Malaysian Journal of Microbiology, Vol 14(2) Special Issue 2018, pp. 137-144

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Proximate and biochemical analysis for marine and freshwater algae

Soopna Puspanadan[1], Wan Maznah Wan Omar[2], Lee Chee Keong[1*]

[1]Bioprocess Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia.
[2]School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia.
Email: cklee@usm.my

ABSTRACT

Aims: In this study, ten indigenous microalgae samples from freshwater and marine waters from Malaysia, cultured and analysed on proximate and biochemical analysis. The proximate and biochemical analysis consists of starch, carbohydrates, lipid, protein, ash and moisture contents. This study was more focused on screening of starch accumulation in marine and freshwater microalgae cultures.
Methodology and results: Based on screening, the results showed that *Chlorella salina* contents highest starch of 4.92±0.33%, followed by *Spirulina* sp. 2.58±1.18%, Isochrysis maritime 0.99±0.33%, and lastly for *Nitzschiapanduriformis* and *Naviculadistans* contents similar percentage of starch (0.44±0.10 and 0.40±0.07%, respectively). Besides starch analysis, proximate analyses (ash, moisture, lipid, protein, and carbohydrates) have been conducted. The results obtained indicated that all the cultures contain more than 4.50% of carbohydrates in average, followed by lipid and protein <1%. The results demonstrate that further optimization and various harvesting stages (early of exponential phase, early of stationary phase and late stationary phase) may increase lipid, carbohydrates, starch, and protein accumulation. *Chlorella salina* and *Spirulina* sp. will be used to further study on optimization of physical and chemical factors for high starch accumulation.
Conclusion, significance and impact of study: In conclusion, this experiment focused more on preliminary screening for further application of starch uses in food and food packaging industries.

Keywords: Marine and freshwater microalgae, proximate analysis, biochemical analysis

INTRODUCTION

The elevating demand for natural products to be utilized in sundry applications has incremented interest in algal biotechnology over the past two decades (Lee and Palsson, 1997; Chisti, 2007). Through algae biotechnology, various high-value compounds could be engendered and isolated from numerous phototrophic and heterotrophic microalgae cultures (Lorenz and Cysewski, 2000; Machado *et al.*, 2004). Microalgae efficiently convert CO_2 to potential biofuels, feeds and high-value by products using a small foot print (Chisti, 2007). Microalgae can grow on non-arable land and use non-potable water without displacing food crops. This growth is considered environmental friendly as microalgal biofuels can take advantage of nutrients in wastewater and CO_2 from power plants; while crop plants cannot use these resources (Chisti, 2007). Other factors, which should be considered simultaneously for sustainable biofuel production include but are not limited to: lipid and high added value chemicals production (e.g. for pharmaceutical or cosmetic industry), extraction economics (solvents, ultrasound application, electromagnetic field use, etc.), incineration/pyrolysis/gasification of residual biomass, its anaerobic digestion for biogas production, etc. microalgae are also excellent candidates for starch accumulation and production (feedstock for bioplastics production). These factors have been reviewed recently in Chisti (2007), Schenk *et al*., (2008) and Šoštarič *et al*., (2012).

Pulz and Gross (2004) reported that successful algal biotechnology mainly depends on culling the right alga with pertinent properties for categorical culture conditions and products. Large scale production of microalgal biomass therefore requires species, which also can tolerant of a wide range of conditions. Thus, microalgae can be harvested within a short span of time as compared to plants and crops and hence can meet the increasing demand of feedstock (Harun *et al*., 2010). Cultivation and environmental conditions are very important factors in microalgae growth and intracellular substance accumulation (Bartual and Gálvez, 2002; Yoon *et al*., 2008). The main motive of this study is to screen the proximate and biochemical analysis in marine and freshwater microalgae.

***Corresponding author**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

MATERIALS AND METHODS

Culture collection

Five marine and 5 freshwater microalgae cultures were obtained from Microalgal Culture Laboratory of School of Biological Sciences and School of Industrial Technology, Universiti Sains Malaysia. For freshwater microalgae cultures there are *Spirulina* sp., *Ankistrodesmus* sp., *Microcystis* sp., *Chlorococcum* sp., and *Chlorella vulgaris*. While for marine microalgae cultures there are *Chlorella salina*, *Tetraselmis* sp., *Isochrysismaritima*, *Nitzschiapanduriformis* and *Naviculadistans*.

Strain activation and inoculum preparation

Three types of media were used which are BG-11 (Stanier *et al.*, 1971), Zarrouk and Conway/Walne's (Walne, 1970) medium. Each microalga from stock solution will be grown in 100 mL of media under illumination at 258 Lux (3.612 µmol photons $m^{-2}s^{-1}$) by heatless white fluorescence lights with 12 h dark:12 h light at room temperature of 28±2 °C.

Screening and growth profile of microalgae

The resulting cultures with initial biomass concentration in the range of 0.50-0.60 OD (at the measurement 680 nm) was used as inoculums by transferring 10% of the inoculums to freshly prepared 900 mL of media. The strains were sampled every day for determination of cell growth by spectrophotometer at OD_{680} for 20 days. After 20 days of cultivation, algal biomass was determined as total dry weight. The entire marine and freshwater microalgae cultures been harvested at the late exponential phase by spun at 6,000 rpm for 10 min (4 °C) (Dragone *et al*., 2011). After that pellet obtained was washed twice with sterile distilled water and sent for freeze drying before analysed for moist and ash, starch, protein, lipid and carbohydrates content.

Moist and ash determination

Moist and ash were determined using the method described in NREL laboratory analytical procedures (Laurens, 2015). Crucibles pre-conditioned in the 575 °C muffle furnace overnight to remove any combustible contaminants. After conditioning is complete, crucibles removed from the 575 °C furnace and cool to room temperature in desiccators (under vacuum). Weight of each crucibles been recorded. About 50 ± 5 mg of prepared algal biomass was weighed out into the pre-weighed crucible. The weight of the crucible and sample were recorded. The samples were placed into a convection drying oven at 60 °C ± 1 °C for 18 h. Thereafter, the samples were taken out from oven and leaved in desiccators to cool to room temperature. The weight was recorded. The percentage moisture on a dry weight basis was calculated as follows equation 1:

$$\%\ Moisture\ = 100\ - \frac{Weight_{crucible\ dry\ sample}\ - Weight_{crucible}}{Weight_{sampleas\ received}} \times 100$$

Equation 1 (Laurens, 2015)

Same sample that been used for the ash analysis. Using an ash burner and a clay triangle on a stand, the crucibles containing the oven-dried sample heated until smoke appears. The smoke immediately ignited and allows the sample to burn until no more smoke or flame appears. The crucible was allowed to cool on a suitable surface before placing in muffle furnace. The cooled samples were placed in the muffle furnace at approximately 575 °C ± 25 °C for 24 h. The pre-ignited samples handled with care while placing them in or taking out to avoid sample loss. The ashed samples were removed from muffle furnace and allow cooling to room temperature in desiccators. The crucible and ashed samples weighed and the weight was recorded. The percentage of ash on a dry weight basis calculated as follows equation 2:

$$\%\ \mathrm{Ash}\ = \frac{(\mathrm{Weight}_{\mathrm{crucible+ash}}\ - \mathrm{Weight}_{\mathrm{crucible}})}{\mathrm{ODW}_{\mathrm{sample}}} \times 100$$

Equation 2 (Laurens, 2015)

Protein determination

Protein extraction

Protein extraction has been done using method by Price (1965) with modifications. 5 mg of freeze-dried microalgae material was weighed out. The analysis was carried out in triplicate. Samples were resuspended by vortexing in 0.2 mL 24% (w/v) TCA. Homogenates were incubated in a water bath at 95 °C for 15 min in an Eppendorf tubes and allowed to cool to room temperature. The samples were diluted to 6% (w/v) with the addition of 0.6 mL deionized water. The homogenates were centrifuged at 15,000 rpm for 20 min at 4 °C and their supernatants discarded. The pellets were resuspended in 0.5 mL Lowry Reagent by repeated pipetting and incubated at 55 °C for 3 h. The samples cooled to room temperature, centrifuge at 15,000 rpm for 20 min room temperature and the supernatant frozen at –20 °C for further analysis.

Protein quantification

Method of Lowry *et al*., (1951) as modified by Price (1965) been used for protein quantification. 50 µL of the protein (supernatant) was added to 1.5 mL centrifuge tubes in triplicate, followed by 950 µL of Lowry Reagent followed by immediate mixing by inverting the tubes. Samples were left in room temperature for 10 min. Then, 100 µL of the diluted Folin-Ciocalteu phenol reagent was added to each tube and mixed by vortex. Samples incubated at room temperature for 30 min. After incubation, the absorbance of each sample was read at 600 nm (Findlay, 1990; Walker, 2002). The percent protein on a dry weight basis calculated as follows equation 3:

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

$$\%\ Protein = \frac{Protein\ content\ from\ BSA\ standard\ (mg)}{Microalgae\ sample\ (mg)} \times 100$$

Equation 3 (Findlay, 1990; Walker, 2002)

Lipid determination

Lipid contains determined by using method by Bligh and Dyer (1959). The weight of 5 mL glass vial been taken followed by 50 mg dry algal sample weighed and added. Volume of 1.5 mL of $CHCl_3$/MeOH (1:2, v/v) was added into the vial and vortexes it vigorously for 5 min. Volume of 0.5 mL of $CHCl_3$ was added and the vial vortexes again for 5 min. Then 0.5 mL of H_2O was added into vial and vortexes it for 5 min. The vial was centrifuged at 3000 rpm for 10 min, and the lower organic layer was transferred to a new glass vial and the solvent was evaporated in a fume hood for 24 h. The vial of extracted lipids was dried in a freeze dryer, and the vial was weighed.

Starch determination

The starch contents of microalgae cultures were determined using the total starch assay procedure from Megazyme (2009). 10 mg dry samples weighed in triplicate into 15 mL centrifuge tubes. Twenty µL aqueous ethanol (80% v/v) and 0.2 mL Dimethyl sulfoxide (DMSO) added and vortex. After vortexing, the tubes placed in a boiling water bath for 5 min. Two hundred ninety µL 3-(*N*-morpholino) propane sulfonic acid (MOPS) and 10 µL thermostable *α*-amylase added, mixed well by vortexing, and the tubes incubated in a boiling water bath for 12 min. Four hundred µL sodium acetate buffer and 10 µL amyloglucosidase added (vigorously vortex) and the tubes incubated at 50 °C for 30 min. Distilled water added to adjust sample volumes to 10 mL. Mixed completely by using vortex, then spin at 3000 rpm for 10 min. One hundred µL of the supernatants transferred to glass vials. Three mL glucose oxidase/peroxidase (GOPOD) reagent added, followed by incubation at 50 °C for 20 min. D-glucose controls (contained 100 µl D-glucose standard solution and 3 mL GOPOD reagent) and reagent blanks (contained 100 µl water and 3 mL GOPOD reagent) and the samples incubated at 50 °C for 20 min. The absorbances of the samples were taken using spectrophotometer at 510 nm against reagent blank. The starch content calculated as per the equation 4:

$$\%\ Starch = \Delta A \times \frac{F}{W} \times FV \times 0.9$$

Equation 4 (Megazyme, 2009)

where,
∆A = Absorbance (reaction) read against the reagent blank
F = 100 µg of D-glucose/absorbance for 100 µg of glucose (conversion from absorbance to µg)
FV = Final volume (10-100 mL)
W = Weight (mg) of sample used

Carbohydrates determination

For carbohydrates determination, 10 mg of freeze dried sample was weighed in a 15 mL centrifuge tube. Then, 0.5 mL of concentrated acetic acid was added and incubated in an 85 °C water bath for 15 min. A volume of 10 mL of acetone was added. The sample was then centrifuged at 4000 rpm for 10 min (25 °C). The upper layer was discarded into a waste jar. A volume of 5 mL of 4 M Trifluoroacetic acid (TFA) was added to the decolorized algal sample, incubated in a boiling water bath for 4 h. During incubating, the sample tube was vortexed every 30 min. After 4 h of incubation, distilled water was added to make up a total volume of 10 mL. A volume of 20 µL of hydrolysed sample was pipette into a glass vial and was placed in a cold water. A volume of 0.9 mL sulfuric acid–phenol reagent was added to the tube and vortex. The tube was incubated in a boiling water bath for 20 min. After that, the tube was placed in an ice bath until the sample is cool. The absorbance of the samples was read using the spectrophotometer at 490 nm against reagent blank (Dubois *et al*., 1951, 1956; Grandy *et al.,* 2000; Laurentin and Edwards, 2003). The carbohydrates content was calculated as per the equation 5:

$$\%\ Carbohydrates = \frac{(Absorbance - Intercept)/Slope}{Sample\ weight\ (mg)/Total\ hydrolyzate\ (ml)} \times 100$$

Equation 5 (Laurentin and Edwards, 2003)

Statistical analysis

Statistical analyses were carried out using the Statistical Package for the Social Science (SPSS) Version 22 for analyses of variance (ANOVA). Significance was defined at $P<0.05$. Three replications were performed for all experiments and analyses.

RESULTS AND DISCCUSIONS

Growth profile of microalgae

Growth profile been studied for all ten strains. The growth curve for all ten analysed strains were shown in Figures 1a and 1b. Almost all freshwater microalgae cultures reached at the late exponential on 9th day except for *Spirulina* sp. on 10th day. On the other hand, for marine microalgae cultures, *C. salina*, *I. maritima* and *Tetraselmis* sp. reached the late exponential phase on 7th day and followed by both diatoms cultures (*N*. *panduriformis* and *N.distans*) reaches late exponential on 9th day. Even though, the growth rate for marine microalgae is much faster than freshwater microalgae, but the biomass is still lower than freshwater cultures (high density growth). This can be seen when *C*. *vulgaris* and *Microcystis* sp. possess similar highest dried cell weight of 0.23g/L followed by *Spirulina* sp. of 0.20 g/L (Table 1). The dried cell weight obtained by the marine microalgae were below 0.12 g/L with the lowest was shown by *I*. *maritima* (0.06 g/L). Overall lower biomass detected in this present study may due to the cultivation conditions at current stage

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

(Screening) are not suitable. Microalgae completely rely on cultivation conditions on determining growth characteristics and composition. There are four major types of cultivation conditions for microalgae which are photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic cultivation (Chojnacka and Marquez-Rocha, 2004) However, the fastest biomass growth can be enhanced at the optimum conditions.

Moisture and ash determination

Table 1: Summary of moist, ash and dry weight analysis.

Cultures	Microalgae cultures (F:Freshwater, M:Marine)	% Ash	Dry weight (g/L)
Spirulina sp.	F	30.82±1.71	0.20
Ankistrodesmus sp.	F	61.45±29.36	0.17
Microcystis sp.	F	85.33±0.35	0.23
Chlorella vulgaris	F	57.48±3.15	0.23
Chlorococcum sp.	F	42.30±9.87	0.14
Chlorella salina	M	41.03±3.45	0.08
Tetraselmis sp.	M	29.92±3.83	0.07
Isochrysis maritima	M	5.80±1.59	0.06
Nitzschia panduriformis	M	27.25±0.62	0.07
Navicula distans	M	25.31±0.85	0.11

*Data reported as means ± standard deviation of triplicates.

As shown in Table 1, freshwater green microalgae contain much higher ash than marine green, diatoms and brown microalgae. Almost all the microalgae cultures rich in ash contents (except for *I. maritima*), ranging from 5.80% to 85.33%. *Microcystis* sp. contained highest percentage of ash contents, and highest biomass. The higher contains of ash in freshwater green microalgae results in its high biomass. Many studies been carried out on discovering characteristics of various species of microalgae. Green microalgae widely being used for the research and the results clearly showed that about 20% DW of the microalgae biomass was left after slow pyrolysis and became char (Wu *et al.*, 2012; Sanchez-Silva *et al*., 2013). Oxidation of the integrated minerals in microalgae into mineral oxides in the open-to-air furnace is the most likely contributor to these significant differences.

Protein determination

Testing for protein is necessary for clear picture on microalgae composition. The results obtained indicated that all the cultures have <1% protein content (Figure 2). Nevertheless, statistical analysis indicated that the protein content obtained by *C. vulgaris* (0.92%) was significantly higher ($P<0.05$) compared with all the tested microalgae strains. On the other hand, *Microcystis* sp. and *Chlorococcum* sp. showed no protein at all. This may due to the protein content was too low to be detected by the assay method. On top of that, it might due to; several substances may interfere with both the Lowry and Bradford method, such as phenol and phenolases (Mattoo *et al*., 1987), glusamine and detergents (Pterson, 1979) and flavonoids (Compton and Jones, 1985). These substances could affect analyses by either increasing the absorbance (overestimating values), or decreasing the measurements by in inhibiting the action of specific reagents (Clayton *et al.*, 1988).

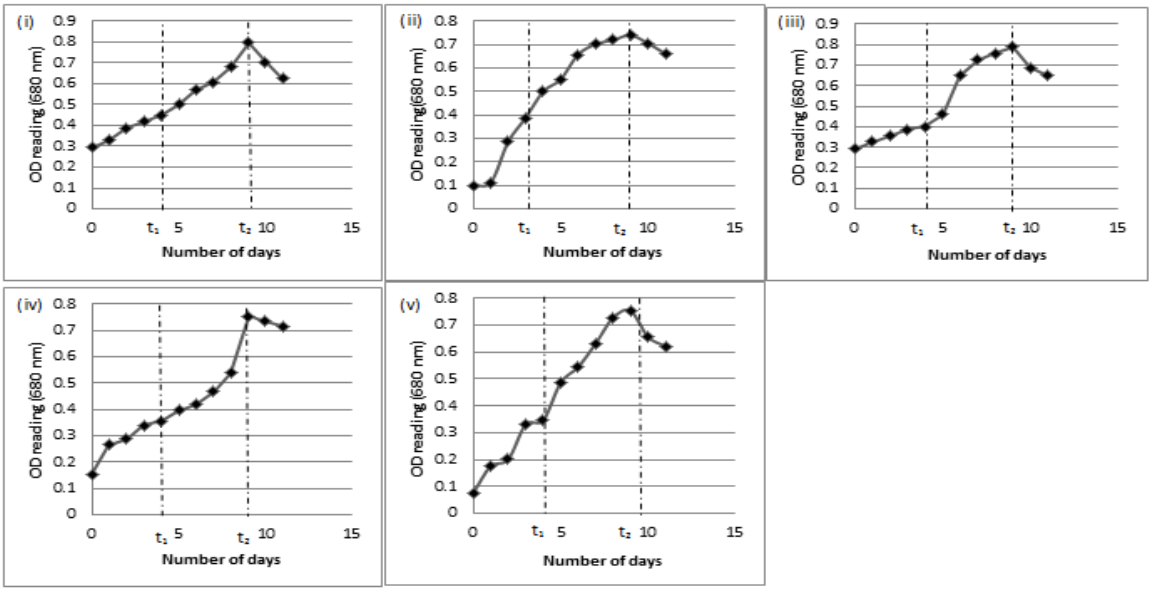

Figure 1a: Growth profile of five species of freshwater microalgae. (i) *Microcystis* sp. (ii) *Spirulina* sp. (iii) *Chlorella vulgaris* (iv) *Chlorococcum* sp. (v) *Ankistrodesmus* sp. t_1 indicates at the early of exponential phase follow by t_2 is late exponential phase. Data was reported as means of triplicates.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

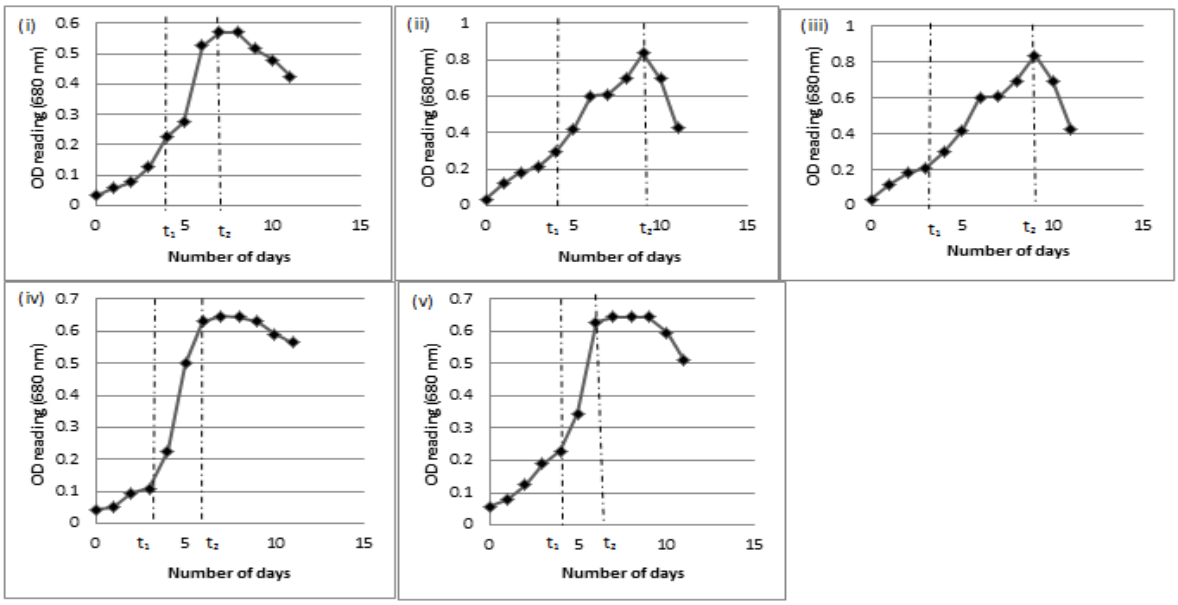

Figure 1b: Growth curves of five species of marine microalgae. (i) *Isochrysis maritima* (ii) *Navicula distans* (iii) *Nitzschia panduriformis* (iv) *Tetraselmis* sp. (v) *Chlorella salina*. t_1 indicates at the early of exponential phase follow by t_2 is late exponential phase. Data was reported as means of triplicates.

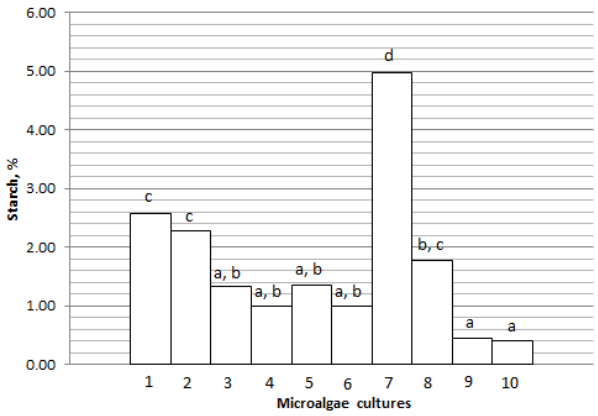

Figure 2: Protein contents (Percentage, %) of marine and freshwater microalgae culture. Data was reported as means of triplicates. Standard deviation has been removed for clearer bar chart. Means with the same letter indicated no significantly difference at 5% level of probability by Duncan Test.*1: *Spirulina* sp., 2: *Ankistrodesmus* sp., 3: *Microcystis* sp., 4: *Chlorococcum* sp., 5: *Chlorella vulgaris,* 6: *Isochrysis maritima*, 7: *Chlorella salina*, 8: *Tetraselmis* sp., 9: *Nitzschia panduriformis*, 10: *Navicula distans*.

Lipid determination

Although the lipid content detected by *Chlorella vulgaris* and *Microcystis* sp. are significantly higher ($P<0.05$) compared with others tested microalgae strains. This is due to fast growing species of algae contain lower amounts of lipids, whereas cells accumulating lipids grow slower (Xiong *et al.*, 2009). In this current research, all the strains contain less than 1% of lipid (Figure 3). This result was expected because most of the biochemical compounds, except for protein, increased when the culture aged, especially carbohydrate and lipid (Fidalgo *et al*., 1998; Lin *et al*., 2007; Chiu *et al*., 2009). Furthermore, phytoplankton may physiologically acclimate in response to variation in temperature, changing their biochemical composition or adjusting their membrane lipid to increase their capacity to grow or survive (Jiang *et al*., 2014). Besides that, data presented in the literature show that the lipid content in marine algae are less than 4% of the dry weight depending on the species (Herbreteau *et al*., 1997; McDermind and Stuercke, 2003). Previous studies show that nutrient limitations and environmental stress such as temperature manipulation, pH stress, different light intensity and high salinity were shown to induce the lipid accumulation in microalgae (Kalpesh *et al*., 2012; Rattanapoltee and Kaewkannetra, 2013).

Starch determination

Accumulation of starch can be induced by nitrogen reduction (Dragone *et al*., 2011), sulphur reduction, high light intensity (Brányiková *et al*., 2011) or a high CO_2 concentration (Izumo *et al*., 2007). The marine *Chlorophyta* phylum which is *C. salina* shows higher percentage (4.92%) and the productivity is 0.392 g/L of starch among all other tested strains ($P<0.05$), followed by freshwater *Cyanobacteria* microalgae *Spirulina* sp. (2.58%) and the productivity is 0.516 g/L (Figure 4). At this screening stage, none of the optimization tests have been

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

conducted. These two cultures were selected for further studied on starch accumulation at the stress stage. *C. salina* is significantly different from all other cultures as it does not appear in any subset together with any other cultures. These cultures been selected for further optimization based on SPSS analysis data.

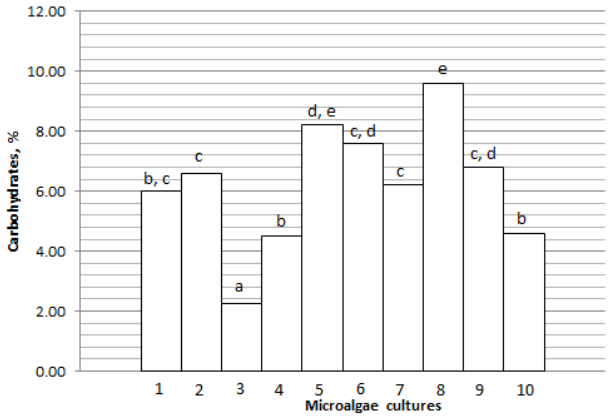

Figure: 3 Lipid contents (percentage, %) of marine and freshwater microalgae culture. Data was reported as means of triplicates. Standard deviation has been removed for clearer bar chart. Means with the same letter indicated no significantly difference at 5% level of probability by Duncan Test.*1: *Spirulina* sp., 2: *Ankistrodesmus* sp., 3: *Microcystis* sp., 4: *Chlorococcum* sp., 5: *Chlorella vulgaris,* 6: *Isochrysis maritima*, 7: *Chlorella salina*, 8: *Tetraselmis* sp., 9: *Nitzschia panduriformis*, 10: *Navicula distans*.

Carbohydrates determination

In this experiment, carbohydrates content obtained by *Tetraselmis* sp. are significantly different (P<0.05) compared with all the tested microalgae cultures except of *Chlorella vulgaris* (Figure 5). It is interesting to be found that, the relationship between growth rate and high carbohydrates correlated. Based on growth profiling, *Tetraselmis* sp. shows fastest growth rate, and in terms of carbohydrates accumulation carries highest percentage of carbohydrates compare to other nine other cultures. In general, the carbohydrate content detected by freshwater and marine microalgae are not varied differently. *Tetraselmis* sp. (marine water culture) contains the highest carbohydrates (9.58%) followed by *C. vulgaris* (8.20%) and the least is shown by *Microcystis* sp. (freshwater culture) with carbohydrate content of 2.27%. The values vary due to environmental conditions and usage of different media composition (location of isolates varies). Previous research shows that, accumulation of carbohydrates will be higher in microalgae is through reduction of nitrogen and phosphorus in the growth medium (Dragone *et al*., 2011; *Behrens et al*., 1989; Brányiková *et al*., 2011).

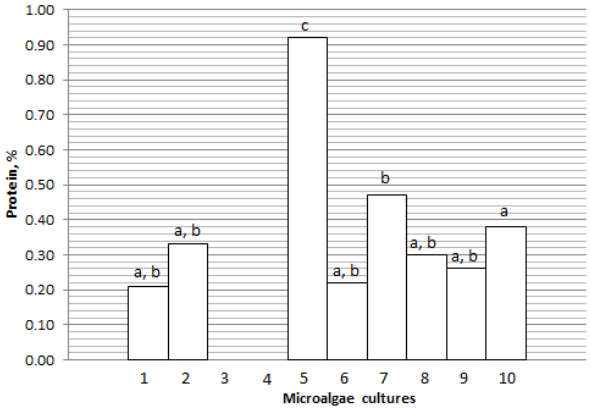

Figure 4: Starch contents (Percentage, %) of marine and freshwater microalgae cultures. Data was reported as means of triplicates. Standard deviation has been removed for clearer bar chart. Means with the same letter indicated no significantly difference at 5% level of probability by Duncan Test.*1: *Spirulina* sp., 2: *Ankistrodesmus* sp., 3: *Microcystis* sp., 4: *Chlorococcum* sp., 5: *Chlorella vulgaris,* 6: *Isochrysis maritima*, 7: *Chlorella salina*, 8: *Tetraselmis* sp., 9: *Nitzschia panduriformis*, 10: *Navicula distans.*

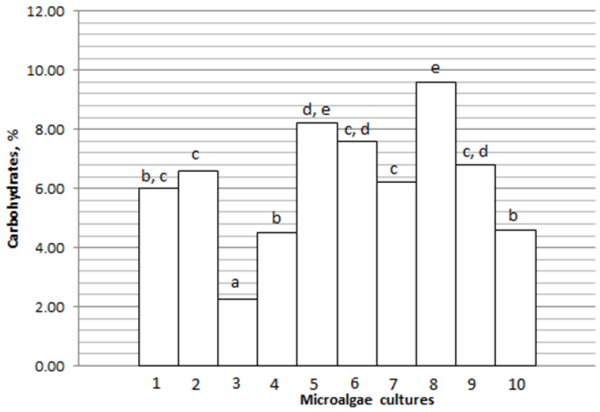

Figure 5: Carbohydrates contents (percentage, %) of marine and freshwater microalgae cultures. Data was reported as means of triplicates. Standard deviation has been removed for clearer bar chart. Means with the same letter indicated no significantly difference at 5% level of probability by Duncan Test.*1: *Spirulina* sp., 2: *Ankistrodesmus* sp., 3: *Microcystis* sp., 4: *Chlorococcum* sp., 5: *Chlorella vulgaris*,6: *Isochrysis maritima*, 7: *Chlorella salina*, 8: *Tetraselmis* sp., 9: *Nitzschia panduriformis*, 10: *Navicula distans.*

CONCLUSIONS

The overall aims of this research are to screen, determined and optimized the cultivation conditions of microalgae for high starch production and accumulation. Our data presented might low compare to literatures,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

however for ash, protein, carbohydrates, lipid and starch contents may also be reliant of seasonal period, geographical location and environmental conditions. The composition variety of the algal content was also reported for various species (Khotimchenko *et al*., 2002; Renaud and Luong-Van, 2006). In present stage of study, *S. platensis* and *C. salina* been chose for further study in order to enhance starch production. Optimization will be done on physical factors and chemical factors (Nutrient availability). So that high starch content will be obtained and consequently the overall cost for starch production will be reduced. With that it can directly lower the cost for bioplastics production.

ACKNOWLEDGEMENT

The author (Soopna a/p Puspanadan) would like to thank Ministry of Higher Education Malaysia (MOHE) for given her Scholarship [MyPhD (Mybrain15)]. The author (Lee, C.K.) would like to express his greatest appreciation and gratitude to USM for given him USM Research Universiti (RUI) grant (1001/PTEKIND/811273). Also, special thanks to lab assistant from School of Industrial Technology for allowing them to use the facility and equipment in the laboratory without any problems.

REFERENCES

Bartual, A. and Galvez, J. A. (2002). Growth and biochemical composition of the diatom *Phaeodactylum tricornutum* at different pH and inorganic carbon levels under saturating and sub saturating light regimes. *Botanica Marina* **45, 491-501.**

Behrens, P. W., Bingham, S. E., Hoeksema, S. D., Cohoon, D. L. and Cox, J. C. (1989). Studies on the incorporation of CO_2 into starch by *Chlorella vulgaris*. *Journal of Applied Phycology* **1, 123-30.**

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37(8), 911-917.**

Bránykiová, I., Maršalkova, B., Doucha, J., Branyik, T., Bišova, K., Zachleder, V. and Vitova, M. (2011). Microalgae-novel highly efficient starch producers. *Journal of Biotechnology and Bioengineering* **108,766-776.**

Compton, S. J. and Jones, C. G. (1985). Mechanism of dye response and interference in the Bradford protein assay. *Analytical Biochemistry* **151, 369-374.**

Chisti, Y. (2007). Biodiesel from microalgae. *Journal of Biotechnology Advances* **25(3), 294-306.**

Chiu, S. Y., Kao, C. Y., Tsai, M. T., Ong, S. C., Chen, C. H. and Lin, C. S. (2009). Lipid accumulation and CO_2 aeration. *Journal of Bioresource Technology* **100, 833-838.**

Chojnacka, K. and Marquez-Rocha, F. J. (2004). Kinetic and stoichiomaetric relationships of the energy and carbon metabolism in the culture of microalgae. *Journal of Biotechnology* **3,21-34.**

Clayton, J. R., Dortch, Q., Thoresen, S. S. and Ahmed, S. I. (1988). Evaluation of methods for the separation and analysis of proteins and free amino acids in phytoplankton samples. *Journal of Plankton Research* **10, 341-358.**

Dragone, G., Fernandes, B. D., Abreu, A. P., Vicente, A. A. and Teixeira, J. A. (2011). Nutrient limitation as a strategy for increasing starch accumulation in microalgae. *Journal of Applied Energy* **88, 3331-3335.**

Dubois, M., Gilles, K, Hamilton, J. K., Rebers, P. A., Smith, F. (1951). A colorimetric method for the determination of sugars. *Nature* **168, 167.**

Fidalgo, J., Cid, A., Torres, E., Sukenik, A. and Herrero, C. (1998). Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalga *Isochrysisgalbana*. *Journal of Aquaculture* **166,105-116.**

Findlay, J. B. C. (1990). Purification of membrane proteins. *In:* Protein purification applications: A practical approach. Harris, E.L.V. and Angal, S. (eds.). Oxword University Press, Oxword. **pp. 59-82.**

Grandy, A. S., Erich, M. S., Porter, G. A. (2000). Suitability of the anthrone–sulfuric acid reagent for determining water soluble carbohydrates in soil water extracts. *Soil Biology and Biochemistry* **32, 725-727.**

Harun, R., Danquah, M. K. and Forde, G. M. (2010). Microalgal biomass as a fermentation feedstock for bioethanol production. *Journal of Chemistry Technology and Biotechnology* **85, 199-203.**

Herbreteau, F., Coiffard, L. J. M., Derrien, Y. and De Roeck-Holtzhauer. (1997). The fatty acid composition of five species of macroalgae. *Journal of Botanica Marina* **40, 25-27.**

Izumo, A., Fujiwara, S., Oyama, Y., Satoh, A., Fujita, N., Nakamura, Y. and Tsuzuki, M. (2007). Physicochemical properties of starch in *Chlorella*change depending on the CO_2 concentration during growth: Comparison of structure and properties of pyrenoid and stroma starch. *Journal of Plant Science* **172, 1138-1147.**

Jiang, Y., Laverty, K. S., Brown, J., Nunez, M., Brown, Lou., Chagoya, J., Burow, M. and Quigg, A. (2014). Effects of fluctuating temperature and silicate supply on the growth, biochemical composition and lipid accumulation of *Nitzschia* sp. *Bioresource Technology* **154, 336-344.**

Kalpesh, K. S., Holger, S. and Peer, M. S. (2012). High lipid induction in microalgae for biodiesel production. *Energies* **5,1532-1553.**

Khotimchenko, S. V., Vaskovsky, V. E. and Titlyanova, T. V. (2002). Fatty acids of marine algae from the pacific coast of north California. *Journal of Botanica Marina* **45, 17-22.**

Laurens, L. M. L. (2015). Summative mass analysis of algal biomass-Integration of analytical procedures, Laboratory analytical procedure (LAP). Golden: National Renewable Energy Laboratory.

Laurentin, A. and Edwards, C. A. (2003). A microtiter modification of the anthrone-sulfuric acid colorimetric

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

assay for glucose-based carbohydrates. *Analytical Biochemistry* **315, 143-145.**

Lee, C. and Palsson, B. (1997). Light emitting diode-based algal photobioreactor with external gas exchange. *Journal of Fermentation Bioengineering* **79, 257-63.**

Li, Y., Han, D., Sommerfeld, M. and Hu, Q. (2011). Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp.(Chlorophyceae) under nitrogen-limited conditions. *Journal of Bioresource Technology* **102, 12-29.**

Lin, Y. H., Chang, F. L., Tsao, C. Y. and Leu, J. Y. (2007). Influence of growth phase and nutrient source on fatty acid composition of *Isochrysis galbana* CCMP 1324 in a batch photoreactor. *Journal of Biochemical Engineering* **37,166-176.**

Lorenz, T. R., and Cysewski, G. R. (2000). Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Journal of Trends Biotechnology* **18, 160-167.**

Lowry, O. H., Rosenbrough, N. J., Farr, A. R. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biology Chemistry* **193, 265-275.**

Machado, D. I. S., Cervantes, J. L., Hernandez, J. L. and Losada, P. P. (2004). Fatty acids, total lipid, protein and ash contents of processed edible seaweeds. *Journal of food chemistry* **85(3), 439-444.**

Mattoo, R. L., Ishaq, M. and Saleemuddin, M. (1987). Protein assay by Coomassie Brilliant Blue G-250-binding method is unsuitable for plant tissues rich in phenols and phenolases. *Analytical Biochemistry* **163, 376-384.**

McDermid, K. J. and Stuercke, B. (2003). Nutritional composition of edible Hawaiian seaweeds. *Journal of Applied Phycology* **15, 513-524.**

Megazymes. (2009). Total starch assay procedure (amyloglucosidase/α-amylase method). Megazyme International Ireland Ltd., Wickdow, Ireland.

Price, C. A. (1965). A membrane method for determination of total protein in dilute algal suspensions. *Journal of Bioresource Technology* **12, 213-218.**

Pterson, G. L. (1979). Review of the folin phenol quantification method of Lowry, Rosebrough, Farr and Randall. *Analytical biochemistry* **100, 201-220.**

Pulz, O. and Gross, W. (2004). Valuable products from biotechnology of microalgae. *Journal of Applied Microbiology and Biotechnology* **65,635-648.**

Rattanapoltee, P. and Kaewkannetra, P. (2013). Nile red, an alternative fluorescence method for quantification of neutral lipids in microalgae. *World Academy of Science, Engineering and Technology: International Journal of Biotechnology and Bioengineering* **7(9), 889-893.**

Renaud, S. M. and Luong-Van, V. J. (2006). Seasonal variation in the chemical composition of tropical Australian marine macroalgae. *Journal of Applied Phycology* **18, 381-387.**

Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., Kruse, O. and Hankamer, B. (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. *BioEnergy Research* **1(1), 20-43.**

Šoštarič, M., Klinar, D., Bricelj, M., Golob, J., Berovic, M., Likozar, B. (2012). Growth, lipid extraction and thermal degradation of the microalga *Chlorella vulgaris*. *Journal of New Biotechnology* **29(3), 325-33.**

Stanier, R. Y., Kunisawa, R., Mandel, M. and Cohen Bazire, G. (1971). Purification and properties of unicellular blue green algae (Order Chroococcales). *Bacteriological Reviews* **35,171-205.**

Walker, J. M. 2002. The proteins protocols Handbook (2nd edn). Humana Press Inc, Totowa, New Jersey.

Walne, P. R. (1970). Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera *Ostrea*, *Crassostrea*, *Mercenaria*, and *Mytilis*. *Fishery Investigation* **26, 162.**

Xiong, W., Gao, C., Yan, D., Wu, C. and Wu, Q. (2009). Double CO_2 fixation in photosynthesis–fermentation model enhances algal lipid synthesis for biodiesel production. *Bioresource Technology* **101, 2287-2293.**

Yoon, J. H., Shin, J. H. and Park, T. H. (2008). Characterization of factors influencing the growth of *Anabaena variabilis* in a bubble column reactor. *Bioresource Technology* **99, 1204-1210.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538