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ARTICLE

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

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ABSTRACT

Freshwater microalgae *Chlorella* sp. was isolated from the 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 were found in 30% (v/v) of POME. The maximal dry biomass 1.56 g/L was obtained and extracted 39.1% lipid, approximating similar productivity as in conventional BG-11 medium 1.65 g/L of biomass and 41.5% lipid. Gas chromatography-mass spectrometry analysis shows the composition of fatty acids such as linoleic acid, oleic acid, palmitic acid, stearic acid, lauric acid, and α -linolenic acid dominant. The highest fatty acid is linoleic acid (41.69%). POME has the potential to be used as a substrate for microalgae *Chlorella* sp. cultivation.

1. Introduction

Microalgae are primarily a diverse group of microorganisms 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 is estimated in ranging from a few micrometres to a few hundred micro-metres (2×10^5 to 8×10^5) in size. Microalgae can be a source of bioactive compounds

in lipids, carbohydrates, protein, vitamins, glycerol, and carotenes (Khan et al., 2018; Ramaraj et al., 2013; 2014). Micro-algae's lipid productivity is higher than other terrestrial plants (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 can absorb nitrogen, phosphorus and carbon from wastewater, including adsorbing metals, and in many cases can be co-cultivated with

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various bacteria in municipal wastewater treatment plants (Zhang et al., 2014; Nwuche et al., 2014). Based on Table 1. The composition of POME contains high nutrients such as nitrogen and phosphorus, which are eventually useful for algae medium.

Nomenclature and abbreviation

POME	Palm Oil Mill Effluent
COD	Chemical Oxygen Demand
TN	Total Nitrogen
TP	Total Phosphorus
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 due to 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 was 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 ($27 \pm 2^\circ\text{C}$), pH (8.0 ± 5), salinity (less than 1000 ppm) was recorded to ensure the compatibility in laboratory scale. The water samples were centrifuged at 2,000 rpm, 15°C for 5 min to concentrate the algal cells and eliminate the solid debris particles. Samples were enriched using a 90% (v/v) sample mixture and 10% (v/v) in BG-11 medium. Samples were aerated with filtered air by using an 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 1×10^{-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 $26 \pm 1^\circ\text{C}$ for the formation of colonies (Bhuyar et al., 2019). After several streaking, the inoculums culture was prepared by culturing with 10ml of the liquid medium in the pre-sterilized test tube. The morphology structures of isolated colonies were examined under the microscope and identified by referring to algae

standard manual and a previous study (Gao et al., 2019; Van Vuuren et al., 2006). Then started to scale up to 100, 500, 1000, and 2000 ml of Erlenmeyer flasks. The purity of culture is checked regularly to maintain monoculture, as shown in Figure 1.

2.2. Characterization of POME

Palm oil mill effluent (POME) was obtained from a palm mill located nearby Gambang, Kuantan. The sample was filtered and eliminated by the solid substance by using a $0.45\mu\text{m}$ 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. Previous studies followed these steps (Hadiyanto and Nur, 2014; Govindan et al., 2019). The characteristics of wastewater have been measured according to the American Public Health Association on standard water and wastewater study methods. 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 concentrations of POME. The 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 was used as a control with 50% (v/v) of culture. The flasks were placed under 2500 lux light intensity, culture aerated by supplying filtered air at $28 \pm 2^\circ\text{C}$ for 24 hours.

2.4. Growth measurement

The growth of *Chlorella* sp. was studied in parameters like optical density and chlorophyll, and dry biomass weight. The optical density of *Chlorella* sp. culture was determined by using a spectrophotometer by measuring absorbance at the 680nm wavelength. Biomass was harvested by centrifuged at 7000 rpm, 20°C for 10 min and measured dry weight every three days from the POME concentration with maximal growth. Chlorophyll extract was measured from the harvested dry biomass on different days (Sukumaran et al., 2014).

2.5. Lipid extraction

Lipid was extracted from the *Chlorella* sp. biomass by the Soxhlet technique. The dry biomass was weighed and soaked in 50 ml of hexane overnight as pre-treatment. The protocol was followed from previous studies (Wiyarno et al., 2014; de Jesus et al., 2014; Bhuyar et al., 2020). The soaked sample was loaded in the thimble and is placed in the Soxhlet extractor. A 500 ml round bottom flask took and cleaned 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 $67 \pm 3^\circ\text{C}$. The hexane solvent was vaporized, diluted, and repeatedly percolated through the dry microalgae samples until it exceeded the maximum extraction. After the extraction process was done for 4h, the condensing unit was removed from the extraction unit and allowed the sample to cool down. The lipid extract and hexane solvent mixture were separated using a rotary evaporator at 110 rpm, 50°C for 15 min. the collected lipid extract was weighed and determined 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 a 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 in three quadrants of the tube into an ultrasonicated water bath at $58 \pm 2^\circ\text{C}$ 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 the 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

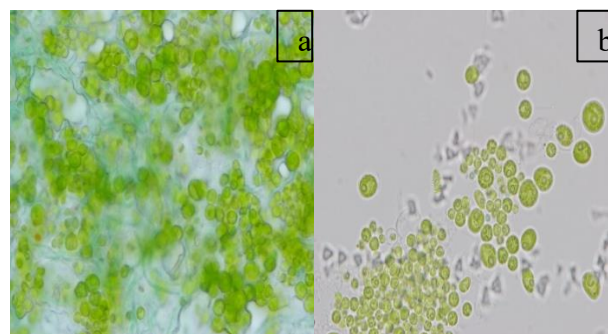
Fatty acid methyl ester obtained from transesterification were analyzed in an Agilent 7890A gas chromatography (GC) system equipped with capillary mega wax MS column (30 m length \times 0.32 mm internal diameter \times 0.50 μm film thickness). The mass spectrometer detector was used with helium as the carrier gas 1ml/min. The oven programmed with the following time-temperature program: 190°C (2 min), $190\text{--}230^\circ\text{C}$ ($5^\circ\text{C}/\text{min}$), 230°C (8 min). Peak areas were quantified with Chrome card for Windows software. The method followed the 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 a homogenous mixture, a 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 was determined by chromatographically comparing with authentic standards. Using heptadecanoic acid as an internal standard, each FAME was quantified from the chromatogram's peak areas.

3. Results and Discussion

3.1. Isolation and identification of *Chlorella* sp.

This study isolated five microalgae species after several re-streaking on the plate from fresh samples collected from Taman Gelora, Kuantan. From freshwater, *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 the light microscope. As shown in Figure 1, obtaining single species of *Chlorella* sp. from the mixture of various microalgae colonies by streaking plate technique was easy to perform under aseptic conditions.

Fig. 1 Microscopic (a) observation of the isolation 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. Figure 2 the growth curve of *Chlorella* sp. in POME medium with different concentrations (v/v). The culture was in the lag phase in all the flasks in the first two days of the experiment, where culture was adapting to the media environment. The maximal growth was found in 30% (v/v) of POME compared to culture in other flasks. Based on Figure 3, maximal dry biomass of *Chlorella* sp. was obtained in the 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 was 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 the early stationary phase of growth. Moreover, *Chlorella* sp. has been seen growing on the inner surface of the flask from day 5. This indicated that *Chlorella* sp. requires a good light source from the surroundings to enhance the growth. The nutrient availability in the 30% (v/v) cause the culture to sustain the growth rate of *Chlorella* sp. Hadianyato et al.(2014) found that greater growth rate ($0.31 \pm 0.06 \text{ d}^{-1}$) and productivity ($0.034 \pm 0.01 \text{ g L}^{-1} \text{ d}^{-1}$) 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 POME concentration would provide ample nutrients for algae to grow and increase light penetration into the medium. These factors simultaneously boost growth until the nutrients in POME have been wholly exploited, and light penetration decreases due to the generated biomass's density.

Most nominal growth of *Chlorella* sp. is found in the POME above 40 and 50% (v/v). The highest concentration of POME does not support *Chlorella* sp. due to the minimal light penetration into the culture in the flask. Increasing POME concentration significantly enhanced the concentration of productivity due to the presents of more nutrient components. However, above 30% of POME did not enhance the biomass productivity in the medium (Sukumaran et al., 2014; Nithin et al., 2020). The high concentration of POME did not support the microalgae growth and production (Sukumaran et al., 2014). The productivity of dry biomass is determined from the optimum 30% (v/v) POME medium, as shown in Figure 3. The dry biomass obtained from the BG-11 medium was 1.38 g/l on day 12. Figure 3 shows the comparison between biomass productivity in (30%) of POME and BG-11 medium. Both productivities are approximately similar but, BG-11 shown greater biomass productivity than (30%) of POME.

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

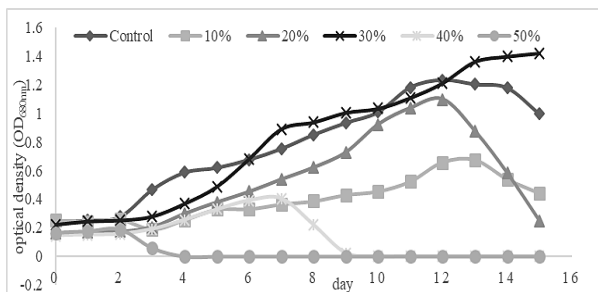


Fig. 2 Absorbance reading of *Chlorella* sp. in different concentrations of POME

Soxhlet technique causes a more significant influence in the lipid extraction from *Chlorella* sp. Greater biomass productivity produced higher lipid extract as shown in Figure 3. The maximal lipid yield extracted from *Chlorella* sp. biomass, harvested from 30% (v/v) culture, is 39.1%. The lipid content obtained from the biomass culture control medium was 41.5%. The advantage of soxhlet is that as time increases, the lipid productivity increases from the biomass. 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).

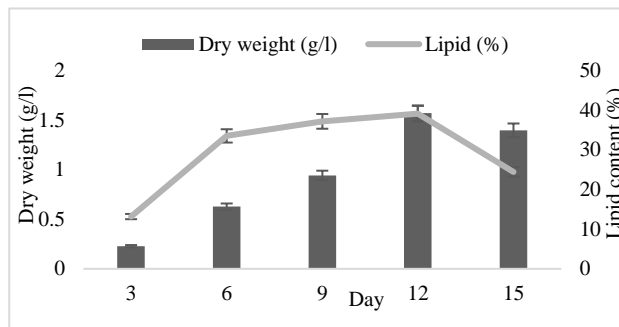


Fig. 3 Dry weight and lipid content of *Chlorella* sp. in 30% (v/v) POME culture

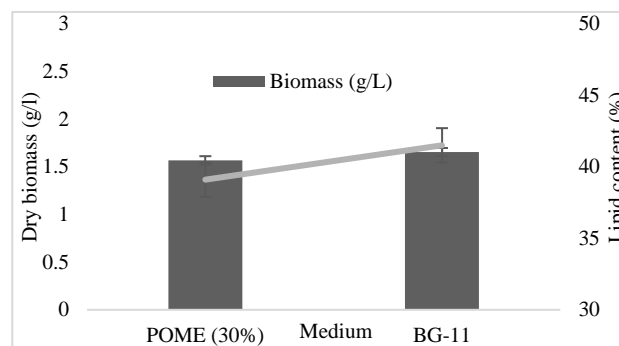


Fig. 4 Comparison of biomass and lipid productivity between POME and BG-11 medium

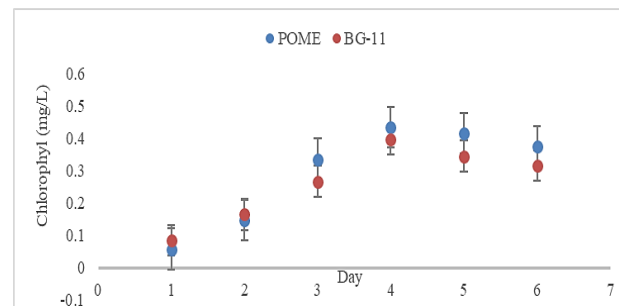


Fig. 5 Chlorophyll content of *Chlorella* sp. in POME (30%, v/v) and BG-11

3.3. Fatty acid methyl ester analysis

The fatty acid composition of *Chlorella* sp. was analyzed in gas chromatography-mass spectrometry. The fatty acid profile of *Chlorella* sp. was analyzed from lipid extract, which was 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, the 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-eicosatrienoic acid, tricosanoic acid, cis-13,16-docosadienoic acid and cis-11,14-eicosadienoic acid. The composition of fatty acids does change 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
Σ 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
Σ Monounsaturated fatty acids	30.10	39.62
α-Linoleic acid	9.18	3.04
Linolelaidic acid	41.69	35.87
Σ 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 could 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. A significant growth rate was found in POME 30% (v/v), an approximately similar growth rate with the BG-11 medium. Increasing concentration of POME medium reduces 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 the POME medium than BG-11.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have

influenced the work reported in this paper.

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