UNIVERSITI PUTRA MALAYSIA

INVESTIGATION ON THE USE OF BLUE AND YELLOW LEDS FOR GROWTH, PROXIMATE COMPOSITION ENHANCEMENT AND MORPHOLOGY OF MARINE MICROALGA *Isochrysis* SP.

NORHAYATI BINTI SUHAIMI

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FACULTY OF VETERINARY MEDICINE

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By

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CERTIFICATION

It is hereby certified that I have read this project paper entitled '**Investigation on the use of blue and yellow LEDs for growth, proximate composition enhancement and morphology of marine microalga *Isochrysis* sp.**'.by Norhayati Binti Suhaimi and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999 - Project.

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ABSTRAK

Abstrak kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD4999 – Projek

PENYIASATAN KE ATAS PENGGUNAAN DIOD PEMANCAR CAHAYA BIRU DAN KUNING UNTUK PERTUMBUHAN, KOMPOSISI PROKSIMAT DAN MORFOLOGI DALAM MIKROALGA LAUT *Isochrysis* sp.

NORHAYATI BINTI SUHAIMI

2016

Penyelia: Prof. Dato' Dr. Mohamed Shariff Bin Mohamed Din

Penyelia bersama: Dr. Sanjoy Banerjee

Mikroalga ialah organisma yang memerlukan cahaya sebagai sumber tenaga utama. Dalam kajian ini, perbandingan dalam pertumbuhan, komposisi proksimat dan morfologi mikroalga laut, *Isochrysis* sp. dilakukan dengan menggunakan diod pemancar cahaya (LED) biru dan kuning, manakala lampu pendafluor dijadikan sebagai kawalan. Pertumbuhan mikroalga diperhatikan selama 13 hari dan kesan perbezaan jenis lampu ke atas bilangan sel, ketumpatan optik dan kadar pertumbuhan spesifik dikenalpasti. Di akhir kajian ini, keputusan menunjukkan *Isochrysis* sp. yang disemai menggunakan lampu pendarfluor mempunyai nilai kadar pertumbuhan spesifik yang tertinggi manakala LED kuning dan biru menhasilkan nilai kadar pertumbuhan spesifik yang sama. LED kuning

dan lampu pendarfluor juga menghasilkan densiti sel yang tinggi. Mikroalga tersebut dituai apabila tiba fasa stasioner diikuti dengan analisis proksimat. *Isochrysis* sp. yang disemai mengunakan LED kuning mempunyai kandungan lipid yang tinggi. Manakala, komposisi protein menunjukkan ketinggian yang ketara di dalam *Isochrysis* sp. yang disemai menggunakan LED biru dan kuning. LED kuning dan lampu pendarfluor juga menghasilkan kandungan karbohidrat yang tinggi berbanding LED biru. Morfologi sel pada peringkat awal dan akhir pertumbuhan telah dikaji menggunakan mikroskop imbasan electron. Lampu pendarfluor mampu menghasilkan sel yang terbesar. Oleh itu, melalui kajian ini dapat disimpulkan bahawa LED kuning merupakan lampu yang optimum untuk penyemaian *Isochrysis* sp. Selain itu, perbezaan jenis lampu juga memberi kesan ke atas morologi sel mikroalga.

Kata kunci: Mikroalga, *Isochrysis* sp., Diod pmancar cahaya (LED), komposis proksimat, morfologi

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of requirement for the course VPD 4999 – Project

INVESTIGATION ON THE USE OF BLUE AND YELLOW LEDS FOR GROWTH, PROXIMATE COMPOSITION ENHANCEMENT AND MORPHOLOGY OF MARINE MICROALGA *Isochrysis* SP.

NORHAYATI BINTI SUHAIMI

2016

Supervisor: Prof. Dato' Dr. Mohamed Shariff Bin Mohamed Din

Co- Supervisor: Dr. Sanjoy Banerjee

Microalgae are photoautotrophic organisms that need light as their main energy source. In this study, the growth, proximate composition and morphology of marine microalga, *Isochrysis* sp., cultured under blue and yellow light-emitting diodes (LED) with fluorescent light as control were compared. Growth of the microalga was observed for 13 days and the effect of the three different light sources on cell count, optical density and specific growth rate were determined. At the end of the experiment, results showed that *Isochrysis* sp. cultured under fluorescent light had highest specific growth rate (SGR)while yellow LED and blue LED cultures had similar SGR. Yellow LED and fluorescent light produced higher cell density in culturing *Isochrysis*sp. The cultures were harvested during the stationary phase and proximate analysis was done. Lipid content was significantly

higher in *Isochrysis* sp. grown under yellow LED, whereas, protein composition was significantly higher in blue and yellow LED cultures. Yellow LED and fluorescent light cultures also had significantly high carbohydrate composition compared to blue LED cultures. Cell morphology was studied under scanning electron microscope during initial and final stage of the microalga cultures and fluorescent light produced the largest cells. Thus, it can be concluded that yellow LED had the optimum wavelength for *Isochrysis* sp. culture. Besides that, different wavelength of lights also affected the morphology of the microalga cells.

Keywords: Microalga, *Isochrysis* sp., LED light, proximate composition, morphology

1.0 INTRODUCTION

Microalgae are of strategic interest for aquaculture as they are an irreplaceable food for aquatic animals like mollusks, shrimps, and fish, especially for early stages (Richmond & Qiang, 2013). Microalgae are the primary food source for a large number of aquatic organisms and play a key role in aquaculture development. According to Cordoba-Matson *et al*. (2013), most of the microalgal species being grown commercially on a large scale are *Spirulina* sp. and *Chlorella* sp. for health food, along with a handful of other species principally used in aquaculture as live food for farmed species.

The microalgae cultivated are not only used as feed for aquatic animals, but have importance in the production of useful compounds, such as biofilters to remove nutrients and other pollutants from wastewaters, in cosmetic and pharmaceutical industry. Microalgae are also potentially good sources for biofuel production because of their high oil content and rapid biomass production (Sirakov *et al*. 2015).

Microalgae need light, carbon dioxide and nutrients for growth. Microalgae are photoautotrophic organisms that need light as their main energy source (Banerjee *et al*. 2011). Photoautotrophic organisms grow through photosynthesis by converting sunlight, carbon dioxide and a few nutrients, including nitrogen and phosphorous, into material known as biomass (Algae Biomass Organization, 2012). The effects of light intensity, temperature, salinity and nutrients on the growth and proximate composition of microalgae have been widely explored (Brown *et al*. 1997). Since there is an increase of demand for microalgae, we need to improve the growth and production of the microalgae. Sunlight is a good source for growth, however, in our tropical country, Malaysia, we do not get

continuous sunlight source especially during rainy season. Numerous investigations have been made on the optimal growth conditions for microalgae in the laboratory. The usage of light-emitting diodes (LEDs) is cost-saving plus the life-span is longer compared to fluorescent light that is commonly used in laboratory.

Thus, the objectives of this study are:

1) To evaluate the effects of different wavelengths of light on the growth and nutritional profile enhancement of *Isochrysis* sp.
2) To determine the morphology of *Isochrysis* sp. using different wavelengths of light.

The hypothesis for this study is:

$H_{o:}$ The different wavelength of light used will not influence the growth, nutritional profile and morphology of *Isochrysis* sp.

$H_{a:}$ The different wavelength of light used will influence the growth, nutritional profile and morphology of *Isochrysis* sp.

2.0 LITERATURE REVIEW

2.1 *Isochrysis* sp.

Isochrysis sp., a haptophytes genus microalga, is a free-living marine unicellular phytofllagelate of the order Chrysomonadales (Kaplan, 1985). According to Kaplan (1985), *Isochrysis* sp. is rich in polyunsaturated fatty acids which are of nutritional value for marine fish larvae and juvenile stages of mollusks. This small golden-brown flagellate microalga is commonly used in the aquaculture industry as it is high in DHA and often used to enrich zooplankton such as rotifers or Artemia. *Isochrysis* sp. are small which is approximately 4–6 μm in diameter, and since they lack of cell wall, they are readily digested by small invertebrates or larva (Cordoba-Matson *et al*., 2013).

According to FAO (2013), the larvae of most bivalve species have similar food preferences; suitable microalga species including *Chaetoceros calcitrans*, *Thalassiosira pseudonana*, *Isochrysis galbana*, and *Tetraselmis suecica* (for larvae > 120 μm in length). Combinations of flagellates and diatoms provide a well-balanced diet which will generally accelerate the rate of larval development compared to unimicroalga diets.

2.2 Growth Phases of Microalga

Microalga has the following growth phases: adaptation (lag phase), exponential growth phase (log phase), stationary phase and logarithmic death phase. The individual phases, shown in figure 1, are not always clearly defined; their length or slope might change according to the culture conditions. During lag phase, the microalga cells are adapting to the new environment conditions, at the end of the lag phase, the cells are well adjusted and then start to multiply rapidly, this is the exponential phase in this phase, the number of living cells, double regularly with time (Becker, 1994).In stationary phase, the cells have

multiplied to such extent that they begin to shade one another so that gradually a high absorption of incident light occurs. This effect, as well as the beginning of limitation of minerals and increasing accumulation of toxic wastes, reduces the specific growth rate and the increase in the microalga biomass becomes almost linear. This phase continues until limitation due to exhaustion of a certain nutrient occurs or the culture reaches a stage where respiration begins to interfere (Becker, 1994). When vegetative cell metabolism can no longer be maintained the death phase of a culture is generally very rapid, hence the term "culture crash" is often used. The steepness of the decline is often more marked than that represented in the accompanying growth figure. Cell density decreases rapidly and the culture eventually collapses. In practice, culture crashes can be caused by a variety of reasons, including the depletion of a nutrient, oxygen deficiency, overheating, pH disturbance, or contamination (Becker, 1994).

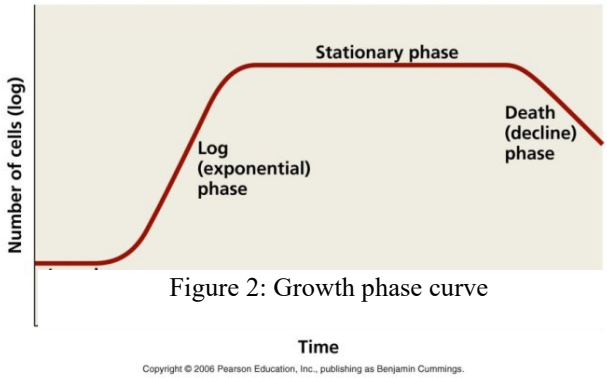


Figure 2: Growth phase curve

2.3 Factors Affecting Microalgae Growth

In photoautotrophic microalgae culture, one of the major energy sources for growth of cells are the photons of light since it can be absorbed by the cells as nutrients, the wavelength and the intensity of the light source are definitely important for the growth of the photoautotrophic microalgae (Wang *et al*. 2007). This also means that the specific growth rate of microalgae could be greatly influenced by the different light source.

Kaplan *et al*. (1985) in his study mentioned that the environmental factors play important roles in providing optimal growth condition of *Isochrysis galbana*. He reported that the optimal temperature for achieving highest microalga yield was 27°C, while temperatures higher than 32°C or lower than 19°C reduced growth markedly. For pH, within the range of 5.0-9.0 the pH in itself had no significant effect on yield and growth rate of the microalga, provided that there was sufficient concentration of Fe^{3+} available in the growth medium while there was a marked pH effect on microalga yield whenever supply of Fe^{3+} was limited, so that no growth occurred at pH higher than 9.1. This effect of pH is enhanced by the presence of carbonates in the medium. The result also showed the effects of increasing light intensity from 50 to 150 $\mu mol/m^2/s^1$ where the growth rate was doubled by increasing the light intensity three times. *Isochrysis galbana* exhibited resistance to a wide range of NaCl concentrations or salinity.

2.4 Growth Rate and Proximate Analysis

Wavelength of light is believed to influence the growth of marine microalgae. Wang *et al*. (2007) used various LEDs with different light wavelengths and illumination intensities to explore the effects of light source on photoautotrophic cultivation of *Spirulina platensis*. The results showed the red LED exhibited the highest specific growth rate of 0.40/day under the condition of 3000 µmol/m^2/s^1 while the blue LED was the least efficient in the conversion of photon to biomass.

Other study on the growth rate, biomass production and composition of *Chaetoceros* sp. were conducted by Sanchez-Saavedra and Voltolina (2006). *Chaetoceros* sp. was used as the model microalga. The light sources used were Cool White (CW), Gro-Lux (GRO) and Gro-Lux wide spectrum agriculture lamps (GRO/WS). The main difference among light sources used is the amount of power emitted in the different wavebands. At the end of exponential and during stationary growth, GRO and GRO/WS lamps gave higher cell numbers and biomass than CW lamps. Biomass was significantly higher with GRO lamps. In all treatments, protein concentration was higher and lipids were lower at the end of exponential growth than in the stationary phase, but proteins remained consistently higher than other treatments with GRO lamps in both growth phases. CW gave the highest lipid and GRO/WS the best carbohydrate production during both phases of growth.

Light quality affected growth and lipid content in the microalga *Chlorella vulgaris* (Hultberg, *et al*. 2014). In a study conducted by Hultberg *et al*. (2014), the species was exposed to monochromatic light at six different wavelengths. A significantly higher

amount of biomass produced in the treatments with yellow, red and white light compared with blue, green and purple light. There were also differences in total lipid content and fatty acid profile between the treatments. The green light regime gave the lowest concentration of lipids but increased the concentration of polyunsaturated fatty acids.

According to Cordoba-Matson *et al*. (2013), there are a limited number of studies utilizing high efficiency light-emitting diodes (LEDs) with microalga, even though LEDs have been proposed as a primary light source in commercial crop cultures due to their lower energy costs compared to standard lighting. For example, Matthijs *et al*. (1996), reported that monochromatic exposure from red LEDs alone can support microalga growth, whereas limited exposure to blue light failed to augment the biomass production of *Chlorella* sp. However, in that study the effect of blue LEDs alone on the growth of *Chlorella* sp. was not studied and the ratio of blue to red LEDs used may have possibly been too low to cause any effect. In another study, Lee and Palsson (1996), reported that in *Chlorella* sp., red LEDs also had produced equivalent biomass growth in comparison to fluorescent light. Oh *et al*. (2008) studied the effects of irradiance with various wavelengths from light-emitting diodes on the growth of the harmful dinoflagellate *Heterocapsa circularisquama* and the diatom *Skeletone macostatum*, they reported light selectivity in stimulating the growth of one over the other. Wang *et al*. (2007) reported that red light-emitting diodes performed better than blue LEDs in the cultivation of *Spirulina platensis*.

2.5 Morphology

Historically, the major groups of microalgae are classified into divisions on the basis of pigmentation, chemical nature of photosynthetic storage product, photosynthetic membranes organization, cell wall structure, arrangement and ultrastructure of flagella, and the occurrence of any other special features (Gualtieri, 2011). Several studies showed that the cell morphology of microalgae change with changing cultivation conditions.

In a study conducted by Cordoba-Matson *et al*. (2013), cell morphology was studied by using microscopic photograph and cell size was measured. After 5 days of light treatments, the result showed that the size of the microalga *Isochrysis aff. galbana* was larger with fluorescent light (mean value of 49μm^2), followed in size with white LED (mean value of 36.5μm^2, $P< 0.05$), and smallest with red LEDs (mean value of 22.6μm^2, $P< 0.05$), which corresponds to sizes that are 1.3x and2.2x smaller, respectively, compared to fluorescent light or control group.

3.0 MATERIALS AND METHODS

3.1 Inoculation of the Microalga

The microalga *Isochrysis* sp. originated from Marine Science Centre (COMAS), Universiti Putra Malaysia. It was brought to the Aquatic Animal Health Unit Laboratory (AAHU) in Universiti Putra Malaysia where it was purified and maintained as stock culture. The seawater was filtered and filled into 2 L Erlenmeyer flasks and covered with aluminium foil. The 2 L Erlenmeyer flasks filled with 2 L filtered seawater were autoclaved at 121°C for 15 min and kept at room temperature for 2 days to allow sufficient time for carbon dioxide equilibration.

The cultures were grown in filtered autoclaved seawater using Conway medium (Walne, 1970) between 24-26˚C, 60 µmol/m^2/s light intensity and 30 ppt salinity. The Conway medium consists of Solution A, Solution B and Solution C. 2mL of Solution A, 1mL of Solution B and 0.2mL of Solution C were added to the filtered and sterilized seawater. Appendix 1 shows the composition of chemicals in each solution respectively. The cultivation of *Isochrysis* sp. started with an initial cell density of 1×10^5 cells/mL in 2 L Erlenmeyer flasks. The volume of stock solution was obtained by calculating the initial cell densities and substituted in the formula below:

Cell density of stock solution: $2.6x10^6$ cells/mL

Initial cell density: $1x10^5$ cells/mL

$$C_1V_1 = C_2V_2$$

$$(2.6x10^6)\ (V_1) = (10^5)\ (24L)$$

$$V_1 = 0.923L$$

*923mL/ 12 flask = 76.92mL/flask

3.2 Setting of Pl

The system consisted of blue LEDs (460nm), yellow LEDs (580nm) and control group illuminated with the normal fluorescent lighting typically used to grow *Isochrysis* sp. in the laboratory. Each treatment consisted of four flasks. The flasks were placed on the 3 level shelf with each level representing each light (Figure 2). The surrounding of the shelf was covered with aluminum foil to exclude external light source and to reflect the respective LEDs on each level and the fluorescent light.

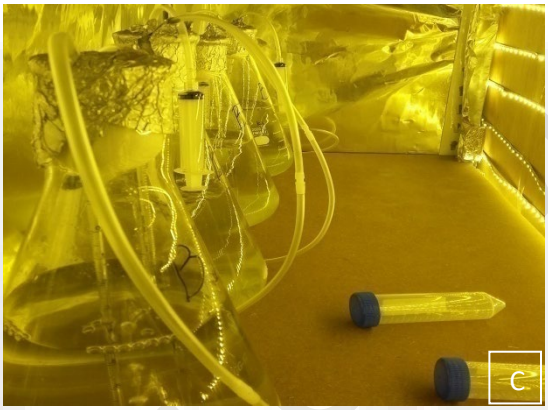


Figure 2: (A) The three levels shelf where blue LED treatment placed on first level, yellow LED treatment on second level, and control treatment on third level. (B) The inside view of blue LED treatment. (C) The inside view of yellow LED treatment.

3.3 Cell Growth

The growth of *Isochrysis* sp. was followed by measuring the optical density at 540nm (Kaplan *et al*., 1985) by using spectrophotometer and obtaining the cell density by cell count using haemacytometer and microscope daily. *Isochrysis* sp. is a motile microalga species so one drop of Lugol solution was added into the sample of the culture to immobilize the cells before it was placed on top of the haemacytometer glass slide, one or two drops of the sample was filled into both chambers using Pasteur pipette. Dilution of the sample was done on 6^{th} day of culture due to high number of cells. A 10x dilution was done by adding 9mL of filtered sea water into 1mL of sample. Cell numbers were determined daily by placing an aliquot of well-mixed culture suspension on a Neubauer haemocytometer. The cells were counted in five small squares in the centre block. The cell number in the culture was calculated by dividing the number of cells counted by the volume

and the dilution. At the end of the experiment, the specific growth rate (SGR) of microalga was calculated using the following equation:

$SGR/day = (In\ X_2 - ln\ X_1)/ (t_2-t_1)$

Where, X_1 is the cell density at the beginning of the selected time interval; X_2 is cell density at the end of the selected time interval; t_2-t_1 is the selected time (in days) for the determination of cell density of microalga species.

3.4 Proximate Analysis

Once the cell density and optical density decreased indicating that cell growth dropped, the samples were harvested for proximate analysis. The samples were first centrifuged at 9000 rpm for 10 min at 10°C, after which the supernatant was discarded and the pellet was transferred into 50mL centrifuge tube. Then, the pellet was rinsed with 40mL autoclaved filtered seawater by mixing them together and centrifuging again as done previously followed by discarding the supernatant. This procedure was repeated twice. Then, the pellet (Figure 3) was kept in fridge at 4°C before being freeze-dried.After freeze-

drying the samples for approximately 2 days (Figure 4), the samples were ground into powder by using mortar and pestle.

Figure 3: The pellet formed after centrifuged process.

Figure 4: Freeze-drying of samples.

3.4.1 Protein Analysis

Protein composition was determined using extraction method by Slocombe *et al*., (2013). First, reagents A, B, and C were prepared which are 2% Na_2CO_3 in 0.1N NaOH, 1% NaK Tartrate in H_2O and 0.5% $CuSO_4$.5H2O in H_2O respectively. These reagents can be stored indefinitely. Reagents D and E were prepared freshly prior to use which are 48:1:1 of Reagent A:B:C and Phenol Reagent – 1 part Folin Ciocalteu-Phenol respectively. Then, stock of 10mg/mL bovine serum albumin (BSA) was prepared as standard solutions. The stock was divided into 1 – 2mL aliquots of concentrations 0.1, 0.5, 1, 2, 3, 4 and 5mg/mL.

For protein extraction, six replicates of 5 mg freeze-dried microalga sample for each treatment were weighed out into Eppendorf tubes after which 200 µL of 24% trichloroacetic acid (TCA) was added to the samples to re-suspend the dried microalga. The samples were incubated at 95 - 100°C for 15 min after which the microalga/TCA samples were removed from water bath and allowed to cool. Once cool, 600 µL of distilled water was added to dilute the samples to 6% TCA and then centrifuged at 13000 rpm at 10°C for 2 min. Then, the supernatant was discarded and pellet was re-suspended in 0.5mL Lowry reagent D and incubated in water bath at 55°C for 1h. After that, the samples were allowed to cool and centrifuged at 13000 rpm for 20 min at room temperature. Then, the supernatant which is the protein/alkaline suspension was transferred to a fresh tube.

For protein quantification, 950 µL of the samples (protein/ alkaline suspension or BSA standard) were pipetted into new centrifuge tube. One mL of Lowry reagent D was added into the tubes and shaken and incubated for 10 min at room temperature. Then, 0.1mL of Folin-Phenol-water (Reagent E) was added to each individual sample after incubation and vortexed immediately the mixture and incubated for a further 30 min. Finally, the absorbance was measured using a spectrophotometer at 600nm with mili-Q water and Reagent D and E as blank. Standard curve was constructed (appendix 2) and the percentage of protein composition was calculated as shown in appendix 3.

3.4.2 Lipid Analysis

Lipid content was determined based on modified method adapted by Bligh and Dyer (1959). The aluminum dishes were labeled first and their weights were recorded. The

samples were weighed out (15 mg) into centrifuge tubes with six replicates for each treatment. Five mL of distilled water followed by 3mL of methanol:chloroform (2:1) was added into the samples. The mixtures were homogenized and then centrifuged for 4 min at 1500 rpm at 4°C. After centrifuging, the mixture clearly separated into three phases as shown in figure 5-A, the supernatants were transferred to a clean tube using Pasteur pipet and the tubes were placed in ice. Then, 3mL of methanol:chloroform (1:2) was added to the centrifuge tube containing the pellet residue and homogenized. The tubes then were re-centrifuged for 4 min at 1500rpm at 4°C. After centrifuging, the supernatant was transferred to the previous supernatant tube (Figure 5-B). Then, 1.5mL of 0.9% NaCl solution was added into combined supernates and shaken. After that, the tubes were kept in the fridge for 45 – 60 min at 4°C until the phases clearly separated. After that, the lower phase chloroform containing lipids was transferred into aluminum dish (Figure 6). Then, the solvent was evaporated by drying the samples in an oven at 60°C overnight. Finally, the aluminum dishes were weighed out and the ratio of weight of aluminum dish containing the lipid residuals and the weight of original aluminum dishes were calculated.

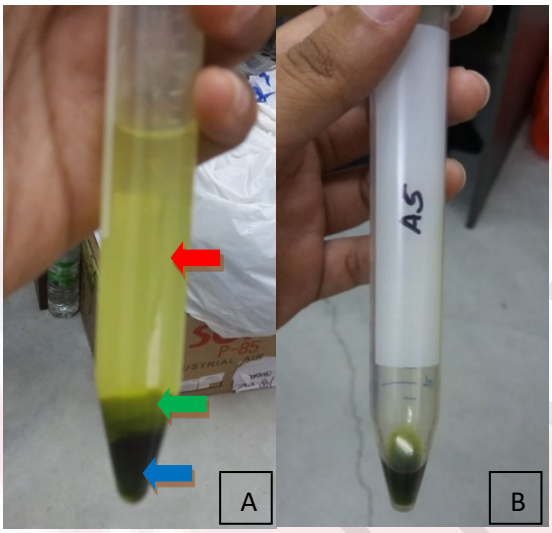


Figure 5: (A) The phases clearly separated after the tube was centrifuged for 4 min at 1500 rpm at 4°C. (⬅)Methanol+water layer,(⬅) tissue residue layer, and (⬅) chloroform containing lipid layer. (B) The lower phase chloroform containing lipids (supernatant)was transferred into new tube.

Figure 6: The aluminum dishes contained lower phase chloroform containing lipids.

3.4.3 Carbohydrate Analysis

Carbohydrate analysis was done by using colorimetric method by Dubois *et al*. (1956). Firstly, Reagents A and B were prepared. Reagent A was 8g phenol in 2g distilled water and heated at 100°C. Reagent B was prepared by taking 5mL of Solution A and added to 100mL distilled water to make 5% phenol solution. For standard solutions, stock solution of 20g glucose in 20mL distilled water was prepared. Six concentrations of standards were prepared which are 0µg/L (only distilled water 10mL), 20µg/L (0.2mL of stock + 9.8mL distilled water), 40µg/L (0.4mL of stock + 9.6mL distilled water), 60µg/L (0.6mL of stock + 9.4mL distilled water), 100µg/L (1mL of stock + 9.0mL distilled water), and 140µg/L (1.4mL of stock + 8.6mL distilled water).

After that, 5 mg of freeze-dried samples of each treatment was weighed out and homogenized with 5mL distilled water and then added in 20mL distilled water. 1mL of homogenized sample and standard solutions were transferred into new test tubes. Each treatment had six replicates. After that, 1mL of phenol 5% solution was added into the sample and after 30 seconds reaction period, 5mL of concentrated H_2SO_4 was added. Then, the tubes were placed in the cold water bath to cool down the mixtures (Figure 7). Finally, the absorbance was measured using a spectrophotometer at 488nm after cooling with 0µg/L standard solution as blank. Standard curve was constructed (appendix 2) and the percentage of carbohydrate composition was calculated as shown in appendix 3.

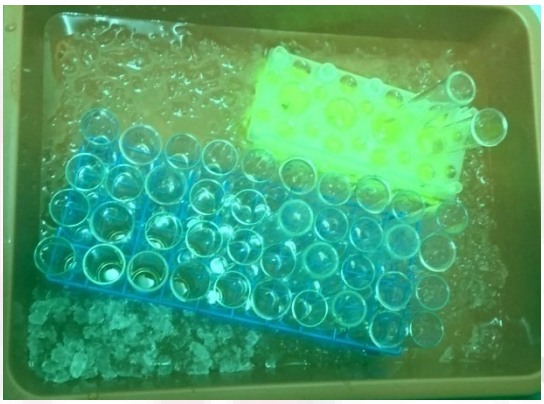


Figure 7: The tubes were placed in cold water bath after 5mL of concentrated H_2SO_4 was added to cool down the mixtures.

3.5 Cell Morphology

The samples were transferred into Eppendorf tubes and centrifuged at 3000 rpm for 3 min until enough amount of pellet was obtained. The supernatant was discarded and equal amount of glutaraldehyde fixative was added to the pellet for the aldehyde groups to react with amino groups and cross-link with the proteins into an insoluble network. The solutions were hand shaken and incubated at 4°C for 4-6h. After that, the cell suspension was centrifuged at 3000 rpm for 3 min into pellet and the fixative was discarded. Then, the pellet was washed with 0.1M sodium cacodylate buffer to clean the surface from contaminants. The pellet was left in the buffer for 10 min. After 10 min, the sample was centrifuged and the buffer was discarded. This process was repeated for three times. After washing, the sample was fixed with 1% osmium tetroxide at 4°C for 2h. This is to preserve the cellular membrane by the reaction of heavy metal with fatty acids. After that, the sample was centrifuged and the fixative was discarded. The washing process was repeated before dehydration process.

For the dehydration process, a series of acetone which are 35%, 50%, 75%, 95% were used where the sample was mixed with each of the concentration and left for 10 min respectively. Finally, 100% of acetone was mixed with the sample and left for 15 min for three changes. This process allows the water in the samples to be slowly exchanged through liquids with lower surface tensions. After that, the cell suspensions were pipetted onto aluminium foil coated with albumin and proceeded for critical point drying. The samples were transferred into specimen basket and put into critical dryer for about ½h. Then, the samples were stuck onto the stub using double sided tape for mounting and lastly coated with gold coating in sputter coater. The samples were ready to view under scanning electron microscope.

4.0 RESULTS AND DISCUSSION

4.1 Growth of *Isochrysis* sp.

There were no significant differences ($P > 0.05$) between the treatments during the first days in terms of cell density and OD for *Isochrysis* sp. cultures. The cultures under blue LED had significantly higher ($P < 0.05$) cell density and OD on day 2 and 3. Starting from day 4, the cultures under all light treatments had no significant difference ($P > 0.05$). However, on day 9, the cultures under blue LED had significantly lowest ($P < 0.05$) cell density and OD, whereas, the cultures under yellow LED was the highest. From the following day, there was no significant difference ($P > 0.05$) in cell density and OD between yellow LED and fluorescent light.

For the cultures grown under blue LED, the highest cell density, 4.75×10^6cells/mL was achieved on day 9 whereas, for yellow LED and fluorescent light were on day 11 and day 12, with cell density 8×10^6cells/mL and 8.19×10^6cells/mL, respectively. Cultures grown under blue LED reached stationary phase earlier and had shorter exponential phase but lower cell density and OD which was on day 10, thus it was harvested 3 days earlier. Cultures grown under yellow LED and fluorescent light reached stationary phase on day 12 and 13, respectively with significantly higher ($P < 0.05$) cell density compared to blue LED during harvest day. At the end of the experiment, the specific growth rate of *Isochrysis* sp. cultured under fluorescent light which was the control was found to be the highest followed by yellow LED and blue LED with similar specific growth rate.

Table 2: Cell count, SGR, proximate composition and day of harvest of *Isochrysis* sp. under blue and yellow LED with fluorescent light (FL) as control.

Parameter	**Blue LED**	**Yellow LED**	**FL (control)**
Cell count (cells/mLx10^6)	4.75±0.41	8±0.79	8.19±0.86
SGR (/day)	0.20	0.21	0.28
Protein (%dw)	20.75±0.39	19.96±0.57	17.09±1.15
Lipid (%dw)	25±0.40	27.94±0.84	23.01±0.41
Carbohydrate (% dw)	5.32±0.23	7.19±0.54	7.99±0.21
Day of harvest	10	13	13

In this study, the growth of microalga was measured using cell count and optical density. The growth rate was determined by specific growth rate. The result showed the growth rate of cells grown under fluorescent light was the highest with high cell count. In a study conducted by Koc *et al*. (2013), *Chlorella Kessler* microalga were grown under red LED, blue LED and fluorescent light, cell concentration was measured under constant light current and light density and the result revealed that the cells cultured under blue LED is lower than under fluorescent. In the experiment, the highest pH was measured in the photobioreactors illuminated with the red LED and fluorescent lights, indicating that the microalga in these photobioreactors were consuming more carbon dioxide and growing faster in number of cells, quantity of biomass, and size (Koc *et al*., 2013) According to Lee and Palsson, (1996) blue light can lead to larger cells as a result of delayed cell division. The delay caused the decrease in cell counts.

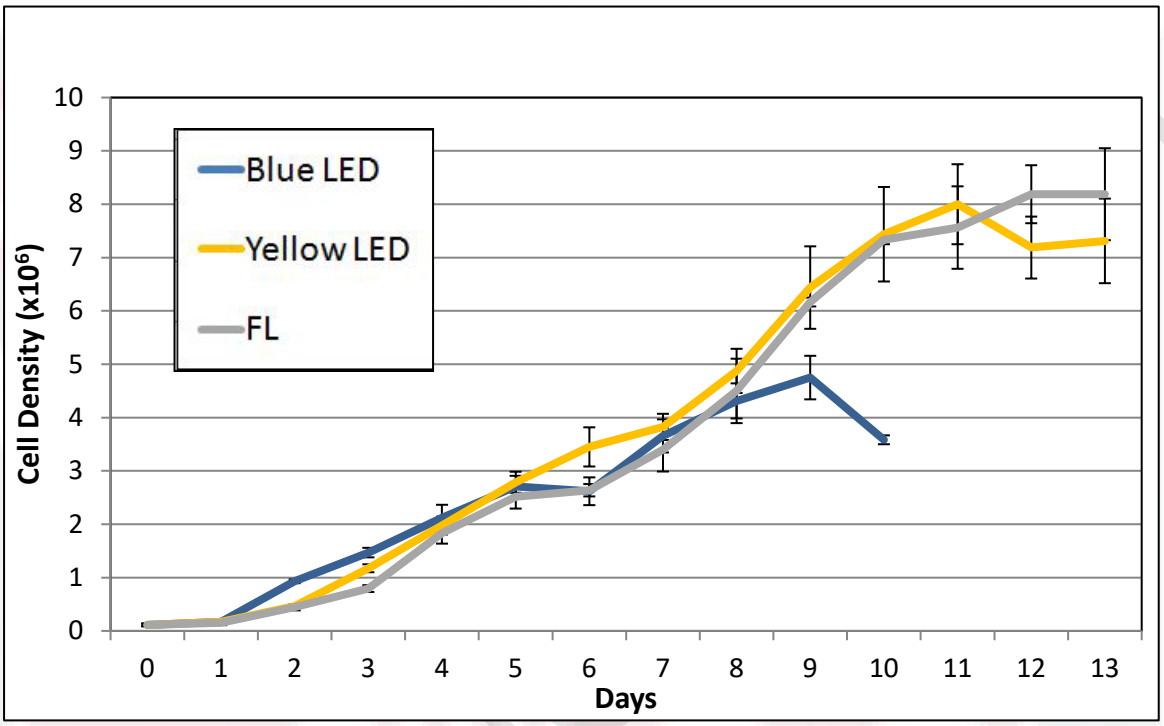


Figure 8: Cell density of *Isochrysis* sp. cultured under blue and yellow LEDs and fluorescent light. Values are mean ± standard error. (n=4)

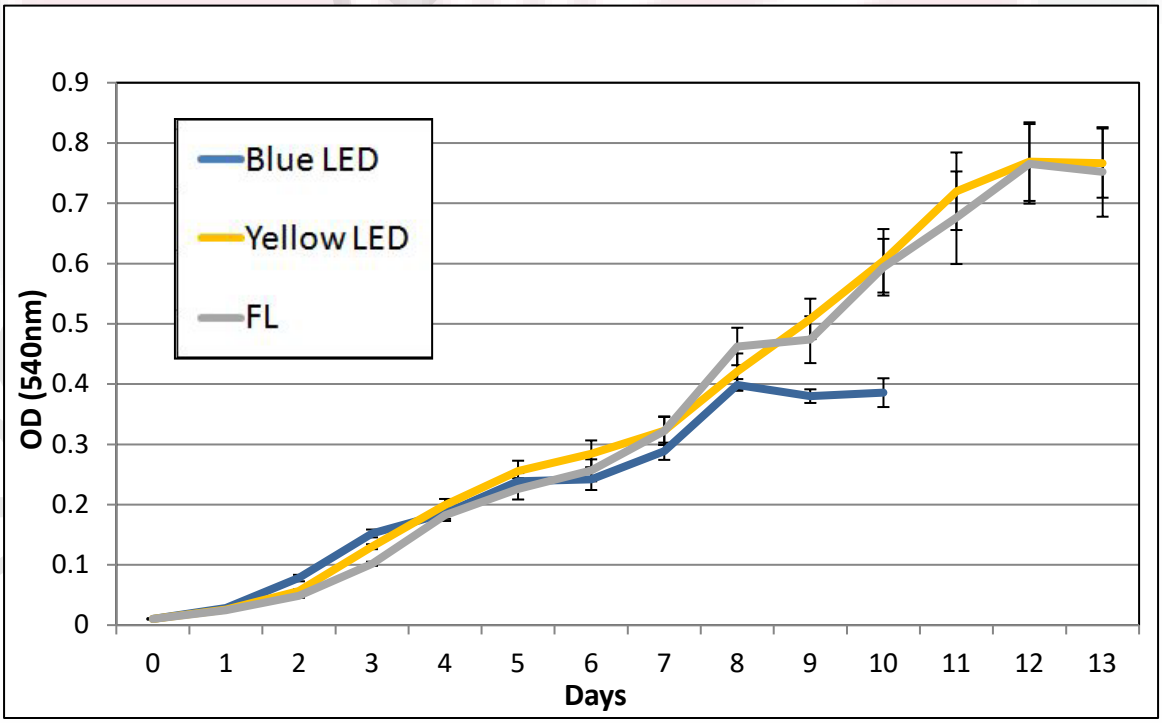


Figure 9: Optical density of *Isochrysis* sp. cultured under blue and yellow LEDs and fluorescent light. Values are mean ± standard error. (n=4)

4.2 Proximate Composition of *Isochrysis* sp.

Protein composition for *Isochrysis* sp. did not show any significant differences ($P > 0.05$) between the yellow LED and blue LED treatments. However, *Isochrysis* sp. cultured under fluorescent light which was the control treatment had significantly lower ($P < 0.05$) protein composition compared to other two LED treatments. The result is consistent with the study conducted by Rivkin (1989) on *Dunaliella tertiolecta* and *Thalassiosira rotula* where the blue light caused higher photosynthetic carbon incorporation into protein when compared with white light thus increase the protein composition.

For lipid composition, no significant differences ($P > 0.05$) were found for *Isochrysis* sp. cultured under control light and blue LED when compared with yellow LED treatment, which was significantly higher ($P < 0.05$) in lipid. The finding was not consistent with a study done by Teo *et al*. (2014), who reported that *Tetraselmis* sp. and *Nannochloropsis* sp. cultivated under blue light has the highest growth rate and also lipid content. Yoshioka *et al*. (2012) found that under blue LED, *Isochrysis galbana* produced maximum lipid content. However, most of these studies compared the blue LED with red, red-blue and white light instead of yellow LED.

Carbohydrate composition for *Isochrysis* sp. did not show any significant differences ($P > 0.05$) between the yellow LED treatment and fluorescent light (control). However, it was significantly lower ($P < 0.5$) in *Isochrysis* sp. cultured under blue LED. Blue light is responsible for endogenous breakdown of carbohydrate reserves (Kamiya and Saitoh, 2002). This can be explained by the results obtained by Marchetti *et al*. (2013) in

his study, where haptophyte *Isochrysis* sp. T-ISO exposed to blue light and grown in a chemostat displayed lower carbohydrate content than when grown under other wavelengths which is consistent with this study. Stocked carbon was utilized to support the higher metabolic rate under blue light, leading to lower carbohydrate content (Marchetti *et al*. 2013).

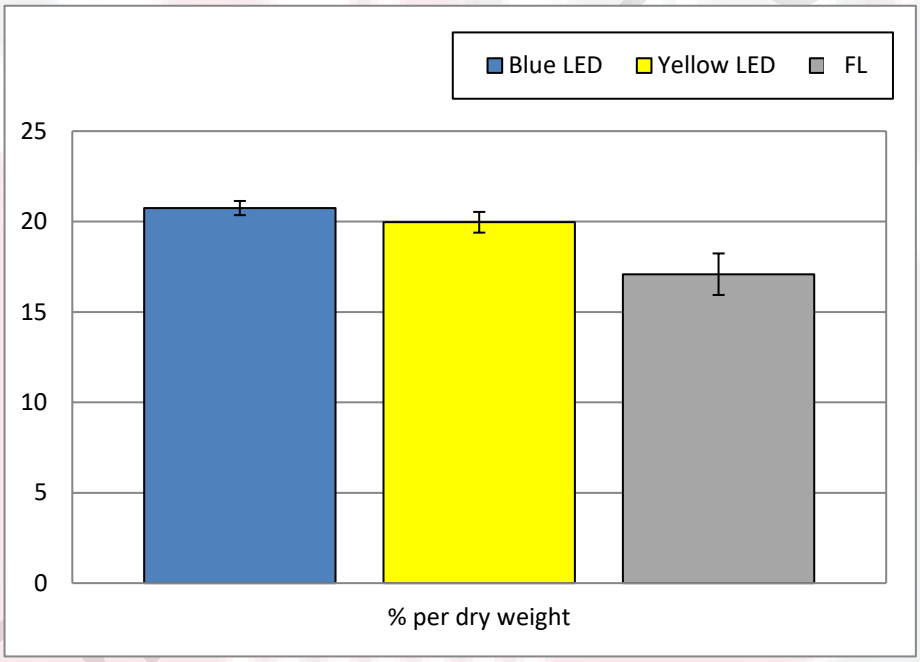


Figure 10: Protein composition in *Isochrysis* sp. cultured under blue and yellow LEDs and fluorescent light.Values are mean ± standard error (n=4)

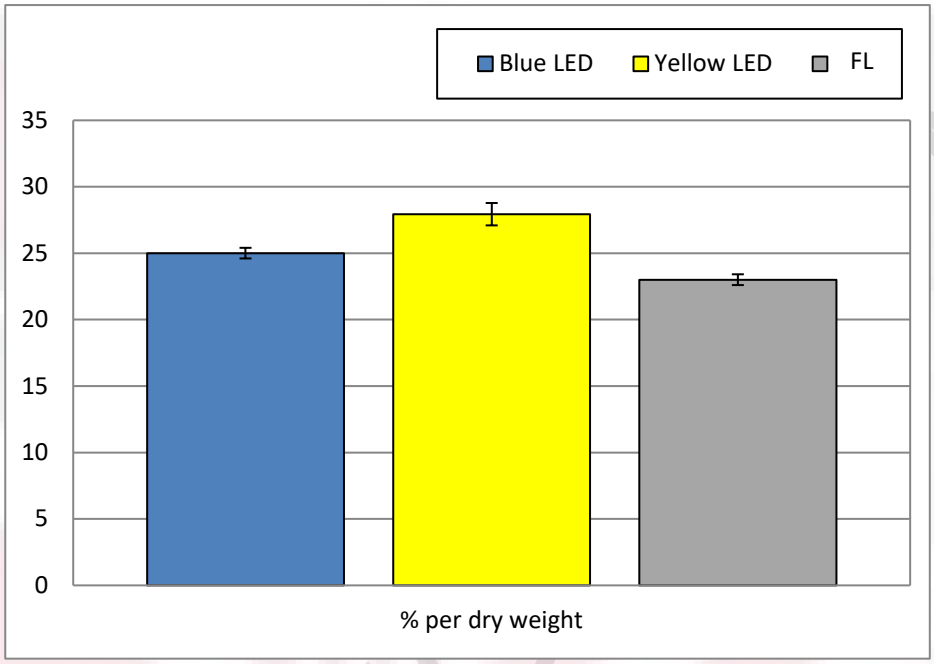


Figure 11: Lipid composition in *Isochrysis* sp. cultured blue and yellow LEDs and fluorescent light. Values are mean ± standard error (n=4)

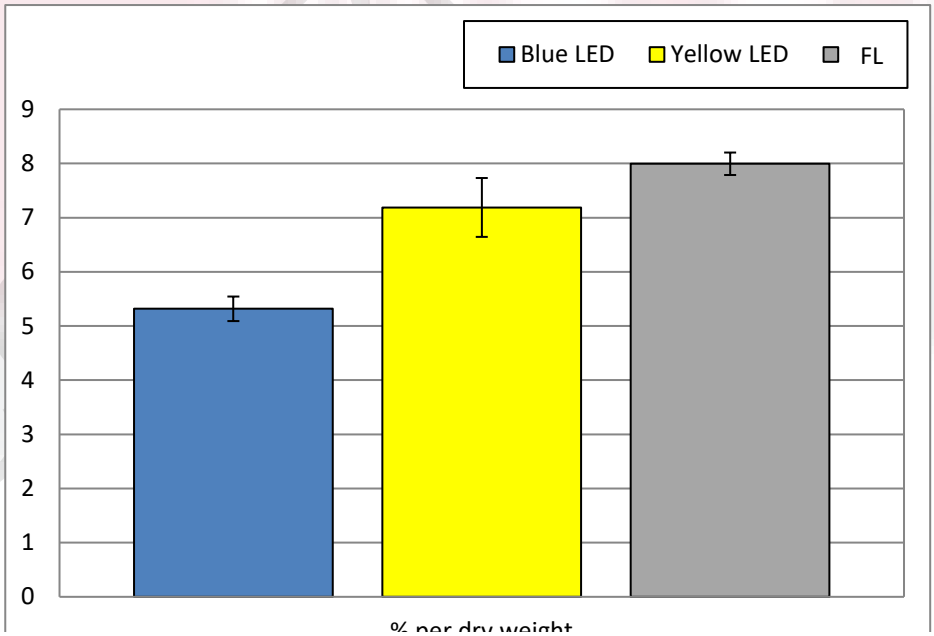


Figure 12: Carbohydrate composition in *Isochrysis* sp. cultured under blue and yellow LEDs and fluorescent light. Values are mean ± standard error (n=4)

4.3 Cell Morphology

The morphology of the cells were studied by viewing under scanning electron microscope after the light treatments for initial (day 3) and final stages (day 10). The result showed that there was no significant difference ($P > 0.05$) in cell size for initial stage and final stage of the cultures (table 2). It may be error during viewing the images where only the largest cells were captured. Besides that, it may be error during measuring since the edge of the cells was unclear in grouping cells.

Table 2: The mean size of *Isochrysis* sp. cultured under blue LED, Yellow LED and fluorescent light (FL=control) during initial stage (day 3) and final stage (day 10).

Light Treatment	**Initial stage (µm²) Mean±STD**	**Final stage (µm²) (Mean±STD)**
Blue LED	4.86±1.12	6.20±1.95
Yellow LED	6.06±0.70	6.65±1.13
FL (Control)	6.47±2.21	9.26±3.37

The cell size during final stage (day 10) was compared between the light treatments and the result showed the size of *Isochrysis* sp. is larger with fluorescent light having mean value of 9.26µm^2, whereas, there was not much difference in size between cells cultured under yellow LED and blue LED having mean values of 6.65µm^2 and 6.20µm^2, respectively. The result was similar to a study conducted on *Isochrysis* aff. *galbana* by Cordoba-Matson *et al*. (2013) who reported that the cell size was largest under fluorescent light instead of white and red LED. Lee and Palsson (1996) had done a study on green microalga, *Chlorella vulgaris* size after exposure to red LED and fluorescent light and they

reported that reduction in size for red LED was due to the presence of the light seemed to facilitate mother cell breakup and early release of autospore instead of using full spectrum light as the cell size increase when the light was switched to fluorescent light.

Overall, the cell shape is rounded with slightly rough surface and presence of indented appearance at the middle of the cell. At the initial stage, the cells seemed to be sticking together indicating the cells were undergoing cell division. For cells under blue LED treatment, the cells were clumped and merged together at the final stage showing that the cells were shrunk and no flagella was found. On the other hand, cells exposed to yellow LED and fluorescent light, the individual cells were well defined and a few cells were found with flagella attached on it. Absence of flagella in most of the cells may be due to the effect during sample processing. However, so far there are no reports regarding the effect of different light wavelength on the morphology of the cells.

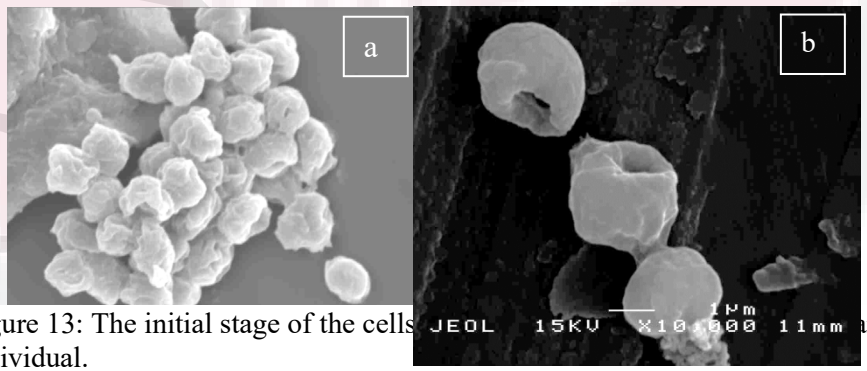


Figure 13: The initial stage of the cells (a) and (b) individual.

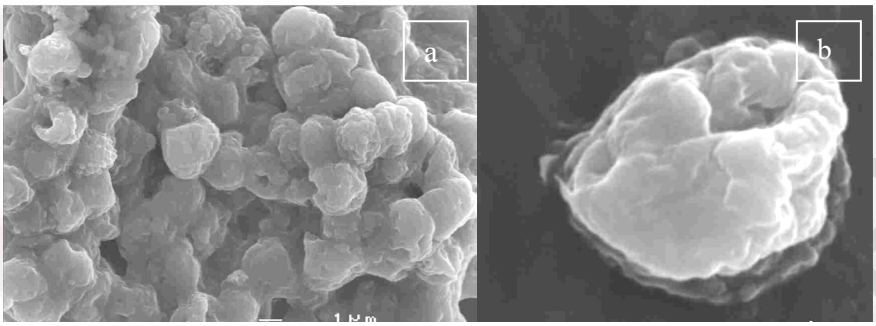


Figure 14: The final stage of the cells under blue LED treatment in (a)group and (b) individual.

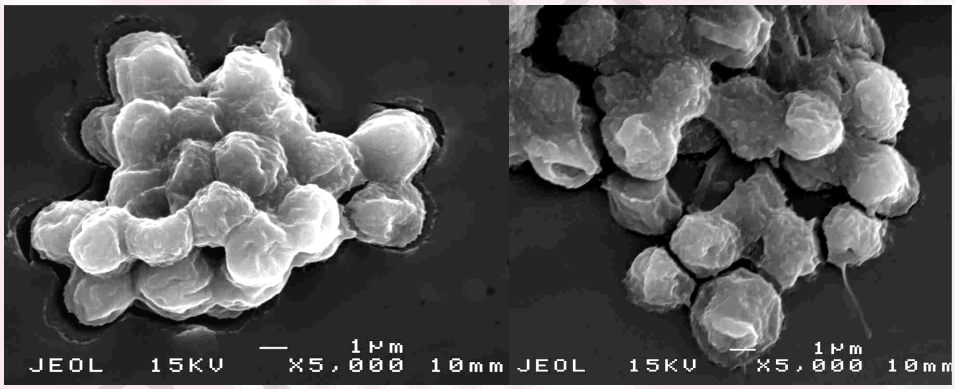


Figure 15: The initial stage of the cells under yellow LED treatment.

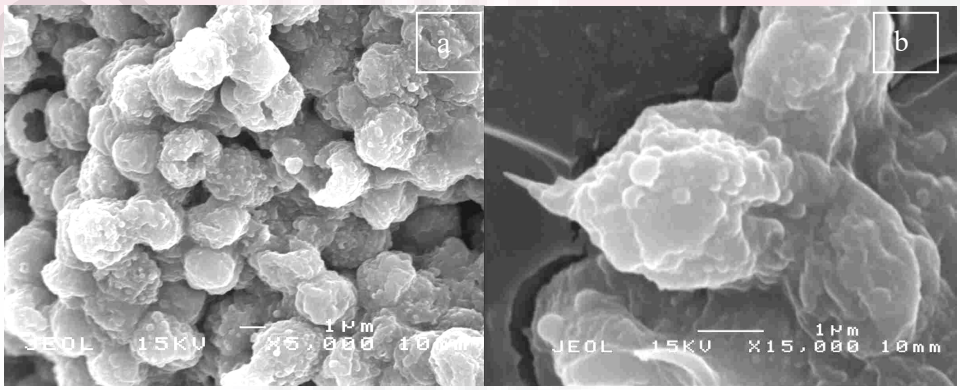


Figure 16: The final stage of the cells under yellow LED treatment in (a) group and (b) individual with flagella.

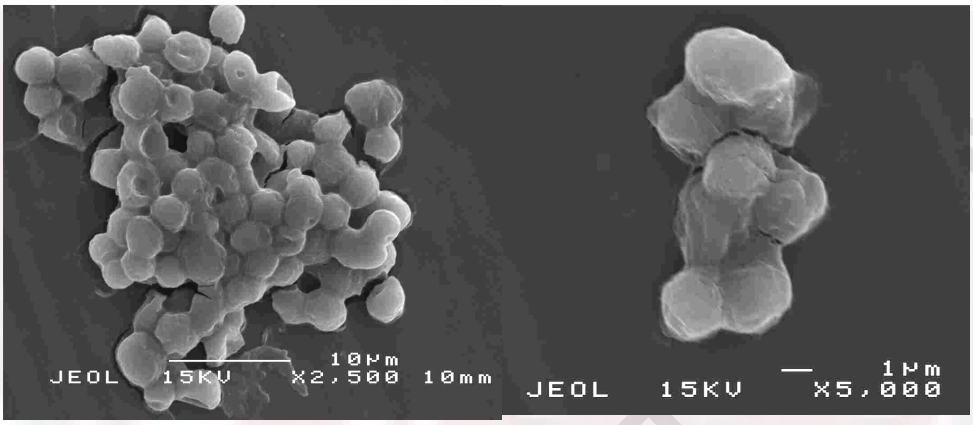


Figure 17: The initial stage of the cells under fluorescent light treatment.

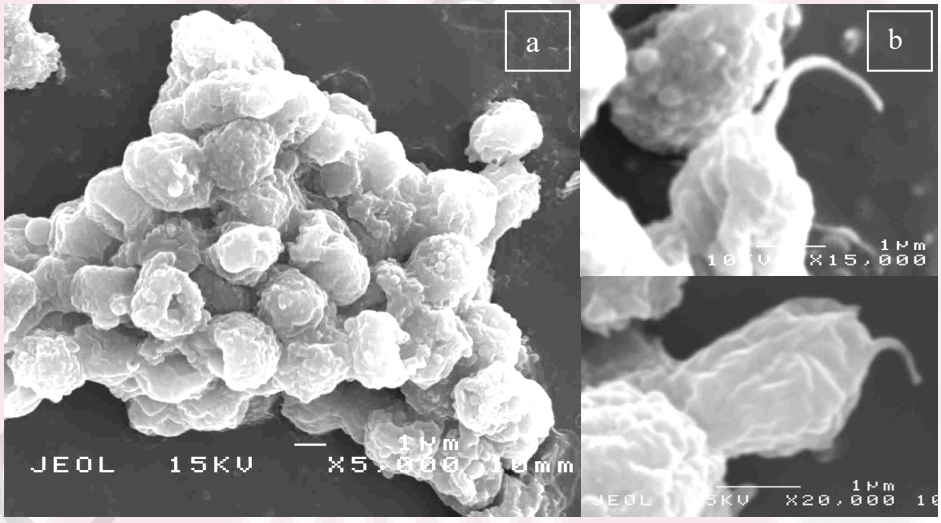


Figure 18: The final stage of the cells under fluorescent light treatment in (a) group and (b) individual with flagella.

5.0 CONCLUSION

The result of the study shows that yellow LED and fluorescent light produce higher cell density in culturing *Isochrysis* sp. while in terms of proximate composition, *Isochrysis* sp. grown under yellow LED has high content in protein, lipid and carbohydrate composition thus yellow LED was the most optimum wavelength for *Isochrysis* sp. Besides that, different wavelength of lights affected the morphology of the microalga cells where the cells exposed under fluorescent light produced the largest cells.

6.0 RECOMMENDATIONS

The study should be done with larger samples so that biomass can be obtained everyday to determine the growth of cells more accurately. The flask cultures were recommended to place on orbital shaker thus the cell cultures can be evenly distributed while taking the sample for cell count and optical density. By doing that, the result will not be biased in case we took the sample at high or low cell density area. Finally, the duration of study should be longer to properly identify the growth curve of the microalga by repeating the culturing and for better understanding.

REFERENCES

Algae Biomass Organization. (2012). Allaboutalgae.com. Retrieved 14 February 2016, from http://allaboutalgae.com/algae-cultivation/

Becker, E. W. (1994). Microalgae: Biotehnology and Microbiology. Cambridge University Press.

Banerjee, S., Wei, E. H., Yusoff, F., Shariff, M. and Khatoon, H. (2011).Growth and Proximate Composition of Tropical Marine *Chaetoceros calcitrans* and *Nannochloropsis oculata* Cultured Outdoors and Under Laboratory Conditions. *African Journal of Biotechnology*, 10, 1375-1383.

Bligh, E. G. and Dyer, W. J. (1959). A Rapid Method of Total Lipid Extraction and Purification.*Canadian Journal of Biochemical and Physiology*, 37, 911-917.

Brown, M. R., Jeffrey, S. W., Volkman, J. K. and Dunstan, G. A. (1997). Nutritional Properties of Microalgae for Mariculture. *Aquaculture*, 151, 315-331.

Cordoba-Matson, M. V., Arrendondo-Vega, B. O., and Carreon- Palau, L. (2013). Evaluation of Growth, Cell Size and Biomass of *Ischrysis aff. galbana* (T-ISO) with Two LED Regimes. *All Results Journals Biol*, 4, 7-15.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry,* 28, 350-356.

FAO (Food and Agriculture Organization of United Nations). (2013). Micro-algae. Retrieved 20 February 2016, from ftp://ftp.fao.org/docrep/FAO/003/w3732e/w3732e02.pdf

Gualtieri, P. (2011). Morphology of Photoreceptor Systems in Microalgae. *Micron*, 32, 411-426.

Hultberg, M., Jonsson, H. L., Bergstrand, K. and Carlsson A. S. (2014). Impact of Light Quality on Biomass Production and Fatty Acid Content in the Microalga *Chlorella vulgaris. Bioresource Technology*, 159, 465-467.

Kamiya, A. and Saitoh, T. (2002) Blue-light-control of the Uptake of Amino Acids and of Ammonia in *Chlorella* Mutants. *Physiologia Plantarum*, 116, 248–254 44.

Kaplan, D., Cohen, Z. and Abeliovich, A. (1985). Optimal Growth Conditions for *Isochrysis galbana. Biomass*, 9, 37-48.

Koc, C., Anderson, G. A. and Kommareddy, A. (2013). Use of Red and Blue Light-Emitting Diodes (LED) and Fluorescent Lamps to Grow Microalgae in a Photobioreactor. *The Israeli Journal of Aquaculture- Bamidgeh*, 797, 1-8.

Lee,C.G. and Palsson, B. (1996). Photoacclimation of *Chlorella vulgaris* to Red Light from Light-Emitting Diodes Leads to Autospore Release Following Each Cellular Division. *Biotechnology Progress*, 12, 249-256.

Marchetti, J., Bougaran, G., Jauffrais, T., Lefebvre, S., Rouxel, C., Saint-Jean, B., Lukomska, E., Robert, R. and Cadoreta, J. P.(2013) Effects of Blue Light on the Biochemical Composition and Photosynthetic Activity of *Isochrysis* sp. (T-iso). *Journal of Applied Phycology*, 25, 109–119

Matthijs, H. C. P., Balke, H., van Hes, U. M., Kroon, B. M. A., Mur, L. C., and Binot, R. A. (1996). Application of Light Emitting Diodes in Bioreactors: Flashing Light Effects and Energy Economy in Algal Culture (*Chlorella pyrenoidosa*). *Biotechnology and Bioengineering*, *50*, 98–107.

Oh, S. K., Kim, D., Sajima, T., Shimasaki, Y., Matsuyama, Y., Oshima.Y., Honjo, T. and Yang, H. (2008). Effects of Irradiance of Various Wavelengths from Light-Emitting Diodes on the Growth of the Harmful Dinoflagellate *Heterocapsa circularisquama* and the Diatom *Skeletonema costatum*. *Fisheries Science*, 74, 137-145.

Richmond, A. and Qiang, H. (Eds.). (2013). Handbook of Microalgae Culture: Applied Phycology and Biotechnology (2nd Ed.). Willey: Blackwell.

Rivkin R.B. (1989). Influence of Irradiance and Spectral Quality on the Carbon Metabolism of Phytoplankton: I. Photosynthesis, Chemical Composition and Growth. *Marine Ecology Progress Series*, 55,291–304

Sanchez-Saavedra, M. and Voltolina, D.(2006). The Growth Rate, Biomass Production and Composition of *Chaetoceros* sp. Grown with Different Light Sources. *Aquacultural Engineering*, 35, 161-165.

Schulze, P., Barreira, L., Pereira, H., Perales, J. and Varelaemail, J. (2014) Light Emitting Diodes (LEDs) Applied to Microalgae Production. *Trends in Biotechology*. 32.

Sirakov, I., Velichkova, K. N. and Stoyanova, S. (2015). The Importance of Microalgae for Aquaculture Industry. *International Journal of Fisheries and Aquatic Studies*, 2, 81-84.

Slocombe, S. P., Ross, M., Thomass, N., McNeill, S. and Stanley M. S. (2013).A Rapid and General Method for Measurement of Protein in Micro-algal Biomass. *Bioresource Technology*, 129, 51-57.

Teo, C. L., Atta, M., Bukhari, A., Taisir, M., Yusuf, A. M. and Idris, A. (2014). Enhancing Growth and Lipid Production of Marine Microalgae for Biodiesel Production via the Use of Different LED Wavelengths**.** *Bioresource Technology***,** 162, 38–44.

Walne, P. R. (1970). Studies on the Food Value of Nineteen Genera of Microalgae to Juvenile Bivalves of the Genera *Ostrea, Crassostrea, Mercenaria and Mytilis. Fishery Investigation*, 26, 162.

Wang, C. Y., Fu, C. C. and Liu, Y. C. (2007). Effects of Using Light-Emitting Diodes on the Cultivation of *Spirulina platensis. Biochemical Engineering Journal,* 37, 21-25.

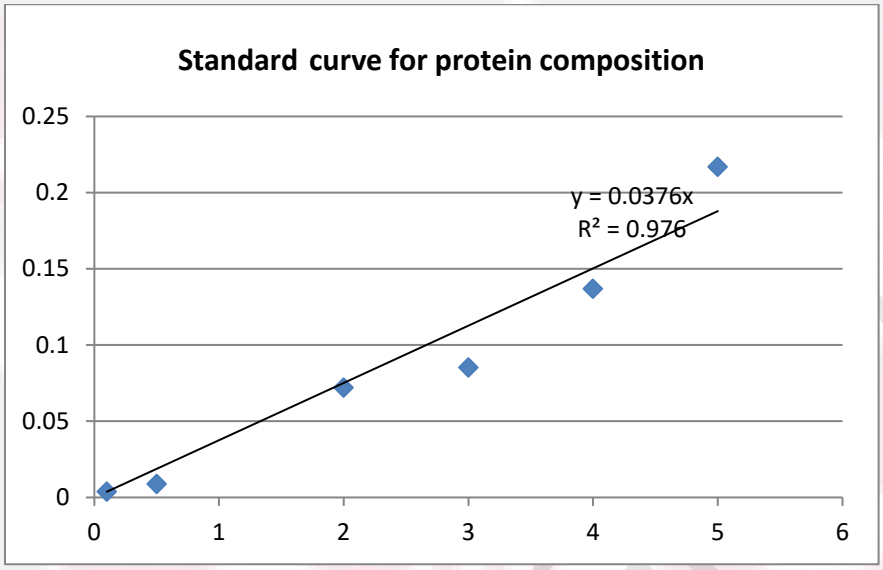
Yoshioka, M., Yago, T., Yoshie-Stark, Y. and Arakawa, H. T. (2012).Effect of High Frequency of Intermittent Light on the Growth and Fatty Acid Profile of *Isochrysis galbana*. *Aquaculture*, 338–341, 111–117.

APPENDICES

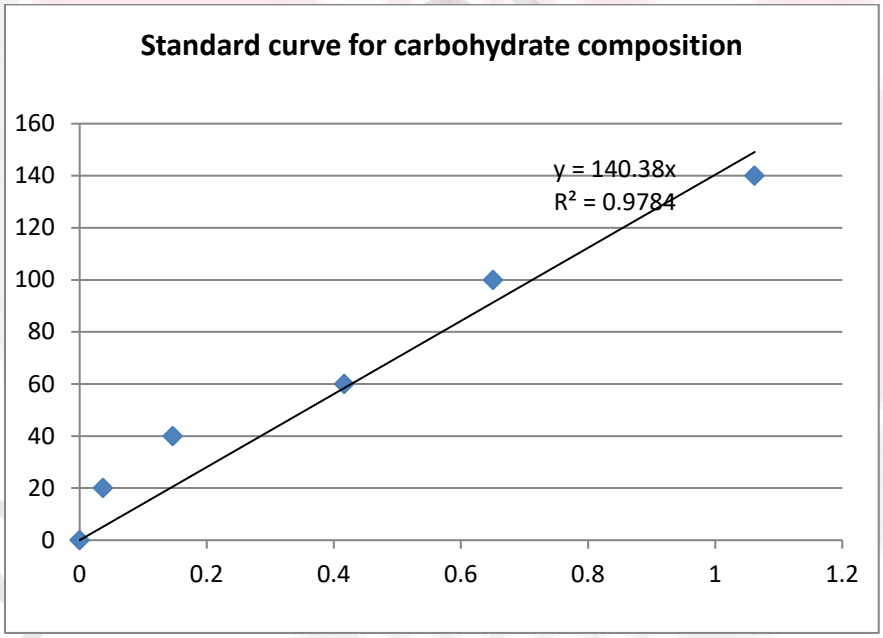
Appendix 1: Chemical composition of Conway medium for marine microalgae culture (Walne, 1970)

Solution A – Stock solution	**Amount**
KNO_3	100 g
Na_3OP_4	20 g
Sodium EDTA	45 g
Boric acid	33.4 g
$FeCl_3$	1.3 g
$MnCl_2$	0.36 g
Distilled water	1000 mL
Solution B – Trace metals solution	
$ZnCl_2$	4.2 g
$CoCl_2$	4.0 g
$CuSO_4$	4.0 g
$(NH_4)MoO_4$	1.8 g
Distilled water	1000 mL
*Note: Acidify with HC1 to obtain a clear solution.	
Solution C – Vitamins solution	
Vitamin B (Thiamin)	200 mg
Vitamin B_{12} (cyanocobalamine)	10 g

Appendix 2: Standard curve for protein and carbohydrate composition



Standard	
[Protein] (mg/mL)	ABS
0.1	0.004
0.5	0.009
1	
2	0.0721
3	0.0854
4	0.137
5	0.2171

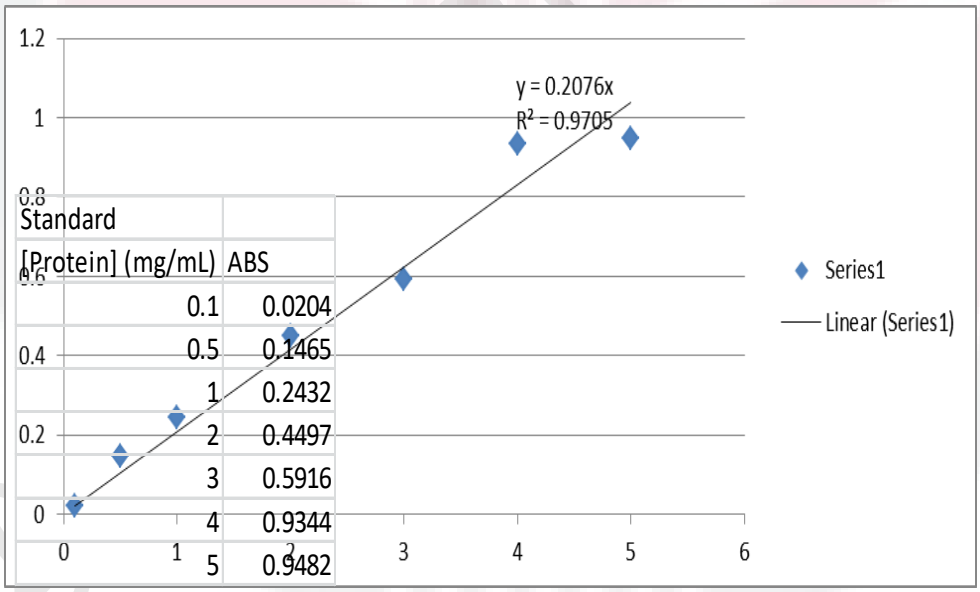


Standard	
[carb] µg/L	ABS
0	0
20	0.0369
40	0.1464
60	0.4164
100	0.6505
140	1.0623

Appendix 3: Calculation for protein / carbohydrate composition in percentage

Sample DW (mg)	ABS (600nm)	Correction factor	Slope of Std. Curve	Intercept
Weight of dry sample used	*Absorbance measurement at 600nm*	*If 25uL used then CF = 1; if 50uL used then CF=2*	*Gradient obtained from standard curve*	*Intercept (c) of standard curve y=mx+c*
Example				
5.1	0.4036	1	0.2076	0

[Protein/ carbohydrate]	%Protein/ carbohydrate
[(Abs – Intercept)/Slope × 1000]/CF	$\frac{100 \times [Protein]}{DW \times 1000} \div 2$
Example	
1944.123314	19.06003249



Standard

[Protein] (mg/mL)	ABS
0.1	0.0204
0.5	0.1465
1	0.2432
2	0.4497
3	0.5916
4	0.9344
5	0.9482