Production of lipids by *Tetraselmis sp.* grown in palm oil mill effluent

Cite as: AIP Conference Proceedings **2454**, 040005 (2022); https://doi.org/10.1063/5.0079106
Published Online: 09 June 2022

Natanamurugaraj Govindan, Pushparani Chadayam, Gaanty Pragas Maniam, et al.

Production of Lipids by *Tetraselmis sp.* Grown in Palm Oil Mill Effluent

Natanamurugaraj Govindan[1,2, a)], Pushparani Chadayam[1, b)], Gaanty Pragas Maniam[1,2, c)], Mohd Hasbi Ab. Rahim[1,2, d)], Ahmad Ziad Sulaiman[3, e)] and Yusuf Chisti[4, f)]

[1]*Algae Culture Collection Center & Laboratory, Faculty of Industrial Sciences & 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]*Faculty of Bioengineering & Technology, Universiti Malaysia Kelantan, 17600 Jeli, Kelantan, Malaysia*
[4]*School of Engineering, Massey University, Private Bag 11 222, Palmerston North, New Zealand*

[a)]Corresponding author: natanam@ump.edu.my
[b)]pushparani826@gmail.com
[c)]gaanty@ump.edu.my
[d)]mohdhasbi@ump.edu.my
[e)]ziad@umk.edu.my
[f)]Y.Chisti@massey.ac.nz

Abstract. Malaysia is one of largest palm oil producer and exporter in the world, which generates million tons of palm oil mill effluent (POME) in a year. Discharge of POME into water bodies had created major environmental pollutions in Malaysia. Other side, POME also comprised of organic and inorganic pollutants that can become as potential medium source for cost-effective wastewater treatment and biofuel production. The fresh water microalgae *Tetraselmis sp.* was isolated from Taman Gelora, Kuantan, Pahang. The isolated pure strain of *Tetrasemis sp.* was cultivated in different concentration of palm oil mill effluent medium (5%, 10%, 15% and 20%, v/v). The results disclose that the maximal growth and biomass productivity was found in 10% (v/v) concentration of POME. The maximal dried biomass 0.832g/mL and extracted total lipid content 37% was obtained. The chemical characteristics of POME before and after cultivation were pH (7.9- 8.1), BOD (38-11mg/L), COD (370-192mg/L), nitrate (247-10mg/L) and phosphate (22-0.7mg/L) respectively. Gas chromatography mass spectrometry depicts fatty acid methyl ester such as palmitic acid 55.03%, stearic acid 30.83 %, palmitoleic acid 7.67% oleic acid 4.84 %, linoleic acid 5.79% and followed by gamolenic acid 3.56 %. It proves that *Tetraselmis sp.* one potential candidate for biodiesel production by utilizing the palm oil mill effluent medium which can contribute to the reduction of the pollution.

INTRODUCTION

Nowadays, the world stress about finding an alternative source of fossil fuel since consumption of fossil fuel every year globally near more than 11 billion tons. Crude oil supply in a year is vanishing at a rate of 4 billion tons [2]. Nevertheless, oil endures the world's leading fuel as the primary energy demand source, computing 33.6% of the overall global shares. Concurrently, the researcher's discovered biodiesel is also eco-friendly and cost-effective due to the less emission of sulphur dioxide than conventional fuel [3]. Expansion of biodiesel blending composition has worked out by the Malaysia government in the present diesel blending to be 7%, 10% and by 2020 expected to be 15% as track of lessening need on petrol-diesel [1]. However, a more significant cropping area was required if Malaysia depended entirely on biodiesel feedstock's palm oil resources. Biodiesel from edible feedstock like palm,

International Conference on Bioengineering and Technology (IConBET2021)
AIP Conf. Proc. 2454, 040005-1–040005-7; https://doi.org/10.1063/5.0079106
Published by AIP Publishing. 978-0-7354-4193-4/$30.00

corn, rapeseed, and soybean is obtained right now. In contrast, biodiesel production from edible oil, not sustainable due to food issues [5]. The solution was proposed to tackle the drawback, which is third-generation biofuel from microalgae cultivation. Microalgae has the capability to convert light and carbon dioxide into high energy lipid and biomass. Besides that, microalgae also have advantageous characteristics such as a high migration rate of carbon dioxide and capabilities to gain nutrients from wastewater. Those characteristics helped increase microalgae's growing rapidly in desirable environment and produce excessive biomass. The reduction of greenhouse gas effect can be obtained from microalgae biofuel that is liable for up to 40% of carbon fixation globally and 70% of oil content recovered by the dry weight [6] [7]. Lipids are stored in microalgae cells during the photosynthesis process in non-polar triacylglycerol (TAG) lipid [8][9]. The microalgae lipid changeover to fatty acid methyl esters (FAME) can be achieved through the transesterification process. FAME is a necessary and flexible form of cornerstone and biodiesel for production [10]. Palm oil industries contributed to high volumes of POME production since its main Malaysian economy activity. As s result from it has become an environmental issue for the country. The raw POME has a significant amount of nutrients. The treatment of POME required little organic loading rate (OLR) and lengthy hydraulic retention time (HTR) [11]. Those available technologies are a fundamental threat to remove low POME organics capacities; thus, it does not convey treated POME characteristics that full-fill straight away release limits into water bodies [12]. The straight discharge of the POME committed to significant health problems and water pollution. Microalgae or macro algae seem possible for phytoremediation applications to transform and remove pollutants, including the toxic chemical and nutrients from wastewater that simultaneously produces [11]. Microalgae usage in POME treatment and biomass production has accepted a serious consideration because of microalgae's ability to digest pollutants to carry the metabolic action within a short time [13]. In these studies, microalgae *Tetraselmis* sp. was isolated and cultivated with POME in different concentrations and optimal growth environment for high biomass production. The harvested microalgae biomass was converted into lipid. The lipid was converted to biodiesel through transesterification process which used to transform crude oils into methyl ester. The component of methyl ester was determined using gas chromatography-mass spectrometry. The palm oil mill effluent waste was characterized according to the American Public Health Association (APHA) which is standard technique for wastewater and water studies.

MATERIALS AND METHODS

Isolation and Identification

The freshwater microalgae sample was collected from the pond of Taman Gelora, Kuantan, Pahang by using 0.5μm mesh size plankton net. The 1000mL of collected samples were placed in bottles for the isolation purpose at optimum condition [16]. Then, details of water temperature, salinity and pH were noted in order to ensure adaptable for laboratory scale. The samples were centrifuged at 2000 rpm for 5 minutes at 15°C to consolidate the microalgae cells and remove the concrete debris. BG-11 medium used to enrich the samples. The samples were aerated with filtered air continuously for 24 hours and cultured until there was a noticeable sign of algae's growth, especially flask of full green. The grown culture was subjected to serial dilution using sterile distilled water. 1mL of diluted sample was streaked on the BG-11 nutrient agar plates. The agar plates were inverted and incubated for 15 days until colonies formation [8]. The grown of microalgae colonies were streaked on new agar plates for further isolation. The streaking method was repeated until the formation of single algal species obtained. The microalgae strains were isolated and determined based on the morphological observation using the Olympus Bx53 Fluorescence Research Microscope in Central Laboratory Malaysia Pahang. The isolated colony was labelled and captured at a magnification of 100X. The microalgae morphology was identified based on the manual 'Freshwater algae' [17].

Characterization of Palm Oil Mill Effluent Medium

Palm oil mill effluent (POME) medium was collected at the anaerobic pond from FGV Palm Oil Mill, Bentong, Pahang. The POME sample was stored in a plastic container with the proper label. The sample was preserved at 4℃ to avoid any activity of biodegradation and contamination [18]. The collected POME medium was centrifuged (Refrigerated Centrifuge 5810R) at 10000 rpm for 10 minutes to remove suspended solids [8]. Then, POME was diluted with 1:1 POME and distilled water. The raw POME was characterized according to the American Public Health Association. The BOD, COD, Nitrate and Phosphate content analysis were done through spectrophotometer

by APHA 5220 test method. The POME was sterilised at 121℃ at 15 Psi pressure for 20 minutes [19]. The characterization of POME was done before and after the treatment.

Cultivation *Tetraselmis* sp. in POME

The *Tetraselmis* sp. growth performance studied in varying POME concentration. *Tetraselmis* sp. in the stock culture 5, 10,15 and 20% (v/v) were added to the POME medium, producing up to 300mL as the total volume Erlenmeyer flask. Medium BG-11 used as a medium, control of 20% (v/v) culture. The flask was placed underneath 2500 lux luminous intensity, culture aerated by filtered air supply 24 hours, (27± 1 °C). The growth of *Tetraselmis* sp. was examined by the optical density using spectrophotometers (UV-vis Genesis). The culture's absorbance reading was taken at a wavelength of 680nm every two days once, and growth curve graph had been plotted [20]. The biomass of the algal cells was collected during the late log phase culture was placed into 250 mL centrifuge tube and centrifuged (Refrigerated Centrifuge 5810R) at 6000 rpm for 15 minutes [21], the supernatant was discarded, washed with distilled water twice and centrifuged again. Then, the remained pellet was placed into -80 °C freezer for overnight. The stored biomass pellet was freeze dried to obtain a powder form of biomass [22]. The dried biomass weight versus concentration of POME (v/v) graph was represented.

Lipid Extraction by Solvent Extraction

The biomass of microalgae was prepared by grinding in the mortar and pastel to a uniform size. Lipid from ground biomass was extracted by using Bligh and Dyer (1959) modified method. 0.5g dry biomass was weighed and soaked in the 10 mL of 1:1 methanol: chloroform solvent overnight. The next day, the mixture was centrifuged at 4000 rpm for 5 minutes to separate the supernatant and pellet [23]. The empty 50mL falcon tube was pre-weighed, and the supernatant was placed into that empty tube. The remaining pellet was added with 10mL of solvent to the falcon tube that contained old biomass. Then, the mixture was ultrasonicated at 60°C for 1 hour. After 1 hour, the mixtures were centrifuged, and the supernatant was collected. The ultrasonication process repeated until the added solvent returned to the organic phase (explicit) [24]. Finally, all the collected supernatant was evaporated under a fume hood. The extracted lipid was weighed and kept for further.

Fatty Acid Methyl Ester Production by Transesterification

The triglycerides in the *Tetraselmis* sp. biomass transformed into fatty acid methyl esters (FAME) extracted to evaluate the fatty acid profile [19]. 50 mg of lipid extract was mixed in 2 ml centrifuge tubes with 0.5 g of 1% potassium hydroxide methanol and vortexed for 30 sec. Then three quadrants of the tube were applied to the ultrasonic water bath at 58±2 °C for two hours [25]. Then 1.0 g of hexane was applied to it and continuously vortexed for 2 min. The mixture transformed into two different solvent layers. In the 2 ml centrifuge tube, the upper layer solvent was extracted, and the hexane was evaporated by bringing it under the fume hood for 30 min. The residual material in the tube is methyl ester fatty acid [19]. Then, the obtained FAME was analysed using GCMS. In the Agilent 7890A gas chromatography mass spectrometry (GC) system with capillary DB wax MS column (60 m length × 250 μm internal diameter × 0.25 μm film thickness), fatty acid methyl ester obtained from transesterification was evaluated. The mass spectrometer detector used 3ml/min as the carrier gas with helium. With the following time-temperature programme, the oven was programmed: 50°C (1 min), 25-200 °C (10 min), 3-230 °C (2 min). With the Chrome card for Windows applications, peak areas were quantified. Approximately 40 mg of methyl ester sample was added to the 500 μL vial of hexane solvent. Samples were injected into GC in a homogenous mixture phase. With a split less, the inlet temperature should be kept at 250°C. The volume of the injection was 1 μl, with split less ratio. FAME was calculated by contrasting it chromatographically with original specifications. The FAME on the chromatogram was quantified from the peak areas with C_{17} as internal standard [20].

RESULTS AND DISCUSSION

Isolation and Identification of *Tetraselmis* sp.

In this study, three species of microalgae were isolated after several re-streaking on the plate from fresh samples collected from Taman Gelora, Kuantan. From freshwater, *Chlorella* sp., Cyanobacteria, and *Tetraselmis* sp. were isolated and identified. These species were observed based on the morphology identification under Olympus Bx53 Fluorescence microscope. As shown in Figure 1 obtaining single species of *Tetraselmis* sp. and was rapidly grown in the lab condition.

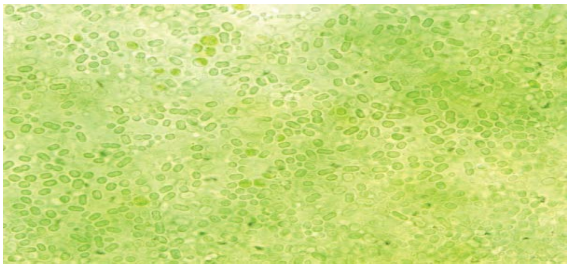

FIGURE 1. Microscopic observation of the isolated microalgae species *Tetraselmis* sp

Cultivation of *Tetraselmis* sp.

In this study, several concentrations of POME (%, v/v) were investigated for determining the growth of *Tetraselmis* sp. Fig. 2 showed the growth curve of *Tetraselmis* sp. in POME medium with various concentration (v/v). In the first two days of cultivation, all the culture was in lag phase where the culture started to adapt with medium environment. [12] reported that nitrogen enhanced the growth of the algae by promoting maximum optical density and shorter lag phases in line. The best growth was found in 10% (v/v) POME concentration as compared to other culture flask. The growth curve pattern in control medium and 10, 20 % (v/v) of POME medium quite similar however 20% (v/v) medium reached stationary phase and started death phase while 10% (v/v) culture medium in the early stationary phase. It is due to the nutrient availability in the 10% (v/v) that caused the sustained growth *Tetraselmis* sp.

Previous study, reported by [23], *Nannochlorpsis oculata* and *Tetraselmis suecica* had enhanced cell growth and lipid accumulation at 10% POME. According to [30], a lower concentration of POME would provide enough nutrient for microalgae to grow and increased light penetration into medium. Apart from that, the light penetration decreased because of the density of the generated biomass. Least growth of *Tetraselmis* sp. found in the POME concentration 5% and 20% (v/v). POME consist of tannic acid that gives darkening colour of POME. The highest concentration of POME does not support the growth of microalgae due to the darkening led to shading minimal light penetration into culture medium and negatively affected chlorophyll formation [18].

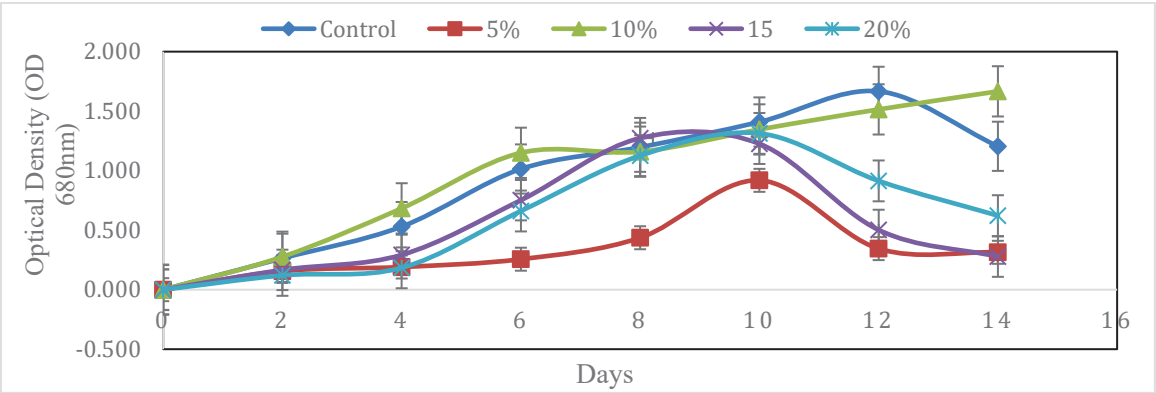

FIGURE 2. Optical density reading of *Tetraselmis* sp. in different concentration of POME

Biomass production was one of the primary concerns in this experiment. It's is due to the criteria for selecting the microalgae strains for production of lipid. Fig. 3 showed dried biomass of *Tetraselmis* sp. in different POME concentration after 14 days cultivation. In the 10% POME concentration, the biomass productivity was 0.322 ± 0.083 g/mL, which is most significant among the control and other biomass productivity concentrations. This is due the nutrient in the POME that consumed by *Tetraselmis* sp during stationary. In past study, microalgae dried biomass in 10% concentration of POME was 0.34g [4] and it indicates that, similarity in present study. It's also showed that 10% POME was the suitable concentration for the cultivation of *Tetraselmis* sp. Thus, increasing of dried biomass influence by the organic load of the effluent. It is due the, metabolism and consumption of the POME components by the microalgae [3]. Nutrient optimization has been made in the manner that concluded the POME medium will enhance the lipid content of microalgae *Tetraselmis* sp.

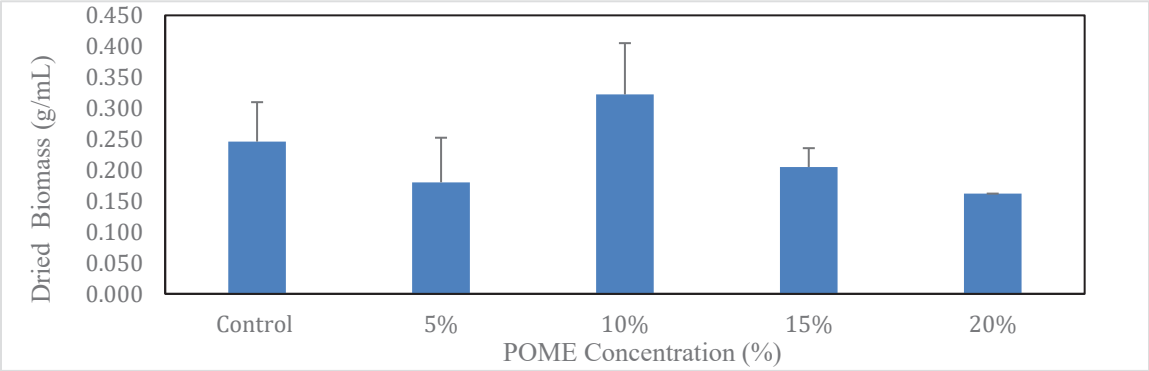

FIGURE 3. Dried biomass productivity of *Tetraselmsis* sp. in different concentration of POME after 14 days cultivation

Lipid Productivity

Tetraselmis sp. harvested and freeze-dried biomass was used for lipid extraction process. The dried biomass was pre-treated before proceeding to further extraction to break the cell wall and ease the extraction process. The ultrasonication approach was used in this process in order to disrupt the cell as for further extraction steps. *Tetraselmis* sp. cells are almost bigger cells which are 10 µm in diameter. According to [24], Chlorella sp. cells had an average about 2 µm diameter while *Tetraselmis suecica* cells are almost 10 µm in diameter. Consequently, the smaller cells diameter leaded to the reduction in the cell disruption efficiency of ultrasonication. It indicated that *Tetraselmis suecica* had significantly higher cell disruption efficiency using ultrasonication. The algae oil extraction method was modified from the method discovered by Bligh and Dyer 1959 [10]. After the extraction process, the mixture has appeared in two layers. The total lipid content extracted from *Tetraselmis* sp. was 13.882 g and total yield was 37%. Pervious study reported the maximum lipid content of *Tetraselmis* sp. 25%.[22]. The effectiveness of using palm oil mill effluent medium for microalgae cultivation not only rely on biomass production but also the lipid content as well. So, the carbon source in wastewater increased and desirable for the growth and lipid accumulation for biofuel production [16]. During the exponential phase, the microalgae cell started to consume nutrient in the POME medium. Consequently, the concentration of biomass higher and accumulate high lipid content [5].

Characterization of Palm Oil Mill Effluent

The optimum pH of microalgae cultivation within the range 7.5-8 [34]. Based on the result obtained, it showed increasing of pH 7.9 into the pH 8.1 before and after cultivation of microalgae. Increasing of pH typically happen due to the assimilation of ammonia or nitrate. Apart from that, the photosynthetic carbon dioxide consumption also the cause increasing of the pH. According to [8], high CO_2 concentration resulted the pH of culture medium decreased which could affect the photosynthesis process and therefore suppress the growth of microalgae while low CO_2 concentration caused increasing of pH. POME rich with high concentration of organic pollutants that required treatment before discharge to water bodies. The BOD of POME before the inoculation was 38 mg/L while after the inoculation was 11 mg/L respectively. By referred to result, there was significant removal of BOD were achieved after cultivation of *Tetraselmis* sp. in POME. It's due to the *Tetraselmis* sp. utilized the organic pollutants in the POME as

a carbon source to enhance their growth. Previous studies revealed that, the BOD removal 81.5-96% were achieved after inoculation *T. suecica* while the BOD removal of 88.5-97% were achieved after addition *N. oculate* [8]. The COD before the cultivation of *Tetraselmis* sp. in POME was 370 mg/L while after the cultivation was 192 mg/L. The removal of COD also varied for before and after. It is due to the, *Tetraselmis* sp. has consumed COD in the POME as organic carbon source. Pervious study by [25], also reported that COD removal efficiency in higher concentration POME media, indicated that *Nannochlorpsis* sp. mixotrophic strategy is suitable and required less light intensity. The reduction of COD in the POME also due to the released of organic compound through carbon dioxide bubbles to the surface. According to [10] organic carbon assimilation process on the growth media occurred during the cellular respiration of microalgae cells. During this process, oxygen and organic compound were used as final electron acceptor and electron donor, respectively. Source of energy from cellular respiration used for treatment and biosynthesis in dark condition. Therefore, during mixotrophic culture mode microalgae cells will utilize carbon dioxide and organic carbon that derived from their growth medium. The nitrate content before and after the cultivation *Tetraselmis* sp. was 247 mg/L and 10 mg/L. It was indicated that, there is assimilation of nitrate by *Tetraselmis* sp. to enhance lipid accumulation. The consumption of ammonia by microalgae can lead to the released of H+ ions which can decrease the pH while utilization of nitrate can increase the pH due to the released of OH- ions [8]. Past study by [5] reveal that in 12.5% (v/v) of POME concentration removed nitrate content up to 72.97%. The phosphate content before the cultivation was 22 mg/L while after the cultivation 0.7 mg/L respectively. Typically, microalgae consumed an inorganic substrate known as orthophosphate. The function of phosphate nutrient in the POME medium as substantial macronutrient for algal growth to produce ATP and phospholipids. Pervious study revealed by [4] on growth of locally isolated microalgae to enhance lipid production. The percentage reduction of the phosphate reached up to 89.2%. It is totally correlated with present study because when the inorganic phosphate inadequate microalgae consume organic phosphate nutrient by converting it into orthophosphate in cell surface by using phosphatases [10].

Fatty Acid Methyl Ester (FAME) Analysis

The fatty acid profile was analysed by using gas chromatography mass spectrometry. The fatty acid methyl ester (FAME) of *Tetraselmis* sp. was analysed and identified from lipid extract which obtained from biomass 10% (v/v) of POME concentration by using gas chromatography mass spectrometry. Typically, the properties of biodiesel determined by the composition of fatty acid due to the fatty acid characteristics such as the level of unsaturation and length of the carbon chain. Oleic, linolenic, stearic, palmitic acids are the most common fatty acids in biodiesel. The, output of GC-MS analysis proves that FAME mainly comprises of palmitic acid 47.36%, palmitoleic acid 7.37% stearic acid 30.83 %, oleic acid 4.84 %, linoleic acid 5.79% and followed by gamolenic acid 3.56 % for *Tetraselmis* sp. transesterified oil. Previously, [25] reported the presences of fatty acid in *Tetraselmis* sp. The fatty acid available is palmitic acid 24.13%, oleic acid 25.67%, and linoleic acid 23.17%. Those fatty acid available also correlated with the presence in study. Most microalgae strains fail to address the properties related with the saturation of the lipid profile, namely, the content of linolenic acid, most important for the oxidation stability. Such oxidative stability is probably related with the FAME profile and consequently produced biodiesel that revealed only trace values of long-chain PUFA, and high contents of palmitic, oleic and linoleic acids. The same is observed when the lipid profile of *Tetraselmis* sp. is compared with that of most common commercial strains of microalgae, such as *Nannochloropsis oculata* and *Phaeodactylum tricornutum* [24].

CONCLUSION

In this study, green microalgae *Tetraselmis* sp. was isolated from Taman Gelora Pond, Kuantan and subjected to the lab conditions. The Blue-green medium (BG-11) and palm oil mill effluent media (POME) were prepared and mainly focus on the POME medium. The isolated microalgae *Tetraselmis* sp. was cultivated in different concentration of POME. The growth parameter of *Tetraselmis* sp. were conducted such as optical density reading and biomass productivity. As a result, 10% (v/v) concentration showed a higher growth rate and biomass productivity. The chemical characteristics such as pH, BOD, COD, nitrate and phosphate were investigated in this study. It is clearly showed that, microalgae utilized the nutrient for their growth and at the same reducing the organic and inorganic pollutants in the POME. *Tetraselmis* sp. lipid production also significantly increased which is 37% as compared to previous studies. Trasesterification of *Tetraselmis* sp. oil was converted into fatty acid methyl ester. The output of GC-MS analysis proves that the potential FAME that mainly comprises palmitic acid 47.36% palmitoleic acid 7.37% stearic acid 30.83

%, oleic acid 4.84 %, linoleic acid 5.79% and followed by gamolenic acid 3.56 % It proves that *Tetraselmis* sp. one potential candidate for biodiesel production.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge University Malaysia Pahang (UMP) for financial support through a Flagship Grant (RDU182205) and Internal Research Grants (RDU1903125 and RDU190337).

REFERENCES

1. N. Hossain, M. H. Hasan, T. M. I. Mahlia, A. H. Shamsuddin, and A. S. Silitonga, Energy Strateg. Rev. **32**, 1–10 (2020).
2. S. Rakesh, J. Tharunkumar, and B. Sri, Highlights Biosci. (2020).
3. M. Hizami, M. Yuso, M. Ayoub, N. Jusoh, and A. Z. Abdullah, 1–18 (2020).
4. S. Vasistha, A. Khanra, and M. P. Rai (2019).
5. H. Alishah Aratboni, N. Rafiei, R. Garcia-Granados, A. Alemzadeh, and J. R. Morones-Ramírez, Microb. Cell Fact. **18** no. 1. BioMed Central, 1–17 (2019).
6. W. Y. Cheah, P. L. Show, J. C. Juan, J. S. Chang, and T. C. Ling, Energy Convers. Manag. **164**, 188–197 (2018).
7. P. Bhuyar, S. Sundararaju, M. H. A. Rahim, R. Ramaraj, G. P. Maniam, and N. Govindan, Biomass Convers. Biorefinery (2019).
8. L. Gouveia et al., Microalgae-Based Biofuels and Bioproducts: From Feedstock Cultivation to End-Products, 2017, pp. 236–258.
9. V. Kumar, M. Nanda, S. Kumar, and P. K. Chauhan, Energy Sources, Part A Recover. Util. Environ. Eff., pp. 1–6, 2018.
10. Q. Emparan, R. Harun, and M. K. Danquah, Appl. Ecol. Environ. Res. **7**(1), 889–915 (2019).
11. A. Ahmad, A. H. Bhat, A. Buang, S. M. U. Shah, and M. Afzal, Int. J. Environ. Sci. Technol. **16**, no. 3. Springer Berlin Heidelberg, 1763–1788 (2019).
12. N. A. Idris et al., J. Oil Palm Res. **29**(2) 291–299 (2017).
13. M. Zabochnicka-Świątek, T. Kamizela, M. Kowalczyk, H. M. Kalaji, and W. Bąba, Glob. Nest J. **21**, 82–89 (2019).
14. S. Hena, S. Fatimah, and S. Tabassum, Water Resour. Ind. **10**, 1–14 (2015).
15. N. Carter, Freshwater Algae. **2** (2015).
16. N. A. Idris et al., J. Oil Palm Res. **30**(1) 141–149 (2018).
17. N. Govindan et al. J. Appl. Phycol. **35**, 375–387 (2020).
18. F. E. Han, Z. H. Zhao, D. P. Li, and L. Zhang, Oceanol. Limnol. Sin. (2019).
19. S. K. Al-Amshawee, M. Y. Yunus, and A. A. Azoddein, IOP Conf. Ser.: Mater. Sci. Eng. **736**(2) (2020).
20. R. Chowdhury, P. L. Keen, and W. Tao, Bioresour. Technol. Reports (2019).
21. S. M. U. Shah, A. Ahmad, M. F. Othman, and M. A. Abdullah, Int. J. Green Energy **13**(2) 200–207 (2016).
22. Z. Chen, L. Wang, S. Qiu, and S. Ge, Biomed Res. Int. 1–17 (2018).
23. N. Hindryawati, G. P. Maniam, M. R. Karim, and K. F. Chong, Eng. Sci. Technol. an Int. J. (2014).
24. C. H-H and K. C-H, J. food drug Anal. (2020).
25. C. Nwuche, Biotechnol. J. **4**(3) 305–316 (2014).