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CULTIVATION OF MICROALGAE IN MEDIUM CONTAINING PALM OIL MILL EFFLUENT AND ITS CONVERSION INTO BIOFUEL

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ABSTRACT

The production of renewable energy has become an important element in worldwide energy policy aimed to reduce greenhouse gases caused by fossil fuels. Biodiesel derived from transesterification of vegetable oil or animal fats, with alcohol in the presence of catalyst has been considered as one of the alternative resources to replace fossil fuels. However, the biodiesel produced from these materials may not be enough for total replacement; hence microalgae are used as another potential alternative due to their high photosynthetic efficiency and biomass productivity. In this study, a microalgae species i.e. Chlorella vulgaris *UMACC 001 having high oil to biomass ratio was cultured, harvested and characterised. It showed very high growth rate, μ (0.29 per day) and biomass productivity (0.14 mg litre per day). In addition, the resulting algal oil showed almost equal amount of saturated fatty acid (48.9%) and unsaturated fatty acid (51.1%). When converted to biodiesel, the ester content was 68.9% and comparable with that produced in previous study (71.0%). Thus,* Chlorella vulgaris *can be considered as a potential feedstock for biofuel production in the future.*

Keywords: biodiesel, microalgae, photosynthetic efficiency, *Chlorella vulgaris*, biomass productivity.

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INTRODUCTION

It was reported that the world oil reserves of about 1.3 trillion barrels shall be shrinking within the next 50 years if the oil utilisation is at a rate of 80 to 90 million barrels a day (Abdullah *et al*., 2007). To overcome this, a new source of renewable energy having high energy content and low carbon dioxide production should be introduced. The United Nation's Intergovernmental Panel on Climate Change has reported on a significant greenhouse gas emissions in the last 30 years, and due to increasing awareness on global warming, development of sufficient recourses for clean energy is important (Rogner *et al*., 2007). Biodiesel is believed to be the best candidate in helping to reduce greenhouse gas emissions as it is carbon natural, environmental-friendly, biodegradable and can be produced from edible or non-edible vegetable oils, animal fats and microalgae.

Biodiesel is a diesel substitute derived from non-toxic renewable sources. It has potential in reducing about 78%, 98% and 50% emissions of CO_2, SO_2 and particulate matters, respectively when it is burnt. It has lower combustion emissions profile, sulphur and aromatic contents that make it a better fuel

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compared to petroleum diesel. It can be used in the existing diesel engine with little or no modification, and can be homogeneously blended in any ratio with the conventional petroleum-based diesel fuel (Demirbas, 2009).

In reality, the first generation biodiesel from food crops cannot satisfy even a small fraction of the existing demand for transportation fuels. This has triggered an increased interest in developing second generation biodiesel from non-food feedstock, such as jatropha (Bhuiya *et al*., 2014) or waste cooking oils (Loh *et al*., 2006). Although second generation biodiesel does not typically affect the human food chain, and has increasingly been produced as a clean alternative fuel, it may still not be enough to cope with the demand for transportation fuels. For this reason, third generation biodiesel derived from microalgae, is increasingly becoming popular and advantageous as microalgae exhibit high photosynthetic efficiency, growth rate and biomass productivity. Microalgae may be a potential renewable feedstock that can meet the global demand for transportation fuels in the future as microalgae produce rapidly in shorter time and are capable of producing 10 times more oil than ordinary vegetable oils and the algal oil/biomass can be converted into biodiesel, bioethanol, biohydrogen and biomethane via thermochemical and biochemical methods (Golueke *et al*., 1956; Gouveia and Oliveira, 2009; Vairappan and Yen, 2009). However, biofuel production from microalgae comes with several challenges such as high energy inputs required for harvesting and dewatering biomass and lipid extraction and conversion processes (Raphael and Ausilio, 2013).

According to previous studies (Ranjith *et al*., 2015), microalgae oil requires extraction and purification by mechanical or chemical methods. The cell wall of microalgae composed of cellulose, chitin, murein, protein, silica and calcium carbonate. Several pre-treatment methods are required to break the algal walls *e.g.* milling, high pressure homogenisation, ultrasonication and microwave digestion before extracting the oil with solvent (Halim *et al*., 2011; Ranjith *et al*., 2015). With a wide range of the lipid polarity, different types of solvent can be used. The lipid fraction extracted from microalgae may change its content and composition based on the solvent polarity (Montes *et al*., 2011). Oil content in microalgae can vary widely according to strains. But, there is an alternative method where the reaction was performed directly that is known as *in situ* transesterification.

In Malaysia, there are many ranges of tropical microalgae. *Chlorella* is one of the most cultivated eukaryotic green microalgae for its robustness and simple growth requirement. It is grown mixotrophically with short doubling time. It contains biomass which is a good bioenergy source. In addition, it is also widely used as a health food and feed supplements as it contains protein, carotenoids, lipids, vitamins, antioxidants and minerals. The genus *Chlorella* contains several species with different lipid production capabilities *i.e. Chlorella vulgaris* (14%-22%), *Chlorella ellipsoidea* (4.49%) and *Chlorella pyrenoidosa* (2%-11%); all in dry weight basis. *Chlorella vulgaris* has been extensively studied for various purposes *e.g.* as a source of valuable chemicals or health food, wastewater treatment and aquaculture feed. Generally, green algae have bulk of saturated and unsaturated fatty acids similar to vegetable oils. The growth of algae is affected by nutrients, pH, salinity, temperature and light (duration and intensity). By varying the growth conditions *e.g.* limiting nitrogen sources, the lipids content may increase (Liang *et al*., 2009).

Under nitrogen stress conditions, many algae alter their lipid biosynthetic pathways resulting in the formation and accumulation of neutral lipids (Li *et al*., 2011). Biomass productivity depends on the feed composition and time needed to cultivate microalgal cells. In nitrogen rich environment, microalgae tend to utilise nutrients such as nitrogen and phosphorus to promote growth and fix CO_2, leading to a high productivity of proteins and carbohydrates. Microalgae are also able to grow in photobioreactor (either open raceway ponds or closed system) for large scale production (Pribyl *et al*., 2012). In particular, *Chlorella vulgaris* has great potential in capturing CO_2 and grow as fast as 0.6 g litre^{-1} per day in 10%-15% CO_2 condition. Besides, it also adapts to extreme environment *i.e.* high temperature (30°C-35°C) and acidic environment up to pH 3 (Converti *et al*., 2009).

If microalgae were to be a suitable biodiesel feedstock, its algal oil should exhibit quality fatty acids. Generally, *Chlorella* contains mainly C16-C18 fatty acids, which are suitable for biodiesel production (Xu *et al*., 2006). The biodiesel stability is influenced by the algal oil saturation level, which can be manipulated via adjusting the growth conditions. High level of unsaturation is undesirable as it tends to undergo oxidation during biodiesel storage (Schenk *et al*., 2008). Hence, the aim of this study was to evaluate the potential of *Chlorella vulgaris* as biodiesel feedstocks through cultivation in an inexpensive carbon rich waste *i.e.* palm oil mill effluent (POME) at different growth conditions.

MATERIALS AND METHODS

Microalgae *Chlorella* Growth and Culture Medium

The microalgae, *Chlorella vulgaris* (UMACC 001) from the Culture Collection of the Universiti Malaya, Kuala Lumpur, Malaysia was cultivated in 5% POME. Ten percent innoculum size from the total culture volume was used having an optical density

(OD_{620}) of 0.4 at 620 nm (equivalent to 0.15 mg litre^{-1} chlorophyll a (Chl*a*), $r^2 = 0.9$). Bold's Basal Medium (BBM) was used as a control. The microalgae was inoculated in a slant culture, followed by flask cultivation for 12 days (*Figure 1*). The flasks were incubated in a controlled environment at 25±1°C, illuminated with cool white fluorescent lamp (40 μmol photon m^{-2} s^{-1}) on 12:12 hr light-dark cycle and supplied with 100 ml filtered ambient air, and then transferred to an enclosed photobioreactor (PBR) in laboratory for further growth study for six days (Vello *et al*., 2013). Next, it was transferred to an outdoor enclosed PBR for 12 days. Two different types of culture system *i.e.* annular PBR and flat panel PBR were used (*Figure 2*). The selection was based on their structures which could facilitate: 1) handling during cultivation, 2) culture accumulation as bigger surface areas were contacted to the light source and no contamination by other competing species.

Microalgae Growth in Nitrogen Stress Condition

The effect of nitrogen stress was conducted based on the study by Lim (2016) where four units of PBR were used and two of them acted as control while the other two were used under stress conditions. For control samples, 400 ml of innoculum was transferred into 3.6 litres of BBM with additional of 10 ml of 1.2 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] as the pH buffer solution. Daylight fluorescent tube lamps were used that operated 12:12-hr light-dark cycle and the level of resultant irradiance was 60 μmol m^{-2} s^{-1}. The PBR was supplied with normal air through air syringe filters to aerate and mix the culture at 1.5 l min^{-1}. Two litres of the algal culture was harvested for every two days and replaced with 2 litres of fresh culture medium and 5 ml of 1.2 M HEPES. The samples were collected on Day 0, 2, 4, 6, 8 and 10 in triplicate for nitrogen content determination. The determination of nitrogen content for stress samples was similar to the control samples but during the harvesting process, 2 litres of algal culture was removed and replaced with 2 litres of modified N-free culture medium (*i.e.* without $NaNO_3$) and 5 ml of 1.2 M HEPES.

Growth Monitoring

The microalgae growth monitoring was conducted based on OD_{620} and Chl*a* concentration. The microalgae was extracted overnight with acetone, filtered and homogenised using 0.45 μm

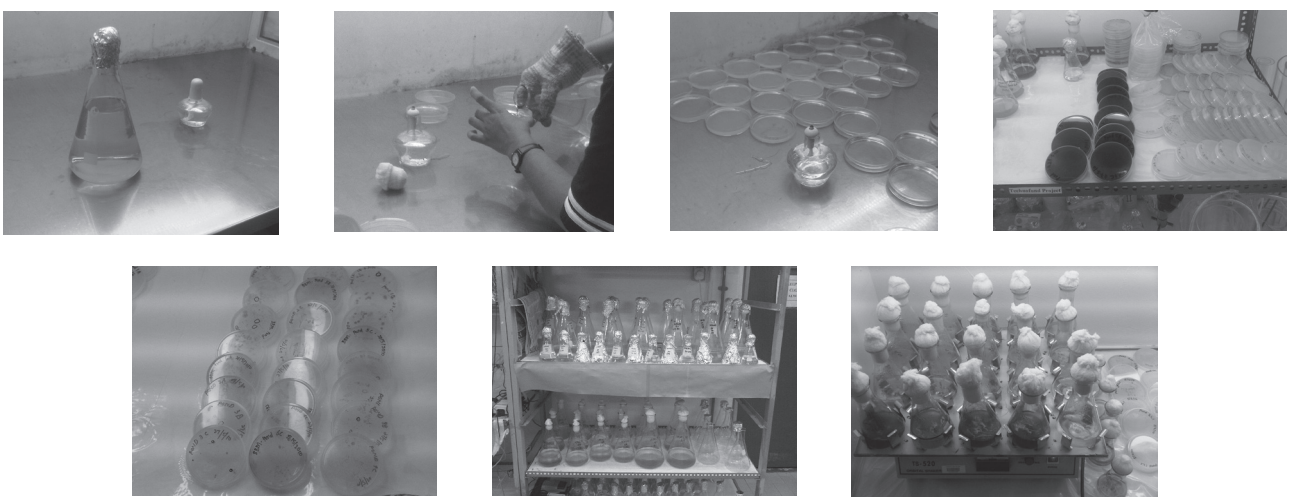
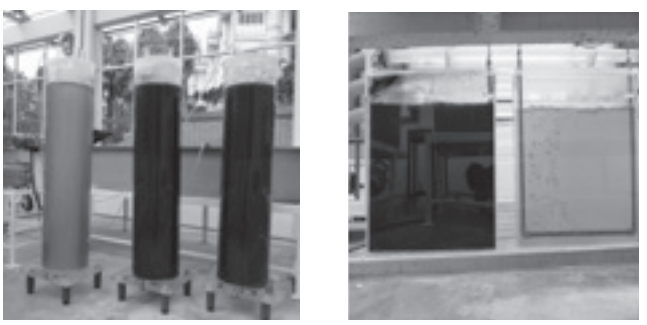


Figure 1. Isolation and culture grown in slant and conical flask.



Annualar PBR Flat panel PBR

Figure 2. Different types of outdoor culture system for Chlorella vulgaris *UMACC 001.*

filters (Whatman Gf/C). The Chl*a* concentration was determined at 665, 645 and 630 nm using a UV-vis spectrophotometer (Shimadzu UV 1700, Japan) based on Equation (1) by Strickland and Parson (1972).

Chl*a* = A x volume of acetone in ml/volume of sample in ml

where A (Absorbance) = 11.6 (OD_{665nm}) - 1.31(OD_{645nm}) - 0.14 (OD_{630nm}) Equation (1)

The specific growth rate (μ, per day) based on Chl*a* concentration was calculated based on Equation (2):

$\mu(day^{-1}) = Ln (N_2/N_1)/(t_2-t_1)$ Equation (2)

where N_2 is chlorophyll content (mg ml^{-1}) at t_2, N_1 is chlorophyll content (mg ml^{-1}) at t_1, and t_1, t_2 are times within the exponential phase.

Cells were harvested between late exponential to early stationary phase to determine dry weight, lipid content and fatty acid compositions (FAC) (Vello *et al*., 2013).

Determination of Biomass and Biochemical Composition

Solvent such as n-hexane was purchased from Sigma Aldrich (St Louis, MO, USA). Algal biomass was harvested via centrifugation and freeze-drying at -51°C, 10 x 10^{-4} atmosphere. Lipids were extracted via an established method *i.e.* in n-hexane using soxhlet extractor until the extracting hexane was colourless and determined by gravimetric method (Elmoraghy and Farag, 2014). The hexane was removed by rotary evaporator leaving the orange coloured soluble lipids as crude algal oil (*Figure 3*). The FAC of the algal oil extracted was analysed via gas chromatography (GC) according to the method used for algal ester as below.

***In situ* Transesterification of *Chlorella vulgaris* UMACC 001**

The dry microalgal biomass (30 g) was converted to fatty acid methyl esters (FAME) through *in situ* acid-catalysed transesterification at methanol to algal biomass ratio of 600:1 while molar ratio of algal biomass to sulphuric acid was 0:0.35. The reaction was performed at constant temperature of 60°C with a stirring rate of 350 rpm. Once the reaction had completed in 1 hr, water was added to neutralise the catalyst and stop the reaction. Then the mixture was centrifuged at 10 000 rpm for 10 min to separate the liquid (Velasques-Orta *et al*., 2012). The ester content of algal ester were analysed via GC. This analysis was determined according to ISO 5508: Animal and Vegetable Fat and Oil Analysis (1990) for FAC of the algal oil. Three drops of oils were accurately dropped into a 1.5-ml GC vial and n-hexane was added up to the level of 1.5 ml. The oil

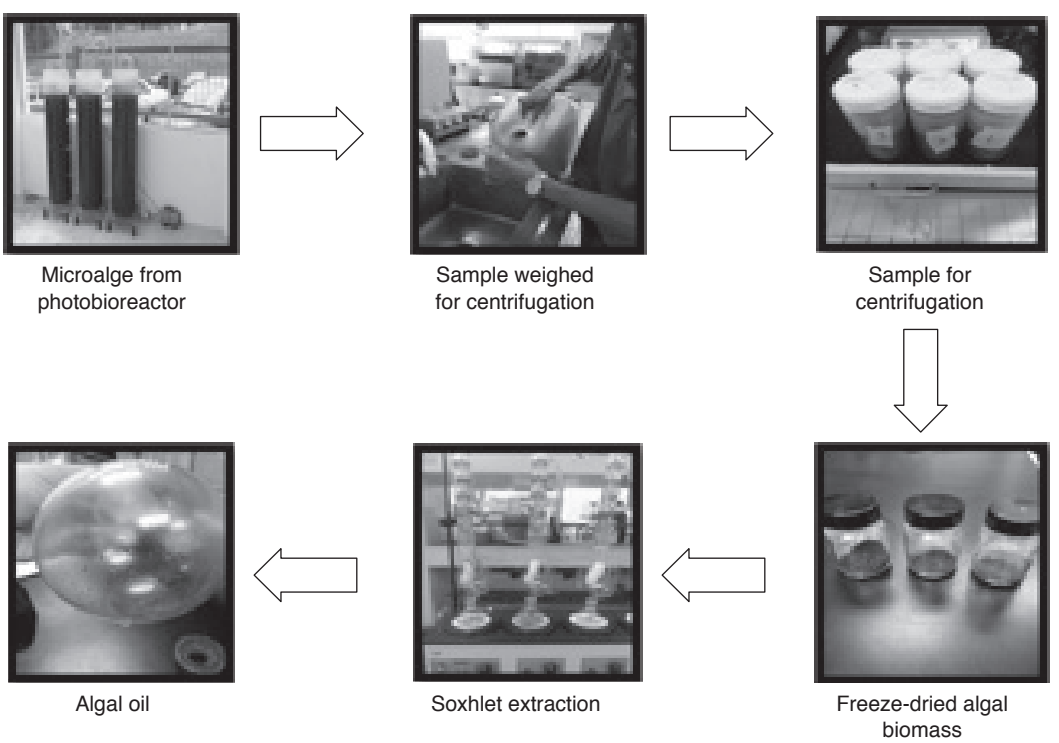


Figure 3. Harvesting and extraction of algal biomass.

mixture was then shaken vigorously until a double layer was formed before 1.0 μl of the upper layer of the oil mixture was injected into the GC.

The FAC was calculated using the following formula:

$$FAC = \frac{Ai}{\sum A} \times 100$$

where A*i* is the area under the peak corresponding to component *i*

$\sum$ A is the sum of the area under all the peaks

The ester content was measured according to EN 14103 (Fat and oil derivatives - Fatty Acid Methyl Esters-Determination of Ester and Linolenic Acid Methyl Esters Contents) by gas-liquid chromatography. Sample was accurately weighed (0.1 g) and methyl heptadecanoate solution was pipetted (2.5 ml) into a 10-ml vial and shaken vigorously and 1.0 μl of sample was injected into GC.

The ester content was calculated based on the following formula:

Ester content = $(\sum A\text{-}A_{E1} / A_{E1}) \times (C_{E1} \times V_{E1} / m) \times 100\%$

where $\sum A$ is the total peak area of the methyl ester from C14 to C24:1

A_{E1} is the peak area corresponding to methyl heptadecanoate;

C_{E1} is the concentration in mg ml^{-1} of the methyl heptadecanoate solution; and

V_{E1} is the volume in ml of the methyl heptadecanoate solution.

Both the analyses were conducted using Hewlett Packard 5890 Series II GC equipped with a flame ionisation detector and split injector. A fused silica capillary column (60 m × 0.25 mm) coated with a highly polar stationary phase, Supelco SP2340 (0.2 μm) was used with programmed temperature profile as follows: 185.0°C, injector temperature: 240.0°C, detector 240.0°C, split ratio: 1:100, carrier gas: helium at 2.0 ml min^{-1}. The whole of the components of the samples was assumed to be represented on the chromatogram, so the total of the area under the peaks represented 100.0% of the constituents.

RESULTS AND DISCUSSION

Chlorella vulgaris UMACC 001 Growth in Different Culture System

The growth of *Chlorella vulgaris* UMACC 001 in BBM in terms of OD_{620} in laboratory and outdoor annular PBR culture systems showed similar trend. The maximum 620 OD obtained were 1.57-1.64 respectively for the two systems. On the other hand, *Chlorella vulgaris* UMACC 001 cultured in 5% POME showed maximum OD_{620} of 1.45 in annular PBR and 1.81 in flat panel PBR. The algae culture showed exponential growth phase at the initial stage and reached the stationary phase at the sixth day of cultivation using PBR in BBM while in POME, the third day for annular PBR and the fourth day for flat panel PBR.

Although light source, medium used, nutrient source, *etc.* were found as the determining factors, there was no clear correlation between OD_{620} of the microalgae grown in different culture systems. Although the lights supply for outdoor conditions were higher compared to that in laboratory condition, their corresponding OD_{620} *i.e.* annular PBR using BBM (1.57±0.03) and annular PBR using POME (1.45±0.00) were lower (*Table 1*). In general, the flat panel PBR showed the highest OD_{620} at outdoor condition in medium enriched with POME due to the ability of *Chlorella vulgaris* UMACC 001 to accumulate biomass at an earlier stage. The maximum concentration of Chl*a* (*Table 1*) in laboratory and outdoor annular PBR were 27.97 μg

TABLE 1. OPTICAL DENSITY (OD_{620}), BIOMASS, CHLOROPHYLL A (Chl*a*) CONTENT AND SPECIFIC GROWTH RATE OF *Chlorella vulgaris* UMACC 001 CULTURED IN PHOTOBIOREACTORS (PBR) IN LABORATORY AND OUTDOOR CONDITIONS IN BOLD'S BASAL MEDIUM (BBM) AND 5% PALM OIL MILL EFFLUENT (POME)

System	Condition	OD_{620}	Biomass content (g litre^{-1}) (dwb)	Chl*a* (μg ml^{-1})	Specific growth rate (per day)	Biomass productivity (g litre^{-1} per day)
Annular PBR	Laboratory (BBM)	1.64±0.02	0.70±0.08	27.97±0.23	0.29	0.12±0.02
Annular PBR	Outdoor (BBM)	1.57±0.03	0.65±0.05	16.58±0.87	0.19	0.05±0.02
RAnnular PBR	Outdoor (5% POME)	11.45±0.00	0.80 ±0.00	15.0±0.00	00.19	0.11±0.00
Flat panel PBR	Outdoor (5% POME)	1.81 ±0.00	1.00±0.00	9.0±0.00	0.39	0.14±0.00

Note: dwb – dry weight basis.

ml^{-1} and 16.58 $\mu g\ ml^{-1}$ in BBM, respectively while annular and flat panel PBR in 5% POME were 5.0 $\mu g\ ml^{-1}$ and 9.0 $\mu g\ ml^{-1}$. The Chl*a* for the microalgae cultured in BBM were higher compared to those cultured in 5% POME. When comparing the Chl*a* using two types of culture medium (BBM and 5% POME) (*Table 1*), the highest specific growth rate of 0.39 per day was attainable for flat panel PBR in 5% POME at outdoor conditions. While the growth rate can be affected by the availability of nutrients and light, the stability of pH and temperature, the initial inoculums density which was dependant on the type of culture conditions used (open or closed system) plays an important role too. A denser inoculums population as in the closed systems employed in this study led to better algal growth and increased nutrient removal efficiency (Natesan *et al*., 2013).

Algal Oil Biomass and Biochemical Composition

The biomass yield (dry weight) were 0.70 g $litre^{-1}$ and 0.65 g $litre^{-1}$ for annular PBR in BBM at laboratory and outdoor conditions while those in outdoor culture with 5% POME, 0.8 g $litre^{-1}$ and 1.0 g $litre^{-1}$, respectively in annular and flat panel (*Table 1*). The biomass productivity was the highest in flat panel PBR with 5% POME (outdoor) (0.14 g $litre^{-1}$ per day) while annular PBR in BBM (outdoor) showed the lowest (0.05 g $litre^{-1}$ per day). Similar culture system (annular PBR) both at outdoor (5% POME) and laboratory conditions (BBM) showed compatible biomass productivity except for that in BBM (outdoor). This probably was due to long lag period in growth that might have been contaminated by other species (Chisti, 2007).

According to Griffiths and Harrison (2009), growth rate and oil content are factors that influence large-scale microalgae cultivation. At higher lipid content, the growth rate might be lower due to limitation of nutrients and light during stationary phase (after Day 3, 4 and 6 in this study) resulted in lipid accumulation. At the same time, the cell division and Chl*a* decreased due to limited source of nitrogen and acetate; thus the carbon source primarily used to build the cell of the algae species could not be realised.

TABLE 2. EFFECT OF NITROGEN STRESS ON BIOMASS AND LIPID PRODUCTION IN SELECTED MICROALGAE

		UMACC 001 (FW)	UMACC 301 (FW)	UMACC 253 (MW)	UMACC 258 (MW)
Specific growth rate (per day)	Control	0.225	0.226	0.177	0.150
	Nitrogen stress	0.190	0.223	0.130	0.145
Lipid content (% DW)	Control	26.88 ± 0.02	25.11 ± 0.02	23.45 ± 0.02	22.49 ± 0.01
	Nitrogen stress	58.98 ± 0.02	47.74 ± 0.02	49.05 ± 0.01	49.87 ± 0.01

Note: FW- freshwater, MW- marine water.

TABLE 3. COMPARISON OF FATTY ACID COMPOSITIONS (FAC) AMONG CRUDE ALGAL OIL FROM *Chlorella vulgaris* UMACC 001, ITS METHYL ESTERS AND OTHER BIODIESEL FEEDSTOCKS

FAC	Palm*	Soyabean (Chowdhury *et al*., 2007)	Rapeseed (Bocianowski *et al*., 2012)	Coconut (Dauqan *et al*., 2011)	Jatropha (Akbar *et al*., 2009)	*Chlorella vulgaris* oil*	*Chlorella vulgaris* methyl ester
C8:0	ND	ND	ND	6.6	ND	ND	ND
C10:0	ND	ND	ND	5.1	ND	ND	0.15
C12:0	0.2	ND	ND	46.5	ND	2.47	4.33
C14:0	0.7	ND	ND	20.6	0.1	1.71	3.15
C16:0	43.0	14.0	7.0	9.2	14.2	40.18	49.73
C16:1	ND	ND	0.2	ND	0.7	0.24	2.56
C18:0	4.1	4.1	7.0	2.9	7.0	4.52	1.74
C18:1	42.4	23.3	61.0	7.2	44.7	39.50	25.8
C18:2	9.5	52.2	20.0	1.6	32.8	9.96	5.05
C18:3	ND	5.6	10.0	ND	0.2	1.41	4.23
C20:0	ND	ND	ND	ND	0.2	ND	0.75
C20:1	ND	ND	1.0	ND	ND	ND	ND

Note: ND- not detected. *Analysed in this study.

Another factor that could possibly affect the growth and lipid content of algae species is the nutrient supplied *i.e.* N_2 and phosphorus (P). During nutrient starvation, the carbon present would be diverted towards lipid accumulation. In this case, the N_2 and P insufficiencies would promote lipid synthesis rather than inorganic carbon fixation and protein synthesis. In this study (Belotti *et al*., 2013), nitrogen stress culture condition was applied for 10 days. The results in *Table 2* show indeed the growth rate reduced while lipid productivity increased during nitrogen stress in selected microalgae from freshwater and marine water species.

Algal Oil and the Derived Biodiesel

Table 3 compares the FAC among several vegetable oils, algal oil from *Chlorella vulgaris* and its methyl esters. The algal oil from *Chlorella vulgaris* UMACC 001 contained predominately C16:0 and C18:1 totalling to 79.7% of the fatty acids present (*Table 3*). The commercial oils are dominated by C16:0 and C18:1 for palm, C18:1 and C18:2 for soyabean, C18:1 and C22:0 for rapeseed, C12:0 and C16:0 for coconut and C18:1 and C18:2 for jatropha. Hence, the algae oil assembled FAC of palm oil with almost an equal amount of saturated fatty acid (48.9%) and unsaturated fatty acid (51.1%). When the oil was converted into FAME via *in situ* transesterification it showed more saturated fatty acids (53.1%) than unsaturated ones (36.2%) dominated by C16:0, C18:0, C18:1, C18:2 and C18:3 (*Table 3*).

This information though was limited could help predicting the performance of the algal oil-based FAME, *e.g*. better oxidative stability and higher cetane number in the presence of more saturated fatty acids. There were changes in the proportion of individual fatty acid in FAME *i.e.* increased composition of C12:0, C14:0, C16:0, C16:1 and C18:3 while those of C18:0, C18:1 and C18:2 decreased compared to its oil counterpart. This was supported by the research of Kaur *et al*. (2012), He *et al*. (2013) and Santhoshkumar *et al*. (2015). The FAME produced had 69.9% ester content. This purity was comparable with those deriving from the same algae species by Velasques-Orta *et al*. (2012), 71.0%; Johnson and Wen (2009), 66.0% and Ehimen *et al*. (2010), 88.0%, respectively.

Generally, *Chlorella* was reported to have high content of C16:0 and C18:2 as the prokaryotic pathway is used for the synthesis of chloroplast lipids similar to higher plant. In this pathway, the C16:0 was desaturated to C16:3 for galactolipid production essentially required for photosynthesis and growth of microalgae. So, the high biomass productivity possessed by *Chlorella* was the result of higher reserve of C16:0 for galactolipid productions (Vello *et al*., 2013). Due to difficulties in extracting the algal oil, the big scale conversion into biodiesel was impossible. Attempted algal oil extraction using bioflocculants deriving from POME as a pre-concentration step has shown promising lipids accumulation in the culture medium (Nurul-Adela *et al*., 2015; 2016). Hence, future work should focus on this effective oil extraction approach to obtain enough biodiesel for fuel properties analysis.

CONCLUSION

The growth of *Chlorella vulgaris* UMACC 001 showed the highest OD_{620} when cultured in 5% POME using flat panel PBR at outdoor condition and so did its biomass productivity and specific growth rate. The FAC of algae oil from *Chlorella vulgaris* UMACC 001 assembled that of palm oil with almost equal amount of saturated (48.9%) and (51.1%) unsaturated fatty acid. The biodiesel produced via *in situ* transesterification had 69.9% ester content and its FAC were mainly C16 and C18. A more effective algal oil extraction method *e.g.* flocculation is required to confirm *Chlorella vulgaris* UMACC 001 as a suitable biodiesel feedstock via a single step conversion.

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