the turbidity levels of palm oil mill effluent were reduced significantly by both species under both light regimes (see Table 3.19). This also further indicates that pollutants were removed from POME by nutrient consumption for cell growth.

Table 3.19

t-test analysis of turbidity in palm oil mill effluent under different light regimes

Species	Light regime 16:8	Light regime 8:16
S.bacillaris		
(1) Difference	- 44.04 %	- 19.08 %
(2) *p*-value	0.013	0.0047
Oscillatoria sp.		
(1) Difference	- 34.69 %	- 36.64 %
(2) *p*-value	0.00435	0.00006

(1) Difference : percentage in increment or decrement of turbidity in palm oil mill effluent before and after treatment
(2) *p*-value : generated from the *t*-test of turbidity in palm oil mill effluent under specified light regime

CHAPTER 4

DISCUSSION

Phycoremediation employs microalgae species to biotransform pollutants, generate biomass for sustainable bioenergy, and potentially produce fish feed and protein sources. It also provides the basis for designing an alternative biological wastewater treatment system. The aim of this thesis was to investigate the growth of 6 microalgae species in industrial palm oil mill effluent under different light/dark cycle conditions to ascertain their potential suitability for efficient pollutant removal and turbidity reduction.

Table 4.1 tabulates previous works at both phycoremediation and bioremediation of wastewater to remove Chemical Oxygen Demand (COD) and total phosphate and nitrate. The selection of strains depends on the purpose of each research, their biochemical properties in the removal of organic pollutants, and their potential to remediate wastewater streams. Most of the previous work focused on green microalgae strains specifically the *Chlorella* species (Aziz & Ng, 1992; Wang *et al*, 2010; Li *et al.*, 2011) due to their higher lipid production relative to other green microalgae taxa. Their medium was domestic, municipal, or agricultural wastewater (Tables 1.3 and 4.1). It was selected due to its more uniform characteristics when compared to the variable constituents of industrial wastewater (Pittman *et al.*, 2011). For example a study by Aziz and Ng (1992) used a mixture of domestic and palm wastewater to investigate the growth and the ability to remove pollution load of *C. pyreinodosa*. Chinnasamy *et al*. (2010) reported a nutrient removal of up to 96% by cultivating the mixture of green microalgae species in 90% industrial effluent and 10% municipal wastewater with 64% higher of lipid to the biomass produced. Shushu and

Chipeta (2006) demonstrated that utilizing *Oscillatoria sp.* removed 100% of the orthophosphate content of domestic wastewater in 6 days, while Sengar *et al.* (2011) showed that *Oscillatoria sp* mixed with other chlorophyta strains removed 82% of COD and 100% of total phosphate and nitrogen in municipal wastewater over a period of 25 days. Su *et al.* (2011) demonstrated a higher COD removal using a mixture of cyanophyta species and bacterial community over a shorter period of cultivation. Microalgae species demonstrably perform better, effectively removing higher pollution load in wastewater than yeast strains.

Table 4.1

Previous works at the removal of organic pollutants

Species	Type of wastewater	COD removal	TP removal	N removal	Retention time	Reference
C. pyrenoidosa	Domestic & POME	82%	60%	70%	15 days	Aziz & Ng (1992)
A. platensis	Industrial	N.A.	99%	100%	15 days	Craggs *et al.* (1997)
Pichia sp.(1)	Pharmaceutical	60-64%	N.A.	N.A.	72 hrs	Recek & Raspor (1999)
Oscillatoria sp.	Domestic	N.A.	100%(2)	N.A.	6 days	Shushu and Chipeta (2006)
Hansenula sp.	*Shochu*	N.A.	95%	N.A.	24 hrs	Watanabe *et al.* (2008)
P. sydowiorum (1)	Chemical	66%	N.A.	55%	7 days	Kanekar *et al. (*2009)
Chlorella sp.	Municipal	83%	91%	82%	10 days	Wang *et al.* (2010)
Chlorella sp.	Municipal	91%	81%	89%	14 days	Li *et al.* (2011)
Cyanophyta + bacteria	Municipal	98%	64%	88%	8 days	Su *et al.* (2011)
Oscillatoria sp.+ mixed strains	Municipal	82%	100%	100%	25 days	Sengar *et al.* (2011)
O. amoena	Municipal	N.A.	92%	100%	N.A.	Xing *et al.* (2011)
S. cerviase + *K. lactis*	Diary	99.21%	N.A.	N.A.	36 hrs	Ghata *et al.* (2013)

N.A Not Available
(1) Yeast strain
(2) Ortophosphate

This thesis focused on less well-characterised taxa, including the exploitation of chlorophyta and cyanophya under 16:8 and 8:16 light regimes. The application of axenic strains i.e. *Stichococcus bacillaris* and *Oscillatoria sp.* in palm oil mill effluent in my research was also novel, as previous works investigated using mixture of microalgae species (with bacterial community) or a mixture of wastewater types. In comparison to the work of Aziz & Ng (1992) (Table 4.1), both strains in my thesis demonstrated higher pollution load removal (78%-85% COD removal; 82%-93% total phosphate removal; 73%-85% nitrate removal) within a cultivation period of 10 days. It also confirms that microalgae species efficiently remove the pollution load better than yeast strains. The results attained in this research could also contribute a wider perspective on phycoremediation technology by supporting the findings of previous work which indicated the utility of microalgae and also by extending our knowledge to two novel species.

4.1 GROWTH

The cultivation of microalgae as a phycoremediation agent with which to treat industrial wastewater offers considerable potential benefits as a low-cost and environmentally friendly means of bioremediation. Cell growth analysis is crucial to investigate the best medium for optimal cultivation. Studies of cell number based on optical density and complementary techniques, such as chlorophyll content and protein determination, are able to reveal the biomass growth of microalgae species under varied conditions. Results from these quantification methodologies can be used as part of the basis for scaling-up into the industrial and commercialization scales.

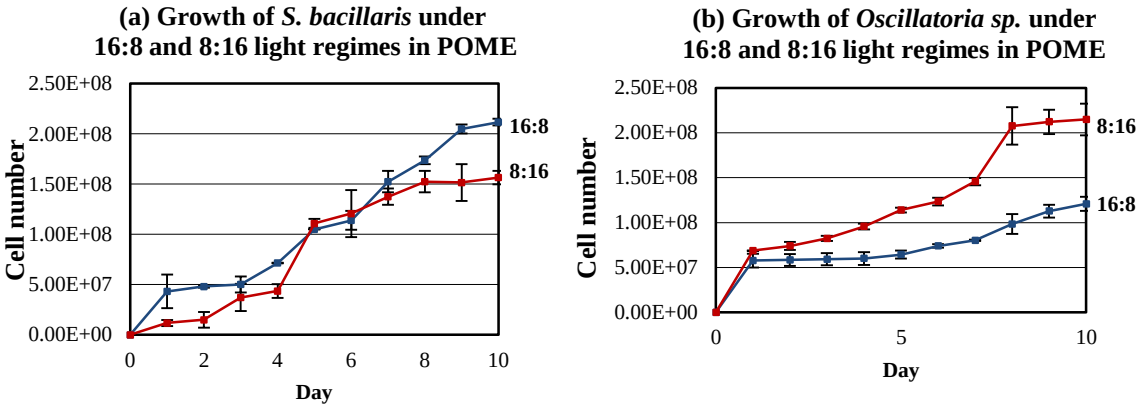
In a preliminary study the selected microalgae species were cultivated in a laboratory-scale photobioreactor under a controlled light supply. Among the selected species, only *S. bacillaris* and *Oscillatoria sp*. demonstrated growth under different light regimes i.e. 16:8, 8:16 and 0:24 in their optimal growth medium (Table 3.4). Additionally, both species also exhibited growth in Palm Oil Mill Effluent (POME). Cell deactivation occurs during light photosynthesis and provides evidences that both species are readily (photo)-heterotrophic (Janssen, 2002). This contributes novel information that *S. bacillaris* and *Oscillatoria sp*. were highly tolerant of the different salinity and pH levels found in POME. Previous works had only discussed the list of standard green and blue-green microalgae species due to the wider literature on their lipid content, as their main focuses were the extraction of oil. Since *S. bacillaris* and *Oscillatoria sp.* have been investigated less, there is limited knowledge of these two strains, especially regarding their ability to grow successfully or to bioremediate industrial wastewater. Therefore, this preliminary study has provided profound evidence that these two strains are able to adapt metabolically in palm waste under different light regimes.

In spite of their excellent osmoregulatory characteristics (John *et al*., 2002; Lee *et al*., 2008), the phototrophic behaviour of green microalgae *Brachiomonas submarina pulsifera*, *Tetraselmis sp*. and *Dunaliella salina* resulted in unsuccessful growth under a regime of total darkness in their optimal growth medium and palm oil waste (Table 3.4). In other hand, the biomass of *E. mutabilis* is reported to be denser in the early morning or early evening as their brilliant eyespots help aid cellular movement according to the light source (John *et al.*, 2002; Lee, 2008). Phang (1988) reported that the euglenophyta outgrew the chlorophyta species when were they were cultivated in 100 litres of digested palm oil mill

effluent during a shorter duration of sunlight (6 to 7 hours per day). In comparison to this research, *E. mutabilis* fell short of maintaining their growth under lower light regimes i.e. 8:16 and 0:24 (Table 3.4) and in the palm oil mill effluent (Table 3.5). This could possibly be due to the light exposure-to-area factor, which provided an inadequate environment for *E. mutabilis* to metabolise. Furthermore, despite of the nutrient-enrichment of palm waste and the toxic and metal compounds it contains, the environmental tolerance of this eukaryotic species could probably be observed better when left to grow and phycoremediate in different type of 'softer' wastewater i.e. domestic, municipal or agricultural wastewater rather than industrial effluent.

Cholorophyta *S. bacillaris*, a freshwater green microalga species, best multiplies vegetatively in an acidic medium. A very highly significant growth of *S. bacillaris (*89.95%) was detected under 16:8 light in palm waste compared to that in the control, with an increment of 87.72% under 8:16 light. The total chlorophyll content of *S. bacillaris* in POME under 16:8 light and 8:16 light was found to be significantly higher than in the optimal growth medium by 63.62% and 63.17% respectively. Total chlorophyll analysis further suggests that *S. bacillaris* procreates better under higher light exposure better in palm oil mill effluent. According to Ruiz-Marin *et al*. (2010), the chlorophytic unicellular microalgae are tolerable to many wastewater conditions. Palm oil mill effluent, known to be high in nutrients enables the chlorophyta *S. bacillaris* to proliferate. Furthermore, protein extracts of *S. bacillaris* also showed greater cell growth in palm waste than those in the control medium both under 16:8 light by 57.1%, and 8:16 light by 65.66%. Hypothetically, the cell growth of *S. bacillaris* could be due to the consumption of abundant nutrients in palm waste as well as the photoheterotrophic characteristic.

Therefore, a higher light cycle would generate higher cell vegetative reproduction when *S. bacillaris* was employed in the acidic palm oil mill effluent. Higher growth in palm waste compared to the control medium further suggested that a chlorophyta strain requires high light exposure to sustain their growth metabolism.



Figures 4.1 (a) and (b) Cell growth of *S. bacillaris* and *Oscillatoria sp.* under different light regimes in POME

When cyanophyta *Oscillatoria sp.* was cultivated in palm waste for 10 days the cell growth was found to be increased under both light regimes, with a higher percentage difference under 8:16 light, in comparison to the growth in the control medium (Figure 3.2(a-b) and 4.1(b)). Although the alkaline BG11 medium enables optimal growth for *Oscillatoria sp.*, the acidic palm oil mill effluent provided an adequate environment. When cultivated in POME, a higher cell number was detected under 8:16 light regime than 16:8 light, by 43.75%. Cells of cyanophyta *Oscillatoria sp.* were deactivated by the light of photosynthetic reactions to reduce photoinhibition as the defensive mechanism and to allow photopigment stimulation. Markou and Georgakakis (2011) suggested that such a complex

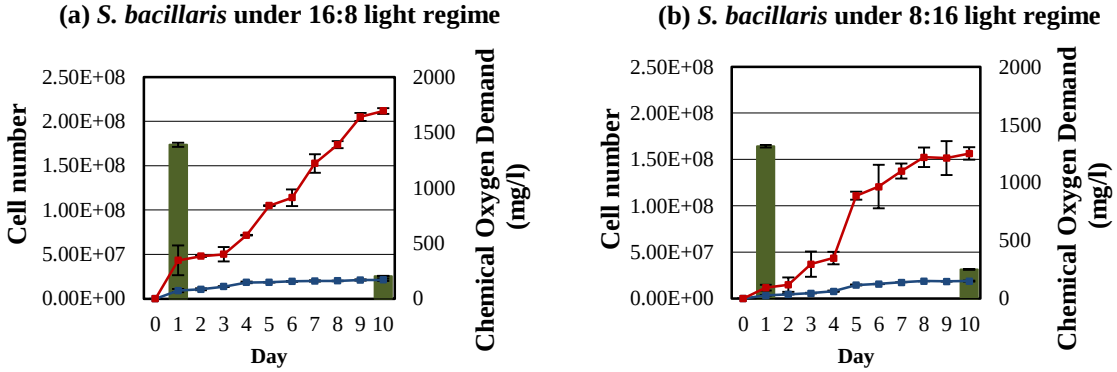
biochemical pathway is supported by carbon fixation which causes a gradual rise in pH, and thus provides a sufficient surrounding for the growth of cyanophyta.

In comparison to the chlorophyta strains which contain chlorophyll *a*, *b*, *c1*, *c2* and *c3*, the photopigmentation of cyanophyta predominantly contains chlorophyll *a* (a green-blue pigment). Cyanophyta absorb light and store the energy in the form of mobilised polysaccharide glycogen. Chlorophyll *a* and other pigments such as phycoerythrin and phycocyanin, which are embedded on photosynthetic lamellae utilise carbon through the Calvin Cycle (Tiwari and Pandey, 2012). My study supported this hypothesis, showing the higher chlorophyll content of cyanophyta after the cultivation in palm waste compared to the control medium, with an increase of 40.8% under the 8:16 light regime and 12.26% under 16:8 light. The presence of sufficient organic substrates in palm waste provided energy and carbon sources during dark cycles. Furthermore, the increasing cell mutual shading effect and avoidance of photoinhibition also contributes to the reduction of growth. A lower light/dark cycle regime therefore provides a suitable environment for *Oscillatoria sp.* to enhance growth up to the maximum point of light saturation (Markou and Georgakakis, 2011). *Oscillatoria sp*. also showed a higher protein % w/w under the 8:16 light regime by 25.53% in palm oil mill effluent compared to the control and 23.57% under 16:8 light. This further suggests that cyanophyta grow best under a lower light regime.

4.2 REMOVAL OF POLLUTION LOAD

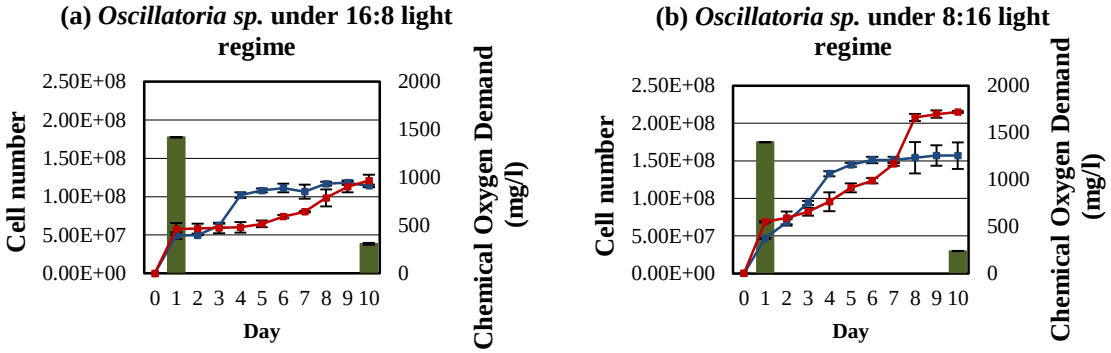
The high concentration of organic load opens up possibilities to utilise palm oil mill effluent in providing nutrients for microalgae cultivation. The consumption of such compounds serves not only to biologically remediate industrial wastewater, but it also produces microalgal biomass that could be recovered for several purposes, including for bioenergy and food production. The level of Chemical Oxygen Demand (COD), total phosphate, nitrate, and turbidity were assessed and discussed as followed.

The high organic compound content of palm oil mill effluent provides abundant nutrients such as microalgal growth, such as carbon. The removal of COD indicates the amount of organic matter that has been consumed by the species, as well as delivering direct information on the wastewater quality.



Figures 4.2 (a) - (b) Reduction of Chemical Oxygen Demand (COD) by *S. bacillaris* after 10 days of cultivation in Palm Oil Mill effluent (POME) under different light regimes. The red and blue lines represent growth of cell number in POME and the control respectively, whilst the green bars represent COD levels.

Chlorophyta *S. bacillaris* reduced COD level after 10 days of remediation. Very highly significant reductions of 85.31% under 16:8 light and 80.88% under 8:16 light were detected and proportionately decreased in relation to the increment of the cell number produced (Figures 4.2 (a) and (b)). The cells consumed the organic compound during light/dark cycles, with preference towards the higher light regime, dictates that *S. bacillaris* metabolised photoheterophically.



Figures 4.3 (a) - (b) Reduction of Chemical Oxygen Demand (COD) by *Oscillatoria sp.* after 10 days of cultivation in Palm Oil Mill effluent (POME) under different light regimes. The red and blue lines represent growth of cell number in POME and the control respectively, whilst the green bars represent COD levels.

The same pattern of reduction was also shown by cyanophyta *Oscillatoria sp*. under the applied light regimes. In comparison to 16:8 light, 82.9% more of the COD was removed under the 8:16 light regime, indicating that *Oscillatoria sp.* feasibly proliferated with preference to lower light exposure. This result supports the review by Markou & Georgakakis (2011) that cyanophyta consume organic compounds during a dark cycle by deactivating the light photosynthetic reactions to reduce photoinhibition as well as to allow photopigmention for biomass growth.

From previous works, green microalgae i.e. the Chlorella species, were reported to remove up to 91% COD from municipal wastewater within 10-14 days of the cultivation period (Wang *et al*., 2010; Li *et al.*, 2011), whereas *Oscillatoria sp.* mixed with other strains removed 82% of COD from municipal wastewater (Senger *et al.*, 2011) within 25 days. Aziz and Ng (1992) demonstrated that the green Chlorella species were able to remove 82% of COD from the mixture of palm waste and domestic effluent. In comparison, the axenic green *S. bacillaris* and blue-green *Oscillatoria sp*. used in this research were more effective in removing COD from pure highly contaminated industrial palm oil mill effluent within 10 days of the cultivation period.

Furthermore, the Mogden Formula covers the costs of industrial effluent treatment prior discharge. The main factors in the formula included COD, but due to other pollutants, such as phosphate, the scheme has also introduced additional add-ons to put more cost pressure onto wastewater treatment industries. Many companies use the formula to calculate the potential cost savings from producing less wastewater that is cleaner and easier to treat (WRAP, 2013). This dictates how important the evaluation of COD removal by microalgae species is, which could reduce the external cost for effluent treatment prior to discharge.

In order to incorporate phycoremediation and the Mogden Formula, further analysis of phosphorus is essential. Phosphorus is utilised by microalgae in the form of orthophosphate. The formation of phosphate provides an increase in pH (Corell, 1998), consequently made palm oil mill effluent sensible for the growth of both microalgae species. The utilization of phosphate is energy dependent and the rate is slower under the dark regime (Falkner *et al*., 1980; Rigby *et al*., 1980; Ritchie *et al*., 1997). It was shown that *S. bacillaris* and *Oscillatoria sp.* removed total phosphate under 16:8 light by 93.07%

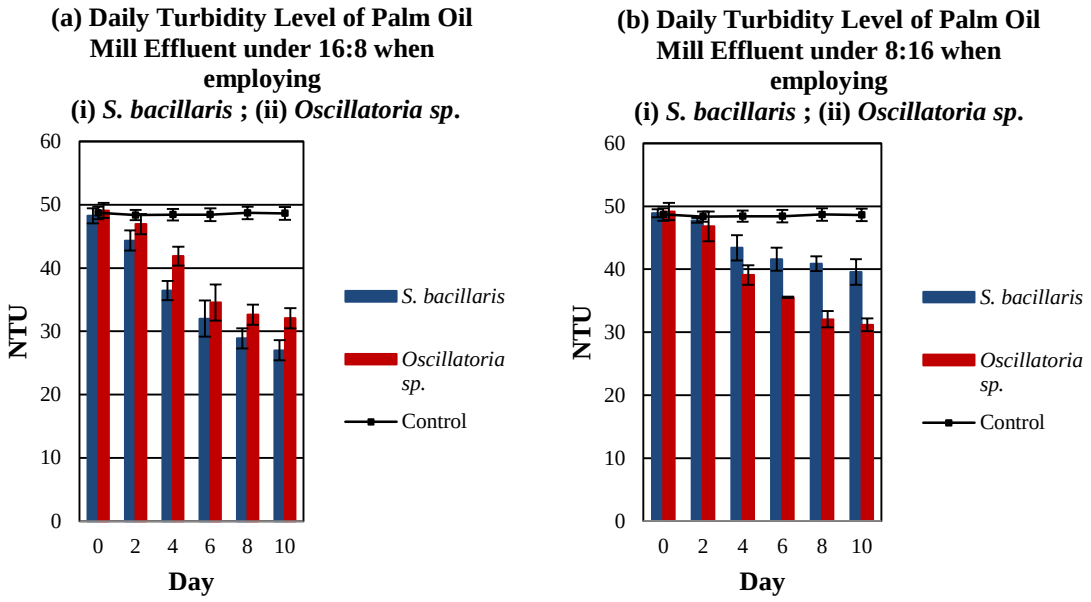
and 88.25% respectively. In comparison, in this research green *S. bacillaris* performed more effectively than those green microalgae species which were investigated by Aziz & Ng (1992); Wang *et al.,* (2010) and Li *et al.,* (2011). Despite the 100% TP removal by *Oscillatoria sp.* cited by Senger *et al.*, 2011, their work was executed with the mixture of other microalgae strains and was cultivated in municipal wastewater.

Nitrate is attained by a reduction process of which nitrogen in palm oil mill effluent is catalysed by the enzyme nitrogenase (Stal, 2000). In this research, the uptake of nitrate is light dependent, which explains its higher removal under 16:8 light compared to 8:16 light by *S. bacillaris* (78.14%) and *Oscillatoria sp*. (85.22%). Nitrate seems to be more important for the growth of cyanophyta (Pouliot *et al*., 1989) due to their diazotrophic characteristic – the capability of utilizing elemental nitrogen as sole nitrogen source (Benemann, 1979). Hence, *Oscillatoria sp*. was able to remove nitrate from palm waste to a higher degree than *S. bacillaris*. In comparison to Aziz & Ng (1992), green *S. bacillaris* was able to remove a higher percentage of nitrates than that by green *Chlorella* species. Craggs *et al*. (1997) indicated that *Oscillatoria sp.* were able to remove 100% Total Nitrate from municipal wastewater when cultivated in a continuous reactor for 14 days. Furthermore, there was the matter of 100% TN removal from municipal wastewater demonstrated by *Oscillatoria sp*. within 25 days, which was recorded by Senger *et al.*, 2011. In comparison to my research, *Oscillatoria sp.* showed greater efficiency in removing TN from industrial effluent after 10 days of remediation.

The reduction of organic carbon, phosphate and nitrate-nitrogen demonstrated that chlorophyta and cyanophyta consumed these nutrients from palm waste as part of their metabolic process, either by nutrient assimilation or precipitation.

4.3 TURBIDITY

Clarity of water is important, mainly for safety, but also for aesthetic reasons. Turbidity can be used to monitor water quality. When investigating the growth of cultivated *S. bacillaris* and *Oscillatoria sp.* in POME, a turbidity test determined the quality of the discharged palm oil mill effluent.



Figures 4.4 (a) and (b) Daily turbidity level in palm oil mill effluent after 10 days of cultivation of *S. bacillaris* and *Oscillatoria sp.* under different light regimes.

The turbidity level of industrial palm oil mill effluent after the cultivation of *S. bacillaris* was found to be very significantly reduced by 44.04% under the 16:8 light regime and 19.08% under the 8:16 light regime after 10 days of treatment. It was proven by the experimental analysis that between Days 2 and 4, the turbidity improved the most (by 17.9 % and 9.2% under 16:8 and 8:16 light regimes respectively), which also implies the period

when the cell growth was the highest i.e. the number of cells present was directly proportional to the reduction in turbidity. In comparison, *Oscillatoria sp*. showed rather constant efficiency in improving the turbidity of POME. The turbidity was reduced by 34.69% under 16:8 light and 36.64% under 8:16 light.

The turbidity of POME was reduced by 99.45% by the natural organic polyelectrolyte derived from chitin (A. Hassan *et al*., 2009). In my research, the free floating microalgae species was able to reduce turbidity by up to 44%. Another possibility to improve the efficiency of these novel species in reducing turbidity is by immobilizing the cells within the polymers using microencapsulation or microbead methods.

CHAPTER 5

CONCLUSION

In 2009, 43.8 million m^3 of palm waste was generated by Malaysia's palm oil mill industry (Malaysian Palm Oil Board, 2010) and this amount is predicted to increase due to a continuous demand for palm oil. The effluent generated contains high levels of pollution and can potentially damage both aquatic life and ground water supplies. The current conventional palm waste treatment is energy intensive, so there is a need for alternative clean-up technologies. Phycoremediation technology potentially provides an economical, sustainable, and environmentally-friendly option.

The overall aims of this study were to investigate the growth characteristics of microalgae species in industrial Palm Oil Mill Effluent (POME) under different light/dark cycle regimes, and also to evaluate which species have the potential to mitigate the harmful effects of the industrial effluent. Experiments were designed to explore the capacity of chlorophyta *S. bacillaris* and cyanophyta *Oscillatoria sp.* to remediate POME, given their ability to grow successfully in the optimal growth medium and palm oil mill effluent under different light regimes. Assessments consisted of comparative studies of the cultivated microalgae species in both the control medium and POME under the different light regimes, including measuring optical density for cell number quantification, spectrophotometric chlorophyll extraction, and the Bradford Assay for protein determination. Pollution load assessments were also performed, such as measuring the removal of Chemical Oxygen Demand (COD) and total phosphate and nitrate, as well as

the reduction of turbidity in Palm Oil Mill Effluent. The following points summarise the conclusions of this research:

- Modification of the light regime has the potential to optimise microalgae growth performance and can also augment the chlorophyll and protein contents. In the preliminary study, among the 6 selected microalgae species only *S. bacillaris* and *Oscillatoria sp*. demonstrated good growth in the control medium and POME under the applied photoperiods: *S. bacillaris* exhibited significant growth in POME under the 16:8 light regime with 26.09% (*p*-value = 0.0005) more growth than under the 8:16 light regime, whereas *Oscillatoria sp*. reproduced more efficiently under the 8:16 light regime than under the 16:8 light regime in POME, displaying 43.75% more growth (*p*-value = 0.000546). Both chlorophyta *S. bacillaris* and cyanophyta *Oscillatoria sp.* showed no growth when cultivated in POME under the 0:24 light regime. The significant increase in chlorophyll and protein content of both *S. bacillaris* and *Oscillatoria sp.* under both 16:8 and 8:16 light regimes also demonstrates the ability of both species to proliferate in POME under these conditions.

  The photoperiod study confirmed the ability of the microalgae species to adapt in order to grow in POME. The light and dark conditions respectively reduced and oxidised the larger pool of membrane soluble plastiquinone molecules, which are responsible for driving the photosystems (Matthijs *et al*., 1994). It is also thought that energy-rich compounds are stored during the light period to be utilised during the dark period (Pirt, 1983), allowing the microalgae species to metabolise under the exploited parameters. An overdose of light energy can lead to the production of toxic species and damage to

the photosystem (Vacha, 1995); light alternation offers light-shading to prevent the cells being bleached, as well as allowing photopigmentation for biomass growth.

- The different photoperiods were shown to have different and significant influences on the microalgae growth. Further assessment included evaluating the remediation of POME by the microalgae species under the different light regimes. The high organic compound content of POME provided abundant nutrients for microalgal growth, such as carbon, phosphate, and nitrogen.

  Chlorophyta *S. bacillaris* reduced COD level in POME after 10 days of remediation by 85.31% (*p*-value = 0.000027) under 16:8 light and 80.88% (*p*-value = 0.000023) under 8:16 light. The COD reduction was also demonstrated by cyanophyta *Oscillatoria sp*. under the applied light regimes. In comparison to 16:8 light, 82.9% (*p*-value = 0.0000015) more COD was removed under the 8:16 light regime, which further indicates that *Oscillatoria sp.* perform better in low light regimes. The results demonstrate that both *S. bacillaris* and *Oscillatoria sp.* were able to grow under the applied photoperiod by utilizing the organic carbon contained in POME. The reduction of COD confirmed that microalgae species were able to remove carbon-based organic matter in POME. This suggests that it was not reduced due to the photosynthetic carbon fixation; the organic carbon was utilised by the microalgae species for growth.

  Nitrogen, on the other hand, can be utilised as nitrate by means of a reduction process in which the enzyme nitrogenase serves as the catalyst (Stal, 2000). Nitrate is more important for the growth of cyanophyta due to their diazotrophic characteristics i.e. their possession of the ability to utilise elemental nitrogen as a nitrogen source

(Benemann, 1979; Pouliot *et al*., 1989). In Chapter 3.5 the uptake of nitrate was shown to be light dependent, which explains the greater removal by both *S. bacillaris* and *Oscillatoria sp*. under 16:8 light when compared to 8:16 light (by 78.14% (*p*-value = 0.00037) and 85.22% (*p*-value = 0.00029) respectively).

S. bacillaris and *Oscillatoria sp.* also significantly removed total phosphate under both light regimes. Inorganic phosphate is another key factor in the metabolism and growth of algae; phosphates in wastewater are transferred by energised transport across the plasma membranes of the algal cells (Cai *et al*., 2013). During cell metabolism, phosphate in the forms of $H_2PO_4^-$ and HPO_4^{2-} is incorporated into organic compounds through phosphorylation, which involves the generation of Adenosine Triphosphate (ATP) from Adenosine Diphosphate (ADP) by means of a form of energy input; this energy comes from the oxidation of respiratory substrates and the electron transport system of the mitochondria (Martinez *et al.*, 1999). This explains how phosphate was effectively removed from POME and consumed by the microalgae cells.

The results of this study further confirmed that phycoremediation is an environmentally amenable and sustainable solution for pollutant removal. The reduction of organic carbon, phosphate, and nitrate-nitrogen demonstrated that chlorophyta and cyanophyta recovered and immobilised these nutrients in POME as part of their metabolic growth processes. The ability of microalgae species to recover resources from industrial wastewater opens up manifold opportunities: for example, the nitrate and phosphate recovered by the microalgae can be used to produce protein-rich animal feed and low-cost fertilisers.

- The turbidity of Palm Oil Mill Effluent after the cultivation of *S. bacillaris* was found to be reduced by 44.04 % (*p*-value = 0.013) under 16:8 light and 19.08% (*p*-value = 0.0047) under the 8:16 light regime after 10 days of treatment. *Oscillatoria sp*. demonstrated consistent rates of turbidity reduction, whereby 34.69% (*p*-value = 0.00435) of turbidity was reduced under 16:8 light and 36.64% (*p*-value = 0.00006) under 8:16 light.

  The reduction of turbidity under both light regimes verified that both chlorophyta and cyanophyta were able to improve the aesthetic qualities of the brown industrial effluent. It also provided further evidence for a relationship between turbidity reduction and pollution load removal, in which nutrients in POME were removed, consumed, or captured by microalgae during cell metabolism. This further suggests that microalgae could play an increasingly significant role for the recovery of resources from wastewater.

5.2 FUTURE WORK

Conventional treatment of POME using pond systems has several unavoidable flaws, such as the irrepressible emission of methane and carbon dioxide from the treatment process and the requirement of both long hydraulic retention time and a vast amount of land mass (Rupani *et al.,* 2010). Furthermore, the existing physicochemical treatments of POME using membrane technology, flocculation, and coagulation are extremely expensive. Phycoremediation offers potential sustainable approaches for designing new biological wastewater treatment systems, coupled with facilitating biomass generation. Further assessment is therefore important to provide comprehensive information in order to design optimal parameters for maximum output at minimum cost.

Wastewater can be used as a sustainable medium for effective algal growth, in which the microalgae can utilise the abundant organic carbon, inorganic nitrogen, and phosphorus resources (Mallick, 2002). Phycoremediation offers efficient recycling of nutrients and production of biomass that can be used as protein-rich animal feed or low-cost fertilisers. The process also reclaims phosphorus, making it a desirable technology given the scarcity and high price of this mineral (Shilton *et al*., 2012). Further research is required to enhance our understanding of how to maximise phosphorus recovery using microalgae species, which could become a key solution in the future.

Other benefits of applying microalgae to wastewater include the removal of heavy metals and the production of renewable biofuel. In the first instance, microalgae can be applied as an inexpensive and sustainable biofilter to remove heavy metals from wastewater (Gonzales *et al*., 1997; Zhang, 1997; Matsunaga *et al*., 1999; Wilkie and Mulbury, 2002;

An *et al*., 2003). Analysis of biosorption and bioaccumulation of heavy metals can be detected using the Atomic Absorption Spectrophotometer (AAS). The production of microalgae biomass by means of phycoremediation technology also offers a viable means for the generation of renewable biofuel, such as Biomass to Liquid (BtL), which can be obtained using the Fischer-Tropsch Process, flash pyrolysis, or catalytic depolymerization. Additional research in the field of genetic engineering is required to facilitate and accelerate the synthesis of microalgae strains which can produce greater amounts of biomass and lipid, while the study of the photosynthetic electron transport system using a mass spectrometer is necessary for further assessment of chlorophyll and protein content.

Phycoremediation is also possible using an engineered algal-bacterial culture (de-Bashan, 2002). After being exposed to light, microalgae produce oxygen (O_2) which can stimulate aerobic degradation in bacteria; conversely the microalgae also consume the carbon dioxide (CO_2) released from the respiration of the bacteria, which also contributes to CO_2 mitigation (Oswald, 1988; Munoz and Guieysse, 2006). The mass transfer of O_2 and CO_2 negates the need for mechanical aeration at a high operational cost (Mallick, 2002). Another advantage of an algal-bacterial culture is that more nitrogen and phosphorus can be absorbed into the algal-bacterial biomass during the treatment process (Su *et al*., 2011). Munoz (2005) demonstrated that 53% of $N - NH_4^+$ from acetonitrile was assimilated into algae and bacterial biomass compared to only 26 % of $N - NH_4^+$ assimilated by the bacteria. These processes produce algal-bacterial biomass, which, when accumulated after treatment, has the potential to be used as agricultural fertiliser (Mulbury *et al*., 2005, Su *et al*., 2011). Pursuing this approach will provide new insights into algal-bacterial culture

enrichment strategies and supplement the available information on diversity in such cultures and also on microbial ecology (Su *et al.*, 2011).

Further work may also include research into the immobilisation of microalgae cells: there is a major limitation in harvesting microalgae biomass after cultivation, which constitutes a potential wastage problem. The immobilization method has been found to be an economical and practical way of managing microalgae cultures, which is usually labour intensive and time consuming (Moreno-Garrido, 2008). An immobilisation system is able to trap microalgae species within polymer matrices due to their pores being smaller than the cells' structures; the medium flows through the system, sustaining the metabolism and eventual growth of the cells. Work on this system would involve the preparation of microbeads or other means of microencapsulation, the evaluation of microalgae biomass, and the qualitative assessment of wastewater after the treatment in-line with the original aims of this thesis.

The implementation of phycoremediation on an industrial and commercial scale is relatively new. Therefore, Life Cycle Assessment (LCA) could provide a notional study to evaluate its potential economic and environmental impact. LCA also aims for change and improvement, either directly, through decision-making, or in more indirect ways, such as through influencing market behaviour or identifying improvement possibilities (Baumann *et al*., 2004). Software tools are available for LCA studies, such as SimaPro 7 and GaBi 6, and, in addition to these, the *International Journal of LCA* provides a platform for researchers to follow the trends in LCA development.

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