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PALM OIL MILL EFFLUENT AS ALGAE CULTIVATION MEDIUM FOR BIODIESEL PRODUCTION

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ABSTRACT

Palm oil mill effluent (POME) - a wastewater from the palm oil milling process is beneficial as a low-cost carbon source for microalgae growth. This does not only help clean the wastewater but also reduce the algal cultivation cost. In this study, the growth rate, biomass productivity and biochemical compositions of Chlorella *sp. grown in diluted POME under outdoor conditions using a 200-ml capacity high rate alga pond (HRAP) and two closed photobioreactors (PBR)* i.e. *annular and flat panel were assessed. The strain,* Chlorella *sp. grown on 5% of POME in a flat panel PBR exhibited the highest specific growth rate of 0.5 per day and biomass productivity (137.5 mg litre^{-1} per day) followed by those in HRAP and annular PBR. Additionally, a good growth of* Chlorella *sp. in POME could sufficiently utilise the nutrients of POME such as phosphate (PO_4), nitrate (NO_3), nitrite (NO_2) and organic substances. The extracted algal oil from the diluted POME culture (5%) showed decrease in the saturated fatty acids and an increase in the polyunsaturated fatty acids compared to those cultured in the standard Bold's Basal Medium (BBM). The biochemical compositions of the algae grown in the flat panel PBR were the highest with lipid, protein and carbohydrate productivity of 17.9 mg litre^{-1} per day, 34.7 mg litre^{-1} per day and 21.4 mg litre^{-1} per day, respectively. The microalgae cultivation in diluted POME had not only shown good potential as a biodiesel feedstock based on the fatty acids profile but also the ability to reduce some pollutants* e.g. *PO_4, NO_3, NO_2 and chemical oxygen demand (COD) in the biological wastewater treatment.*

Keywords: wastewater treatment, photobioreactors, biomass productivity, specific growth rate, COD reduction.

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INTRODUCTION

In Malaysia, the palm oil industry is an important economic force contributing significantly to the nation's revenue streams. The industry involves a planted area of 5.74 million hectares and a processing capacity of 453 palm oil mills. In 2016, the crude palm oil (CPO) production was 17.3 million tonnes (MPOB, 2016). Malaysia is one of the largest CPO producers and exporters accounting for 12% of the world's oils and fats production and 27% of world export (MPOC, 2013). Generally, the palm oil extraction process requires a lot of water and consequently generates large amounts of palm oil mill effluent (POME) (Wang *et al*., 2015; Loh *et al*., 2013). For each tonne of CPO, 5-7.5 t of water are

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required and more than 50% of the water ends up as wastewater known as POME.

POME is a thick brownish liquid that contains high level of suspended solids, oil and greases with chemical oxygen demand (COD) and biological oxygen demand (COD) (Hancsók *et al*., 2007) that can pollute a water source if discharged untreated (Loh *et al*., 2013). Conventionally, it is treated in a series of anaerobic and aerobic ponds that require more than 100 days of treatment. This type of treatment system is not able to meet the required discharge limits. The high content of organic materials and nutrients in POME makes it possible as a low-cost substrate for cultivating microalgae. Microalgae utilises the organic carbon material and nutrients in POME for its growth, thus removing some of these contaminant sources of pollution (Azmi and Yunos, 2014). Microalgae can survive in various types of wastewater. They are able to perform different types of metabolism *i.e.* autotrophic, heterotrophic, mixotrophic and photoheterotrophic depending on the surrounding environment (Brennan and Owende, 2010).

Additionally, microalgae offers an interesting alternative feedstock for the production of biofuels. Microalgae has a high total biomass yield and utilises lower land areas which are unsuitable for food production. It also has the potential to utilise any emitted CO_2 for photosynthesis which then offers a carbon neutral biofuel (Chen *et al*., 2011). However, the microalgae poses a sensitive characteristic *i.e.* vulnerable to the changes in culture conditions which might become a major drawback in the production of algal oil that is capable of meeting the biodiesel standards. Other environmental factors such as climatic conditions especially temperature and culture management techniques may influence the up-scaling of microalgae for mass cultivation in outdoor or closed photobioreator (PBR) (Moreno-Garcia *et al*., 2017; Mata *et al*., 2010; Idris *et al*., 2017).

Chlorella, one of the most studied microalgae, is commercially cultivated by more than 70 companies worldwide mainly as dietary supplements and nutraceuticals (Guccione *et al*., 2014). It is commercially grown under photoautotrophic conditions in open ponds (both raceway and circular), or heterotrophically in fermenters (Guccione *et al*., 2014). However, most *Chlorella* strains can grow mixotrophically in short doubling times and simple growth requirements (Rai *et al*., 2014). Previous studies using *Chlorella* strains cultured in several types of PBR have demonstrated a high lipid productivity up to 330 mg litre^{-1} per day due to a high biomass production rate (Pribyl *et al*., 2012; Idris *et al*., 2017). This indicates that most *Chlorella* strains are able to produce biomass in high quantity. Quality feedstock from microalgae for conversion to biodiesel demands not only high biomass content and lipid productivity but also a suitable fatty acid compositions (FAC). To date, high production cost is a serious setback for the production of biofuel from microalgae. Among the potential microalgae species, *Chlorella* is of major interest for biofuels owing to its ability to accumulate large amounts of lipids under stress (Idris *et al*., 2017). Thus, this study aimed to compare the growth of *Chlorella* UMACC 283 in different cultivation system using POME-enriched medium and assess the resulting algal oil as biodiesel feedstocks.

MATERIALS AND METHODS

POME

POME was collected from the raw and anaerobic ponds from Tennamaram Estate Processing Mill and characterised for pH, COD, ammoniacal-N, phosphate-P, nitrate, nitrite, total solid (TS), total suspended solid (TSS) and total volatile solid (TVS). Algae from POME were isolated and purified for further characterisation.

Cultivation Condition

The microalgae, *Chlorella* (UMACC 283) from the University of Malaya culture collection was cultured in 6.25% (v/v), 12.5% (v/v), 25.0% (v/v) and 50.0% (v/v) POME in 600-ml flasks at laboratory conditions as mentioned by Vello *et al*. (2013), while that of 5.0% (v/v) POME cultivation at outdoor conditions was chosen based on the previous studies (Idris *et al*., 2017; Tan *et al*., 2016) showing optimum values as follows: chlorophyll a (Chl-a) concentration (27.4 mg litre^{-1}), dry weight (1296.7 mg litre^{-1}) and specific growth rate of 0.84 per day (*Figure 1*). Three types of outdoor PBR were used namely high rate algal ponds (HRAP), annular and flat panel PBR (*Figure 2*). Bold's Basal Medium (BBM) was used as a control. For the outdoor cultivation, the algal culture was harvested and replaced with fresh culture medium every two days. Samples were also collected for growth analysis by assessing the optical density (OD_{620}), Chl-a, carotene content and biochemical compositions *i.e.* protein, carbohydrate and lipid contents (Vello *et al*., 2013).

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 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)

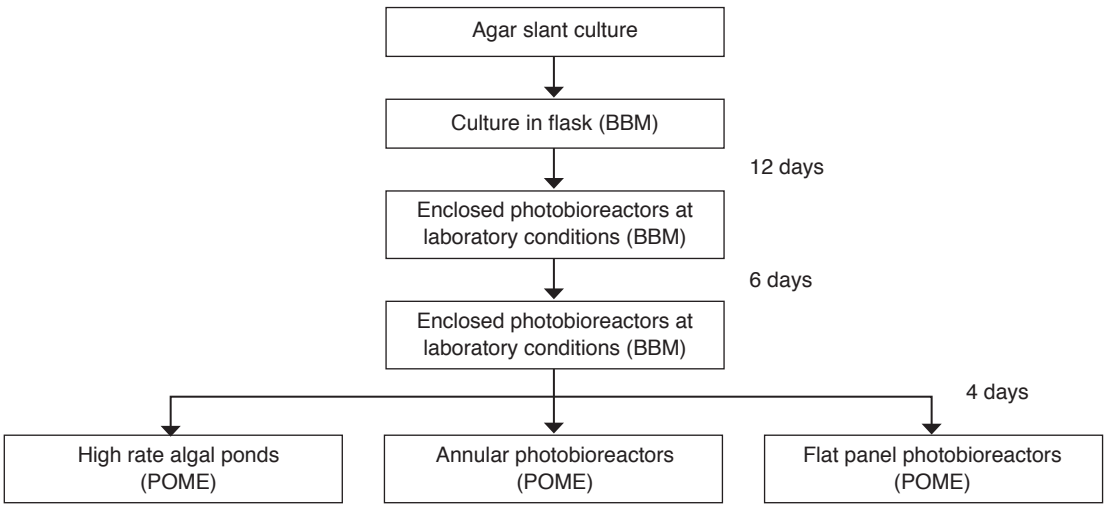


Figure 1. Cultivation condition for Chlorella *UMACC 283 using 5% palm oil mill effluent (POME).*

Figure 2. Different outdoor photobioreactor (PBR) for Chlorella *UMACC 283.*

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_{665}) - 1.31 (OD_{645}) - 0.14 (OD_{630}) 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 cultured in PBR was harvested via centrifugation, then freeze-dried at -51°C and $10x10^{-4}$ atm. The dried biomass was extracted in n-hexane using soxhlet extractor until the hexane was colourless and the lipid content determined via gravimetric method (Elmoraghy and Farag, 2014). The hexane was removed by rotary evaporator leaving the orange lipid as crude algal oil.

FAC Analysis

The lipids were transesterified in 1.2% (w/v) HCl in MeOH, toluene, and water (100°C, 1 hr) (Ichihara and Fukubayashi, 2010). The extracted fatty acid methyl esters (FAME) were stored in an inert atmosphere (N_2) in a freezer at -18°C. The

composition of FAME in the sample (1 µl) was analysed using the Agilent 7820A GC equipped with a capillary column (SLB-IL100, 30 m × 0.25 mm × 0.20 µm, Supelco, USA) and a flame ionisation detector with temperatures of the injector and detector set at 250°C and 260°C, respectively. The following thermal ease of 5°C min^{-1} until it reached 260°C. Helium was used as carrier gas at 4.41 ml min^{-1}. The hydrogen gas and purified air were flowed at 30 and 450 ml min^{-1}, respectively. The quantification of FAME was performed in triplicate by adding an internal standard (C7:0 Sigma®, USA).

RESULTS AND DISCUSSION

The characteristic of POME (*Table 1*) clearly showed that POME contained high N and P nutrients that are of interest and potential for microalgae growth. These nutrients could support photosynthesis reaction to produce algal biomass consisting of lipid, protein and carbohydrate for conversion into valuable products *e.g.* biofuel, bioethanol or feed supplement (Hadiyanto *et al*., 2012; Hadiyanto and Nur, 2012). According to Griffiths and Harrison (2009), a large-scale cultivation of microalgae for biofuel production is dependent on growth rate and oil content (in % dwb). As POME is rich in COD, BOD, N and P, it has been used in culturing different microalgae (Kamyab *et al*., 2015; Vairappan and Yen, 2008).

Microalgae cultivated in different POME concentration showed higher biomass and lipid content (440 mg $litre^{-1}$ to 627 mg $litre^{-1}$ and 15.9% to 30.9%, dwb) compared to that in standard medium (BBM) with 257 mg $litre^{-1}$ of biomass and 23.8% (dwb) lipid content (*Table 2*). The POME concentration that gave the highest growth rate of 0.32 per day for *Chlorella* UMACC 283 was 12.5%, followed by that in 6.25% POME at laboratory scale. POME contains a variety of biocompounds that can stimulate microalgal growth (Nwuche, 2014). Besides, POME is also high in inorganic components such as nitrate (NO_3), and phosphate (PO_4) that are easily accessible as nutrients in microalgae cultivation (Vairappan and Yen, 2008). It is undeniable that lipid productivity is an important factor in microalgae species selection for biodiesel production (Huerlimann *et al*., 2010). It correlates with biomass productivity and can be a useful indicator to select the feedstock for biodiesel production (Vello *et al*., 2013).

The higher the concentration of POME, the lower the growth of *Chlorella* due to inadequate light penetration due to the darker colour of POME. The problematic colour appearance in POME may probably caused by the present of tannic acid (Hadiyanto and Nur, 2012). Moreover, algae cultured in highly concentrated POME took a longer time to reach the stationary phase and generated lower biomass due to longer lag phase (Nur and Hadiyanto, 2013). It was found also that separation of the algal biomass from POME was difficult when higher POME concentrations were employed. Besides, the lipid content was found the highest in the medium with 12.5% POME.

TABLE 1. CHARACTERISTICS OF PALM OIL MILL EFFLUENT (POME) USED IN THIS STUDY

Parameter*	Concentration (mg $litre^{-1}$)
pH	4.9±0.1
Chemical oxygen demand (COD)	51 233±246
Total solid	55 356 ± 257
Total suspended solids	35 648 ± 214
Chlorophyll a	0.19 ± 0.01
Carotenoid	0.08± 0.01
Ammoniacal nitrogen	26 ± 1
Nitrate	753 ± 15
Nitrite	3.5 ± 0.3
Phosphate	102 ± 2

Note: * Units in mg $litre^{-1}$ except for pH.

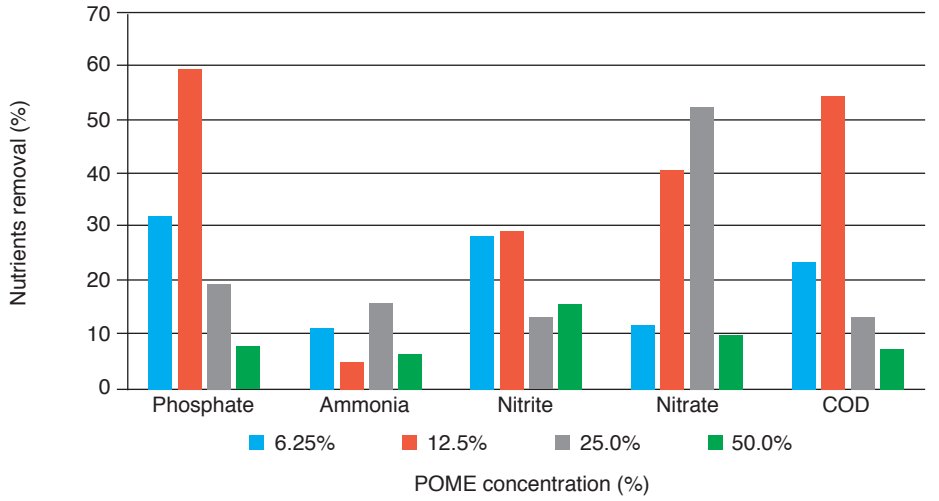
TABLE 2. GROWTH OF *Chlorella* UMACC 283 IN DIFFERENT CONCENTRATION OF PALM OIL MILL EFFLUENT (POME) IN LABORATORY CONDITIONS

Treatment	Biomass (mg $litre^{-1}$)	Lipid content (%)	Specific growth rate (per day)	Biomass productivity (mg $litre^{-1}$ per day)	Lipid productivity (mg $litre^{-1}$ per day)
BBM (control)	256.7±35.1	23.8±3.9	0.28±0.06	10.4±2.2	0.32±0.13
6.25% POME	440.0±36.1	26.8±5.6	0.31±0.03	19.5±3.1	0.71±0.28
12.5% POME	590.0±26.5	30.9±10.1	0.32±0.06	27.1±1.4	1.00±0.34
25% POME	533.3±62.6	15.9±1.3	0.07±0.08	16.1±10.1	0.34±0.17
50% POME	626.7±63.9	20.9±3.9	0.19±0.06	11.7±7.1	0.13±0.29

Note: BBM - Bold's Basal Medium.

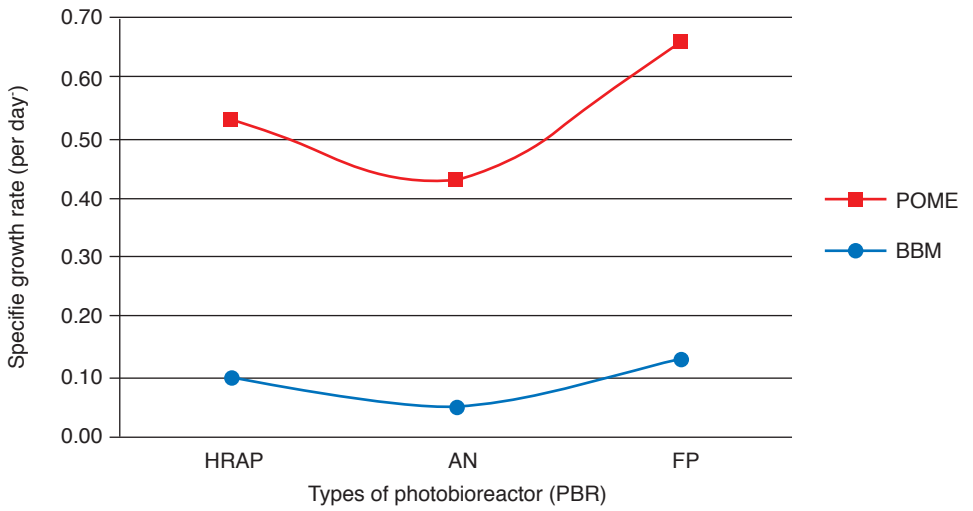
Nutrient removal is important in wastewater treatment because rich nutrient streams may resulted in eutrophication and phosphorus accumulation (Dalrymple *et al*., 2013). Cultivation of *Chlorella* UMACC 283 in 12.5% POME concentration showed potential in pollution reduction (*Figure 3*) as most of the 'polluting nutrients' *e.g.* PO_4, NO_3, NO_2 and organic substances in the form of COD were successfully reduced with removal efficiencies of 59%, 29%, 40% and 55%, respectively. This may to certain extent help in improving the quality of wastewater in use.

In cultivating *Chlorella* UMACC 283 at outdoor PBR using 5% POME, the exponential growth phase was achieved in Day 3. The growth of this *Chlorella* in Day 4 is shown (*Figure 4*). Basically, POME medium showed better carbon source for *Chlorella* UMACC 283 growth than the BBM. The highest biomass production was obtained when *Chlorella* UMACC 283 was cultured using 5% POME in annular PBR (2.27 g litre^{-1}) followed by HRAP (1.06 g litre^{-1}) (*Table 3*) and flat panel PBR (0.87 g litre^{-1}), respectively. However, the growth of *Chlorella* UMACC 283 in flat panel PBR showed the highest specific growth rate (0.53 per day) and Chl-a (7.62 mg litre^{-1} per day). In addition, Badar *et al*. (2017) performed a study in a laboratory scale PBR at room temperature under continuous illumination for 14 days and 10% v/v POME discovered that the specific growth rate for *Chlorella sorokiniana* UKM3, *Coelastrella* UKM4 and *Chlorella* UMACC324 were 0.20 per day, 0.22 per day and 0.19 per day respectively. The growth of *Chlorella* UMACC 283 in this study was much higher. The variation of growth rates is usually related to the algal growth conditions and requirements (Juneja *et al*., 2013).



Note: COD - chemical oxygen demand.

Figure 3. Nutrients removal efficiencies of Chlorella *UMACC 283 cultured in palm oil mill effluent (POME).*



Note: HRAP - high rate algal pond. BBM - Bold's Basal Medium.
AN - annular.
PP - flat panel.

Figure 4. Specific growth rate of Chlorella *UMACC 283 in 5% palm oil mill effluent (POME) via outdoor cultivation.*

For biochemical study, the highest productivities for carbohydrate (21.4 mg litre^{-1} per day) protein (34.7 mg litre^{-1} per day) and lipid (17.9 mg litre^{-1} per day) were obtained from flat panel PBR in 5% POME (*Table 4*). Hence, flat panel PBR was found the highest performing system in culturing *Chlorella* UMACC 283 in 5% POME. The biochemical productivities from HRAP also were not bad, but with a reduced 47% in carbohydrate (11.4 mg litre^{-1} per day), 37% in protein (21.8 mg litre^{-1} per day) and 30% in lipid (12.5 mg litre^{-1} per day). Besides, although the HRAP open pond system showed good biochemical compositions, it has several limitation such as high contamination risk and space requirement (Narala *et al*., 2016) making it less suitable for large scale production.

The lipid and biomass productivity during microalgae cultivation should be assessed appropriately to facilitate obtaining a suitable species for biodiesel production (Vello *et al*., 2013). The choice of utilising algal biomass depends on its constituents and may impart the economic of biodiesel production (Hempel *et al*., 2012) *e.g. Chlorella* and *Spirulina* (Arthrospira) contain high amounts of protein (50% to 60%, dwb) that favour coproduction of food, feed supplements and fertiliser (Lammens *et al*., 2012). *Chlorella* was also reported to accumulate high amount of carbohydrate *i.e.* > 40%, dwb and has potential for bioethanol and biogas production (Lee *et al*., 2015). This should be investigated in-depth to reduce the overall cost of production to make microalgae an alternative in wastewater treatment more feasible.

The FAC profiles of the lipid extracts of *Chlorella* UMACC 283 (*Table 5*) grown in 5% POME were compared with those grown in BBM. Both cultivation medium showed that the palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) were the predominant fatty acids in the microalgae grown. The highest amount of C16:0 was obtained when the microalgae was cultivated in annular PBR followed by flat panel PBR while for C18:0 by HRAP, all in BBM. Cultivation using 5% POME showed mainly the changes in unsaturated fatty acid compositions.

The algal oil from all the different cultivation systems exhibited more saturated fatty acids than the unsaturated ones (*Table 6*). Cultivation in diluted POME (5%) could reduce the saturation portion of fatty acids while that of unsaturation increased slightly. In BBM cultivation, the saturation level of the algal oil varied and could not be predicted. In-depth study is required to confirm if the different culture system using POME had indeed influenced

TABLE 3. THE GROWTH STUDY OF *Chlorella* UMACC 283 IN 5% PALM OIL MILL EFFLUENT (POME) VIA OUTDOOR CULTIVATION

Day	System	Biomass content (dwb) (g litre^{-1})	Chlorophyll a (mg litre^{-1})	Specific growth rate (per day)
0 (control)	BBM	0.07±0.01	1.5 ± 0.03	-
0 (control)	POME	0.81±0.01	1.6 ± 0.03	-
4	HRAP (BBM)	0.13±0.00	1.67±0.05	0.10
4	HRAP (POME)	1.06±0.04	5.57 ± 0.03	0.43
4	AN (BBM)	0.12±0.00	1.30 ± 0.19	0.05
4	AN (POME)	2.27±0.06	4.68± 0.42	0.38
4	FP (BBM)	0.11±0.00	2.08± 0.09	0.13
4	FP (POME)	0.87±0.01	7.62± 0.5	0.53

Note: dwb - dry weight basis. BBM - Bold's Basal Medium. HRAP - high rate algal pond. AN - annular. FP- flat panel.

TABLE 4. THE BIOCHEMICAL COMPOSITIONS OF *Chlorella* UMACC 283 IN 5% PALM OIL MILL EFFLUENT (POME) VIA OUTDOOR CULTIVATION

Day	System	Carbohydrate productivity (mg litre^{-1} per day)	Protein productivity (mg litre^{-1} per day)	Lipid productivity (mg litre^{-1} per day)
4	HRAP (BBM)	2.29	2.44	2.94
4	HRAP (POME)	11.4	21.8	12.5
4	AN (BBM)	0.83	1.25	1.69
4	AN (POME)	10.4	9.17	6.56
4	FP (BBM)	3.03	4.29	5.22
4	FP (POME)	21.4	34.7	17.9

Note: BBM- Bold's Basal Medium. HRAP- high rate algal pond. AN- annular. FP- flat panel.

TABLE 5. FATTY ACID COMPOSITION (wt%) OF *Chlorella* UMACC 283 IN 5% PALM OIL MILL EFFLUENT (POME) VIA OUTDOOR CULTIVATION

Day		C4:0	C6:0	C:8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C20:0	C22:0	C24:0	C14:1	C16:1	C18:1	C20:1	C18:2	C18:3	C20:3	C20:4	C20:5	C22:2
4	HRAP (BBM)	0.4	0.2	0.3	0.2	0.4	0.9	43.0	35.0	0.5	0.1	0.1	0.4	2.6	3.1	0.6	3.7	7.0	0.0	0.1	0.1	1.4
4	HRAP (POME)	0.4	0.2	0.5	0.0	0.4	0.8	42.0	18.7	0.6	0.3	0.4	1.0	2.6	15.1	0.4	11.4	4.9	0.3	0.0	0.0	0.0
4	AN (BBM)	0.1	0.1	0.0	0.0	1.0	1.6	48.5	12.3	0.7	0.2	0.4	0.7	1.3	22.4	0.3	6.8	3.6	0.2	0.0	0.04	0.04
4	AN (POME)	0.6	0.2	0.0	0.0	0.7	1.3	38.5	21.4	0.5	0.2	0.3	2.1	4.0	6.5	0.3	9.8	13.7	0.0	0.0	0.1	0.0
4	FP (BBM)	0.7	0.8	0.6	0.9	0.5	2.3	45.3	26.2	0.5	0.0	0.0	1.0	3.7	4.8	0.6	5.4	6.4	0.1	0.4	0.0	0.0
4	FP (POME)	0.4	0.3	0.3	0.2	0.3	3.2	42.3	21.7	0.5	0.3	0.7	1.4	4.1	6.5	0.3	8.8	8.6	0.2	0.1	0.0	0.0

Note: BBM - Bold's Basal Medium. HRAP - high rate algal pond. AN - annular. FP- flat panel.

TABLE 6. SUMMARY OF SATURATED AND UNSATURATED FATTY ACID OF *Chlorella* UMACC 283 IN 5% PALM OIL MILL EFFLUENT (POME) AT DIFFERENT CULTIVATION SYSTEM

System	Saturated fatty acid (wt%)	Unsaturated fatty acid (wt%)
HRAP (BBM)	81.1	17.9
HRAP (POME)	64.2	35.8
AN (BBM)	64.8	35.2
AN (POME)	63.6	36.4
FP (BBM)	77.7	22.3
FP (POME)	70.1	30.0

Note: BBM - Bold's Basal Medium. HRAP - high rate algal pond. AN - annular. FP - flat panel.

the production of compositional algal lipid suitable as biodiesel feedstock.

In all cases, the high saturated fatty acids of the algal oil were indicator of a good quality biodiesel with high cetane number, higher energy yield and superior oxidative stability which might cause less problems in fuel polymerisation during combustion (Knothe, 2012; Wood *et al*., 2015). Probably, these is a need to blend this algal oil with two (binary) or even more different microalgae oils to attain an optimum FAC for biodiesel production as suggested by Cha *et al*. (2011). On the other hand, the algal oil cultivated in diluted POME may have beneficial cold-flow properties with the increasing of unsaturation level.

CONCLUSION

POME was evaluated and found to be a potential medium in microalgae cultivation. The microalgae, *Chlorella* UMACC 283 cultivated in 5% POME produced high content of lipid and biomass showing good potential as a biodiesel feedstock. It's good growth in POME contributed to the reduction of nutrients such as PO_4, NO_2, NO_3 and COD which was important in wastewater treatment. In addition, the flat panel PBR showed better performing system in culturing *Chlorella* UMACC 283. The FAC of the algal lipid extracts showed an abundant of C16:0 and C18:0 with potential increase in unsaturation suitably used as a non-food type biodiesel feedstock.

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