

APPLIED PHOTOPERIODS FOR THE STUDY OF PHYCOREMEDIATION

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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 (6) microalgae species, *Stichococcus 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. Besides the importance of applied photoperiods for 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 techno-economic advantages of phycoremediation and the key parameters dictating pollution load reduction.

Introduction

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 consisting 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.

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.

Literature Review

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.

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.

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 170m³/ha in comparison to 100-130 m³/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 (2) 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).

Despite 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.

Aims 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 utilisation 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).

To this end, the aims and objectives 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.

Methodology

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 utilisation 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.

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 this research, six (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 1.1** summarises the strains chosen for this research.

Table 1.1

Summary of the selected microalgae species

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

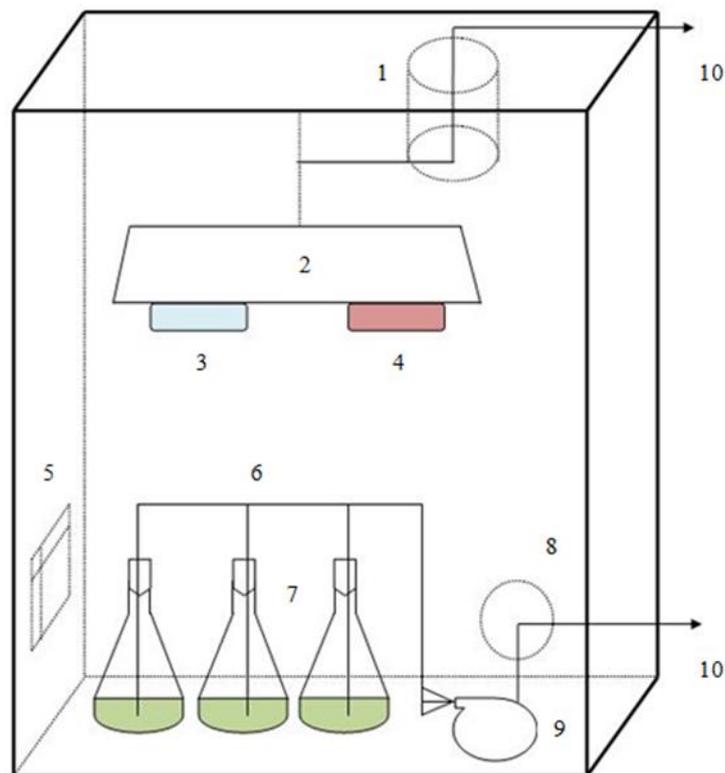
Six (6) microalgae species were selected and purchased from The Plymouth Algal Culture Collection of The Marine Biological Association (MBA), U.K., and Culture Collection Algae and Protozoa (CCAP), Scotland. Stock cultures remained in non-aerated isolation in lit conditions and under low temperature before being inoculated into the specified growth

medium as a starter culture. Besides the optimal growth medium for each species, three (3) main physical parameters were taken into consideration for inoculation: light source, temperature, and aeration (air/ CO_2). The cultures were maintained for between one (1) and two (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).

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. 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).

Figure 1.1 *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 Erlenmeyer 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

Optimal Growth Medium

Cultures are maintained in order to preserve healthy populations, which are physiologically, morphologically, and genetically representative. The growth medium provides a nutrient-rich environment for the microalgae.

Four (4) 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.

Industrial Wastewater

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 (5) 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 1.2**.

Table 1.2 *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 1.3 summarises the methods used to evaluate changes of parameters in POME in order to investigate the reduction of pollution load within POME.

Table 1.3 *Methods of test 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

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. The growth curves for each species within the standard growth medium were generated before those for the species when applied under different parameters.

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 analysed 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 analysed. Readings were again done in triplicate. The growth curves of *S. bacillaris*, *B. submarina pulvisifera*, *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. Growth of each selected microalgae species in POME was also determined using the same methods.

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*, *c1*, *c2*, *c3*, and *d* pigments, can be used as a standard basis to represent the productivity of microalgae species.

The primary photosynthetic pigments, Chlorophyll *a* (Chl *a*) and/or Chlorophyll *b*, *c1*, *c2*, or *c3* pigments, can be used as a standard basis to represent the productivity of microalgal biomass. In this research, two (2) ml of each culture sample was withdrawn daily, and centrifuged at 3,000 rpm for 10 minutes. When cells were pelleted, the supernatant, which had become fairly clear, was pipetted off, avoiding disturbance of the pellet. Cells were then re-suspended in two (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 (2) 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:

$$\text{Chlorophyll } a \text{ extract} = (11.85 * A_{664} - 1.54 * A_{647} - 0.08 * A_{630}) * l$$

$$\text{Chlorophyll } b \text{ extract} = (21.03 * A_{647} - 5.43 * A_{664} - 2.66 * A_{630}) * l$$

$$\text{Chlorophyll } c \text{ extract} = (24.52 * A_{630} - 1.67 * A_{664} - 7.6 * A_{647}) * l$$

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

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

The total concentration of chlorophyll in the sample ($\mu\text{g}/\text{ml}$) was obtained by adding up the chlorophyll *a*, *b*, and *c* samples:

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

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

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). 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).

For this research, 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 Coomassie 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 analysed using a Magellan 6 v6.4. The results obtained were plotted using MS Excel.

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 -80°C 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 spun 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):

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

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).

Turbidity of POME

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 research, the turbidity of POME 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).

Results and Discussion

This research focuses on less well-characterised taxa, including the exploitation of chlorophyta and cyanophyta under different light period ie16:8, 8:16, and 0:24 light regimes. The application of most axenic strains in palm oil mill effluent in this research is 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), the dominant strains in this thesis i.e. *Stichococcus bacillaris* and *Oscillatoria sp.* demonstrated growth only under the presence of light ie 16:8 and 8:16, 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 these two novel species.

Growth

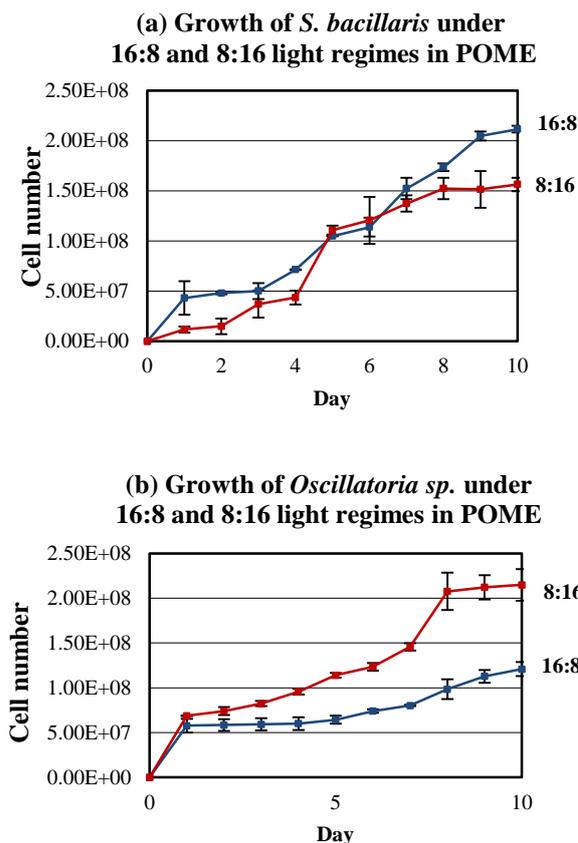
The cultivation of microalgae as a phycoremediation agent with which to treat industrial wastewater offers considerable potential benefits as a low-cost and environmentally friendly means of bioremediation. Cell growth analysis is crucial to investigate the best medium for optimal cultivation. Studies 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 commercialisation 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. 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 both 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. 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 and in the palm oil mill effluent. 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.

Chlorophyta *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.



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. 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). This research 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.

The student *t*-test was used to make pair-wise comparison of the differences in growth ie cell number, chlorophyll content, and protein content between *S. bacillaris* and *Oscillatoria sp.* in palm oil mill effluent and the control medium under each light regime.

Table 1.2 *t*-test analysis of cell number

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

Table 1.3 *t*-test analysis of chlorophyll content

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

(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

Table 1.4 *t*-test analysis of protein content

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

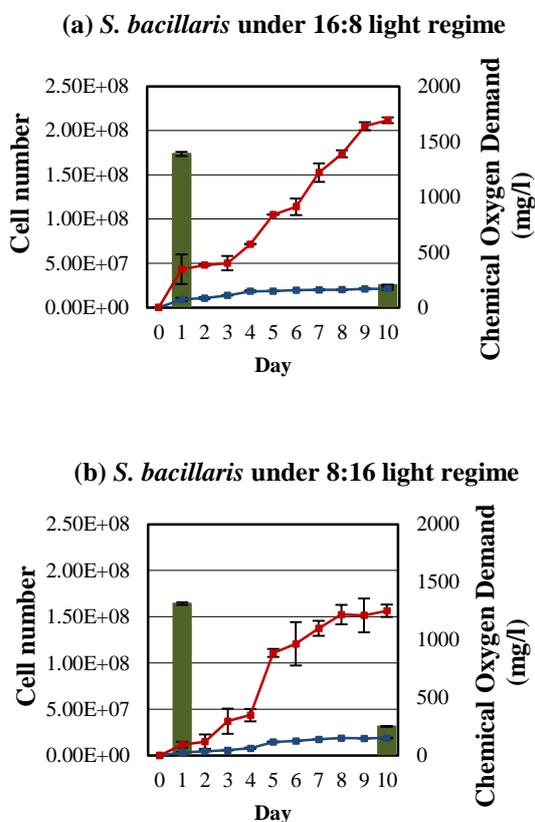
(1) Difference: percentage in increment or decrement of cell number at the end-of-point between POME and control
(2) *p*-value: generated from the *t*-test of cell number in optimal growth medium and palm oil mill effluent

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.

Figure 1.3 *Reduction of Chemical Oxygen Demand (COD) by S. bacillaris after 10 days of cultivation in Palm Oil Mill effluent (POME) under different light regimes.*

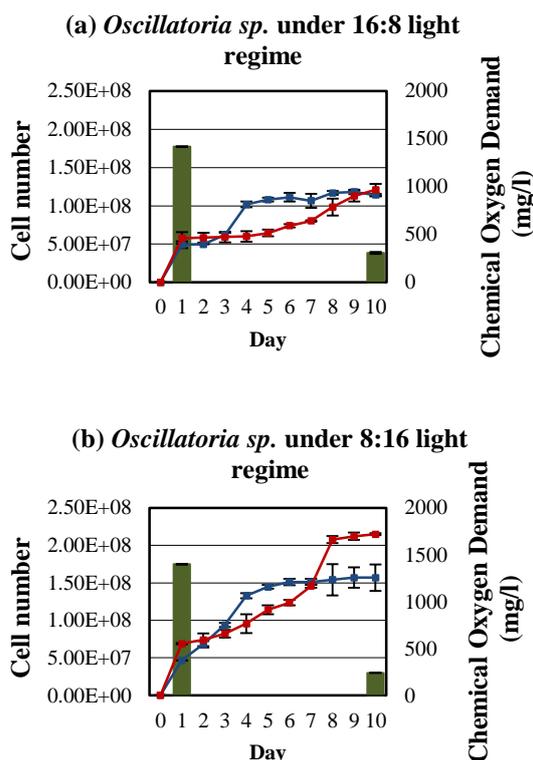


The red and blue lines represent growth of cell number in POME and the control respectively, whilst the green bars represent COD levels.

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. The cells consumed the organic compound during light/dark cycles, with preference towards the higher light regime, dictates that *S. bacillaris* metabolised photoheterotrophically.

Figure 1.4

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 photopigmentation 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 utilisation 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.

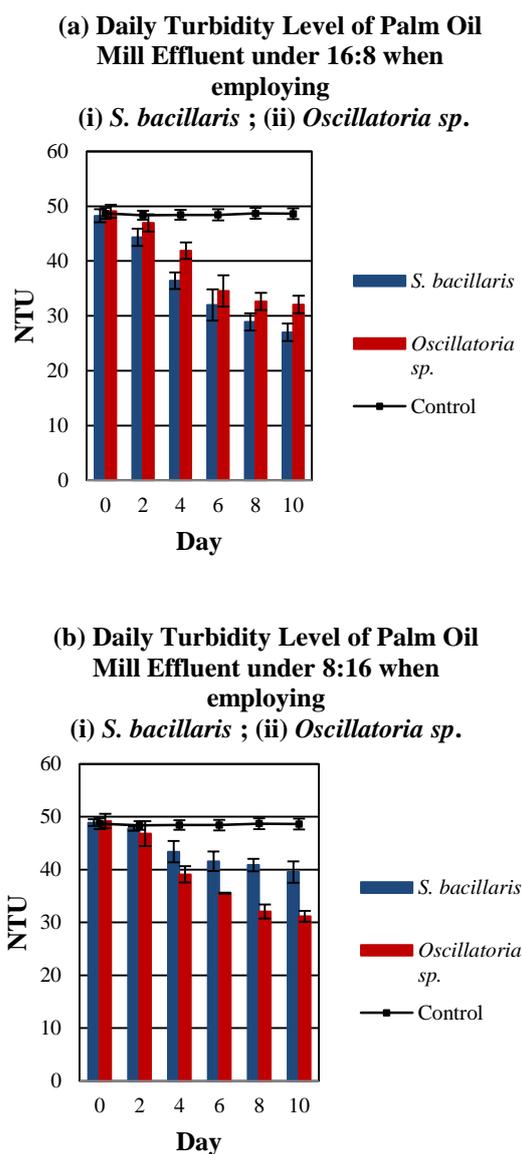
Nitrate is attained by a reduction process of which nitrogen in palm oil mill effluent is catalysed by the enzyme nitrogenase (Stal, 2000). In this research, the uptake of nitrate is light dependent, which explains its higher removal under 16:8 light compared to 8:16 light by *S. bacillaris* (78.14%) 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 utilising 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.

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.

Figure 1.5 Daily turbidity level in palm oil mill effluent after 10 days of cultivation of *S. bacillaris* and *Oscillatoria sp.* under different light regimes.



(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.

Conclusion

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 six (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 plastoquinone 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). 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.

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