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ARTICLE

Palm oil mill effluent as a potential medium for microalgae *Chlorella* sp. cultivation for lipid production

Karthick Murugan Palanisamy[1], Primilla Paramasivam[1], Gaanty Pragas Maniam[1,2], Mohd Hasbi Ab. Rahim[1], Rameshprabu Ramaraj[3], Natanamurugaraj Govindan[*1,2]

[1]Algae Culture Collection Center & Laboratory, Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, Lebuhraya Tun, Razak, 26300 Gambang, Kuantan, Pahang, Malaysia
[2]Earth Resources & Sustainability Centre, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang, Malaysia
[3] School of Renewable Energy, Maejo University, Chiang Mai, Thailand

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ABSTRACT

Fresh water microalgae *Chlorella* sp. was isolated from water body of Taman Gelora, Kuantan, Pahang. The isolated monoculture was cultured in different concentration of POME (10%, 20%, 30%, 40% and 50%, v/v) as substrate. The maximal growth and biomass productivity found in 30% (v/v) of POME. The maximal dry biomass 1.56 g/L was obtained and extracted 39.1% lipid which approximate similar productivity as in conventional BG-11 medium 1.65 g/L of biomass and 41.5% lipid was obtained. Gas chromatography mass spectrometry analysis shown composition of fatty acid such as linolelaidic acid, oleic acid, palmitic acid, stearic acid, elaidic acid and α-linoleic acid as dominant. Highest fatty acid has found to be linolelsidic acid (41.69%). POME has potential to be used as a substrate for microalgae *Chlorella* sp. cultivation.

1. Introduction

Microalgae are primarily a diverse group of microorgan-isms consisting of prokaryotic cyanobacteria and eukaryotic photoautotrophic protists (known as blue-green algae) (Ramaraj et al., 2010; 2016a,b; Venkatesan et al., 2015). Microalgae are single-cell species usually available in fresh and marine water (Khan et al., 2018; Tsai et al., 2016). The size estimated between in ranging from a few micrometres to a few hundred micro-metres (2×105 to 8×105) in size. Microalgae have the potential to be source of bioactive compounds in the form of lipids, carbo-hydrates, protein, vitamins, glycerol and carotenes (Khan et al., 2018; Ramaraj et al., 2013; 2014). Lipid productivity of micro-algae is higher as compared compare to other terrestrial plant (Chia et al., 2013; Khalid et al., 2018; Ramaraj et al., 2014; 2015a,b,c).

From the concept of resource conservation and nutrient recovery emerges the idea of growing microalgae in wastewaters (Tipnee et al., 2015; Unpaprom et al., 2015; Zhang et al., 2014; Saengsawang et al., 2020;). In addition, microalgae are capable of

* Corresponding author.
E-mail address: natanam@ump.edu.my (Natanamurugaraj Govindan)

absorbing nitrogen, phosphorus and carbon from wastewater, including adsorbing metals, and in many cases can be co-cultivated with various bacteria in municipal wastewater treatment plants (Zhang et al., 2014; Nwuche et al., 2014). Based on the Table 1. Composition of POME contain high nutrients such as nitrogen and phosphorus, which eventually useful for algae medium.

Nomenclature and abbreviation	
POME	Palm Oil Mill Effluent
COD	Chemical Oxygen Demand
TN	Total Nitrogen
TP	Total Phosporus
FAME	Fatty Acid Methyl Ester
NH_3-N	Ammoniacal-Nitrogen

The cultivation of microalgae in wastewater offers a low-cost tertiary treatment method, reduce the nutrient cost for cultivation and at the same time increases the production of microalgae biomass by supplying the nutrients and organic compounds required for the metabolism of microalgae (Hadiyanto and Nur, 2014). Organic effluent load decreases as a consequence of digestion and microalgae taking up the wastewater materials (Nwuche et al., 2014; Hadiyanto and Nur, 2014; Chin et al., 2020).

Therefore, this study focused on the biomass productivity for fatty acids by cultivating microalgae *Chlorella* sp. with POME wastewater. An optimal concentration condition for cultivation found and the biomass productivity and lipid composition were analyzed.

Therefore, the main aim of this study was to identify the POME enhances the growth of green algae (*Chlorella* sp.) and how based on the composition measured for control (*Chlorella* sp. culture without POME) and experiment (*Chlorella* sp. culture with POME). Besides that, the green algae reduce the chemical oxygen demand of POME also is investigated. Thus, the microalgae can expect to remove the pollution of POME.

2. Materials and methods

2.1. Isolation and identification

Freshwater microalgae samples were collected from the pond of Taman Gelora, Kuantan. The detail of water temp (27±2℃), pH (8.0±5), salinity (less than 1000 ppm) was recorded to ensure the compatible in laboratory scale. The water samples were centrifuged at 2,000 rpm, 15℃ for 5 min to concentrate the algal cells and eliminate the solid debris particles. samples were enriched by using a mixture of 90% (v/v) sample and 10% (v/v) in BG-11 medium. Samples were aerated with filtered air by using air pump for 24 h continuously. They were cultured until visible signs of algae growth especially flask with full green. The growth culture was diluted to 1 $\times 10^{-5}$ by distilled water. Streaked the diluted sample on BG-11 nutrient agar plate, sealed with parafilm wax strip and incubated for 15 days under 2000 lux light intensity and 26±1℃ for the formation of colonies (Bhuyar et al., 2019). After several streaking, the inoculums culture prepared by culturing with 10ml of liquid medium in pre-sterilized test tube. The morphology structures of isolated colonies were examined under microscope and identified by referring algae standard manual and previous study (Gao et al., 2019; Van Vuuren et al., 2006). Then started to scaled up to 100, 500, 1000, and 2000 ml if Erlenmeyer flasks. The purity of culture checked regularly in order to maintain as monoculture as shown in Figure 1.

2.2. Palm oil mill effluent characteristics

Palm oil mill effluent (POME) was obtained from a palm mill located nearby Gambang, Kuantan. Sample was filtered and eliminated the solid substance by using 0.45µm pore size membrane. The POME media was prepared by 500 ml of unsterilized POME diluted with 500 ml of distilled water and adjusted the pH 8.0±0.5 by adding 3.0g of sodium bicarbonate and 1M of NaOH buffer solution. These steps were followed as referring in previous studies (Hadiyanto and Nur, 2014; Govindan et al., 2019).The characteristics of wastewater have been measured according to the American Public Health Association on standard methods for water and wastewater study. The COD, TN and TP analysis of wastewater was performed through a spectrophotometer by APHA 5220 test method (Van Vuuren et al., 2006).

Table 1
Characteristic of POME by analysis.

Parameters	**Concentration (mg/l)**
pH	6.2
Chemical oxygen demand, COD	1671
Ammoniacal nitrogen, NH_3-N	14.7
Total nitrogen	135.5
Total phosphorus	12.1
Total suspended solid	77.5

2.3. Cultivation of Chlorella sp. in POME

The growth performance of *Chlorella* sp. was studied in different concentration of POME. In stock culture of *Chlorella* sp. POME medium was added in 10, 20, 30, 40 and 50% (v/v), making 1L as total volume Erlenmeyer flask. BG-11 medium used as control with 50% (v/v) of culture. The flasks were placed under 2500 lux light intensity, culture aerated by supplying filtered air at 28±2℃ for 24 hours.

2.4. Growth measurement

The growth of *Chlorella* sp. was studied in parameter like optical density and chlorophyll and dry biomass weight. Optical density of *Chlorella* sp. culture was determined by using spectrophotometer. The absorbance reading of the culture was takes at 680nm wavelength. Biomass was harvested by centrifuged at 7000 rpm, 20℃ for 10 min and measured dry weight every three days once from the POME concentration with maximal growth.

Chlorophyll extract was measured from the harvested dry biomass in different days (Sukumaran et al., 2014).

2.5. Lipid extraction

Lipid was extracted from the *Chlorella* sp. biomass by Soxhlet technique. The dry biomass was weighed and soaked in 50 ml of hexane for overnight as pre-treatment. The protocol was followed from previous studies (Wiyarno et al., 2014; de Jesus et al., 2014).The soaked sample was loaded in the thimble and it placed in the Soxhlet extractor. A 500 ml of round bottom flask took and clean it then filled with 250 ml of hexane. The whole setting was placed on a heating mantle and allowed the hexane to boil. The extraction process was carried out for 4 h. The refluxing started at 67 ± 3 ℃. The hexane solvent was vaporized, diluted, and repeatedly percolated through the dry microalgae samples until it exceeded the extraction was maximum. After extraction process was done for 4h, the condensing unit was removed from the extraction unit and allowed the sample to cool down. The mixture of lipid extract and hexane solvent were separated by using rotary evaporator at 110 rpm, 50℃ for 15 min. the collected lipid extract was weighed and determine the lipid productivity.

2.6. Transesterification of lipid

The lipid extract from *Chlorella* sp. contained the large macromolecules compounds and it breaks into micro molecules compounds by transesterification process. In 2 ml centrifuge tube, 0.5 g of lipid extract mixed 0.5 g of methanol KOH and vortexed for 30 sec. Then the tubes were emerged three quadrants of tube into ultrasonicated water bath at 58±2 ℃ for 2 h. Then, 1.0 g of hexane was added with it and vortexed for 2 min continuously. The mixture turns into two separated layers of solvents. The upper layer solvent was collected in the 2 ml centrifuge tube and evaporated the hexane by placing it under fume hood for 30 min. The remaining substance in the tube is fatty acid methyl ester (Hindryawati et al., 2014; Boey et al., 2009).

2.7. Fatty acid methyl ester content analysis

Fatty acid methyl ester obtained from transesterification were analysed in Agilent 7890A gas chromatography (GC) system equipped with capillary mega wax MS column (30 m length × 0.32 mm internal diameter × 0.50 µm film thickness). The mass spectrometer detector used with helium as the carrier gas 1ml/min. The oven programmed with the following time–temperature program: 190°C (2 min), 190–230°C (5°C/min), 230°C (8 min). Peak areas were quantified with Chrome card for Windows software. The method followed from previous study by Boey et al. (2009) and Chiu et al. (2020) to determine the fatty acid profile. About 40 mg of methyl ester sample was weighed in vial 1000 µL of internal standard dilute on octane 15 mg/ml concentration was added. In homogenous mixture form sample was injected into GC. The inlet temperature was maintained at 230°C with a split ratio of 50:1. The injection volume was 1 µl, with a split ratio of 50:1. FAME were determined by chromatographically comparing with authentic standards. By using heptadecanoic acid as an internal standard, each FAME were quantified from the peak areas on the chromatogram.

3. Results and discussion

3.1. Isolation and identification of Chlorella sp.

In this study, five species of microalgae were isolated after several re-streaking on the plate from fresh samples which collected from Taman Gelora, Kuantan. From fresh water, *Anabaena* sp., *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp., and *Tetraselmis* sp. were isolated and identified. These species were choose based on the growth rate and morphology identification under light microscope. As shown in Figure 1 obtaining single species of *Chlorella* sp. from mixture of various microalgae colonies by streaking plate technique was ease to perform under aseptic condition.

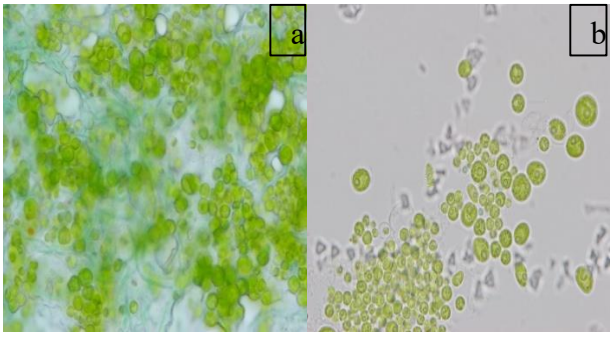

Fig. 1. Microscopic (a) observation of the isolated of various microalgae species and (b) identified microalgae strain *Chlorella* sp.

3.2. Cultivation of Chlorella sp.

Several concentrations of POME (%, v/v) were studied for determining the growth of *Chlorella* sp. and lipid production. As shown in Figure 2 the growth curve of *Chlorella* sp. in POME medium with different concentration (v/v). The culture was in lag phase in all the flasks in first two days of experiment where culture was adapting in the media environment. The maximal growth was found in 30% (v/v) of POME as compared to culture in other flaks. Based on Figure 3 maximal dry biomass of *Chlorella* sp. was obtained in early stationary phase was 1.56 g/l at day 12 in 30% (v/v) POME. The growth curve pattern of cultures in control medium, 20 and 30% (v/v) of POME medium were almost similar but culture in control and 20% (v/v) medium reached stationary and start the death phase whereas 30% (v/v) medium culture at early stationary phase of growth. Moreover, *Chlorella* sp. have seen growing on the inner surface of flask from day 5. This indicated that, *Chlorella* sp. requires sufficient light source from the surroundings to enhance the growth. The nutrient availability in the

30% (v/v) cause the culture to sustained the growth rate of *Chlorella* sp. Hadianyato et al.(2014) found that greater growth rate (0.31±0.06 d^{-1}) and productivity (0.034±0.01 g $L^{-1}d^{-1}$) of *Nannochloropsis* sp. cultured in 30% (v/v) fresh POME. Cheah et al. (2018) found that, the maximal biomass concentration obtained at 5% and 20% (v/v) of POME while culturing of microalgae *Chlorella vulgaris* (3.46 g/l) and *Chlorella sorokiniana* (3.30g/l). Ding et al. (2016) proposed that a lower concentration of POME would provide ample nutrient for algae to grow and increase light penetration into medium. These factors will simultaneously boost growth until the nutrients in POME have been completely exploited and the penetration of light decreased due to the density of the generated biomass.

Least growth of *Chlorella* sp. found in the POME above 40 and 50% (v/v). The highest concentration of POME does not support the growth of *Chlorella* sp. due to the minimal light penetration into the culture in flask. Increasing POME concentration significantly enhanced the concentration of productivity due to presents of more nutrient components. However, above 30% of POME did not enhance the biomass productivity in the medium (Sukumaran et al., 2014).The high concentration of POME did not support the microalgae growth and production (Sukumaran et al., 2014). The productivity of dry biomass determined from the optimum concentration of 30% (v/v) POME medium as shown in Figure 3. The dry biomass obtained from the BG-11 medium was 1.38 g/l. at day 12. Figure 3 shown the comparison between productivity of biomass in (30%) of POME and BG-11 medium. Both productivity approximately similar but, BG-11 shown greater biomass productivity than (30%) of POME.

Chlorophyll content of *Chlorella* sp. was measured from both medium. According to Figure 5, chlorophyll content found higher in BG-11 culture in first 6 days, however culture in POME 30% contain higher from day 9. The color changes in the biomass due to the process of chlorophyll degradation. During chlorophyll degradation mechanism, phaeophytin formation takes place. Phaeophytin compound is a form of chlorophyll and does not contain Mg^{2+}. The Mg^{2+} ion replaced by hydrogen ion, so the color expressed become yellowish (Prihantini et al., 2019).

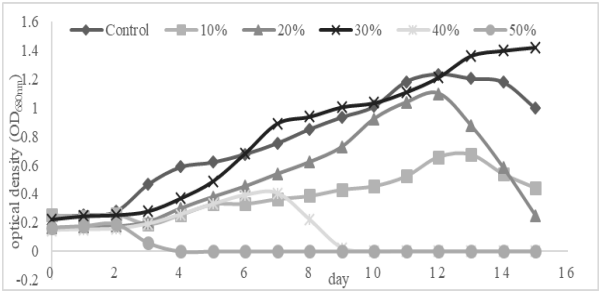

Fig. 2. Absorbance reading of *Chlorella* sp. in different concentration of POME

Soxhlet technique cause greater influence in the lipid extraction from *Chlorella* sp. Greater biomass productivity produced higher lipid extract ad shown in Figure 3. The maximal lipid yield extracted from *Chlorella* sp. biomass which harvested from 30% (v/v) culture is 39.1%. the lipid content obtained from the biomass culture control medium was 41.5%. The advantages of Soxhlet is as time increases, the lipid productivity increases from the biomass. This is because increased interaction between solvent and biomass paste by periodic siphoning allows the liquid to refresh itself continuously, maintaining a gradient of oil concentration between solvent and sample (Mahmood et al., 2017). It allows the oil to be solubilized. The advantage of immersion is that the solvent is more effective, because solvents with low polarity cannot remove all polar lipids when the method is applied for a short time (Mahmood et al., 2017; Kumar et al., 2017; Purkan et al., 2019).

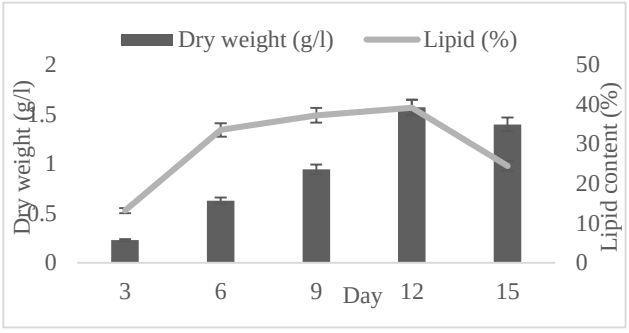

Fig. 3. Dry weight and lipid content of *Chlorella* sp. in 30% (v/v) POME culture

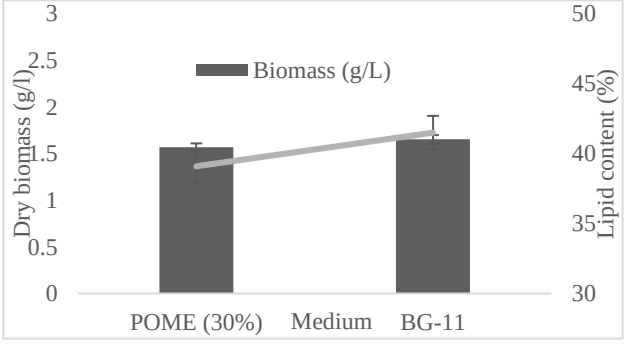

Fig. 4. Comparison of biomass and lipid productivity between POME and BG-11 medium

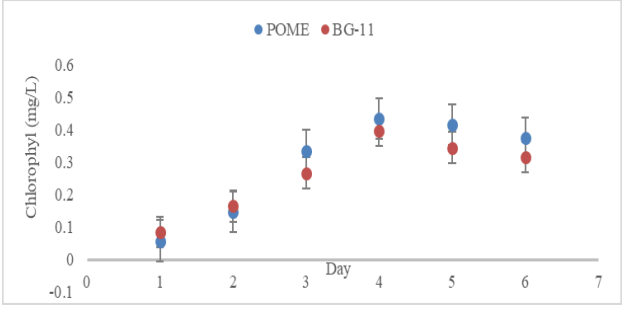

Fig. 5. Chlorophyll content of *Chlorella* sp. in POME (30%, v/v) and BG-11

3.3. Fatty acid methyl ester analysis

The fatty acid composition of *Chlorella* sp. was analysed in gas chromatography mass spectrometry. The fatty acid profile of *Chlorella* sp. was analysed from lipid extract which obtained from biomass of POME 30% (v/v) and BG-11 medium. Nine fatty acid methyl esters were identified as shown in Table 2. POME medium (50.87%) biomass contained higher polyunsaturated fatty acid than BG-11 medium (38.91%) whereas monounsaturated fatty acids more in BG-11 medium. On the other hand, growing under BG-11 yields higher saturated fatty acids (21.47%) and monounsaturated fatty acids (39.62%), as compared to the growth under POME medium, 19.03% and 30.10%, respectively (Table 2). The observation revealed that for producing polyunsaturated fatty acids, POME medium is comparatively the better choice. Chi et al. (2019) identified 11 fatty acids from *Chlorella* sp. such as capric acid, lauric acid, palmitic acid, palmitoleic acid, oleic acid, linolenic acid, linoleic acid, cis-11,14,17-eicosotrienoic acid, tricosonoic acid, cis-13,16-docosodienoic acid and cis-11,14-eicosodienoic acid. Composition of fatty acid do changing in cultivation nutrient media.

Table 2
Fatty acid profile of *Chlorella* sp. cultured in POME and BG-11 medium.

Fatty acids	POME medium	BG-11 medium
Tridecanoic acid	0.13	0.05
Pentadecanoic acid	0.95	0.41
Palmitic acid	12.63	14.30
Stearic acid	5.32	6.71
∑ Saturated fatty acids	19.03	21.47
Heptadecenoic acid	1.83	11.54
Elaidic acid	3.05	8.63
Oleic acid	25.22	19.45
∑ Monounsaturated fatty acids	30.10	39.62
α-Linoleic acid	9.18	3.04
Linolelaidic acid	41.69	35.87
∑ Polyunsaturated fatty acids	50.87	38.91

4. Conclusions

This study was conducted to determine the viability of POME as a medium for growth for microalgae. The experiment result showed that POME has potential to be used as a substrate for microalgae *Chlorella* sp. cultivation. The cultivation of microalgae in wastewater offers a low-cost tertiary treatment method, reduce the nutrient cost for cultivation and at the same time increases the production of microalgae biomass by supplying the nutrients and organic compounds required for the metabolism of microalgae. Significant growth rate was found in 30% (v/v) of POME which approximately similar growth rate with BG-11 medium. Increasing concentration of POME medium reduce the growth rate due to inhibition in light intensities penetrations and effect of nutrients. The results revealed that polyunsaturated fatty acids are found comparatively higher in POME medium as compared to BG-11.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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