Research article

Saidu, H., O. A. Salau, and S. E.Mohamad, Investigating the Effect of Several Palm Oil Mill Effluent (POME) Dilutions on Biomass And Specific Growth Rate of *C. sorokiniana*. International Journal of Life Sciences and Biotechnology, 2021. 4(2): p. 192- 203. DOI: 10.38001/ijlsb.827309

Investigating the Effect of Several Palm Oil Mill Effluent (POME) Dilutions on Biomass And Specific Growth Rate of *C. sorokiniana*

Haruna Saidu [1]*, Olanrewaju Abiola Salau [2], Shaza Eva Mohamad[3]

ABSTRACT

Palm Oil Mill Effluent (POME) contained high amount of nutrients necessary to support the growth of microalgae. However, high amount of turbidity was identified as one of the important factor affecting the successful cultivation of microalgae in POME. In this study, *Chlorella sorokiniana* was cultivated in POME, under different dilutions of 80, 60, 40 and 20% (v/v) with distilled water. Standard methods were used for the analysis of the wastewater parameters such as nitrate, phosphate, turbidity, pH, total carbohydrate, phenols and heavy metal. The results indicated that, *C. sorokiniana* grew well on both sterilized and unsterilized condition. Result of the batch experiment showed that, POME turbidity of 45 NTU produce the maximum specific growth rate of 0.14 d^{-1}, which correspond to highest biomass production of 4.06±0.16 g/L in sterilized POME. Whereas in unsterilized POME, turbidity of 45 NTU produces specific growth rate of 0.2 d^{-1} which correspond to biomass production of 5.94±0.12 g/L in unsterilized POME. Biomass production of 5.88±0.31 was obtained when cultured in control experiment. This study concluded that 80% dilution was identified as the optimal medium for enhance biomass production of algae in POME which could add value for sustainable bioenergy production.

ARTICLE HISTORY
Received
17 November 2020
Accepted
1 February 2021

KEYWORDS
Turbidity, *Chlorella sorokiniana*, POME dilution, bioenergy

Introduction

Malaysia is a palm oil producing country accounting for about 41% world palm oil trade making it the second largest producer of palm oil in the world [1]. Due to increase in palm oil production of about 16.3 million tonnes in a year, large volume of palm oil mill effluent (POME) of about 2.5 to 3.5 tonne are generated [2]. POME contains large amount of chemical oxygen demand (COD), Biochemical oxygen demand (BOD), nitrate, phosphate

[1] Department of Biology, Faculty of Science, Gombe State University, Gombe State, Nigeria
[2] Department of Natural Science, Gombe State College of Education, Billiri, Gombe State, Nigeria
[3] Department of Environmental and Engineering and Green Technology, Malaysia Japan International Institute of Technology (MJIIT), Universiti Teknologi Malaysia, Kuala Lumpur, Malaysia
* Corresponding Author**: Haruna Saidu**, E-mail: saiduharunn@gmail.com

and turbidity. However, these pollutants are dangerous to human and aquatic life especially when they are discharge into the environment in an untreated form [3].

Currently, there was a developing interest in the biotechnological exploitation of POME for the production of value added materials [4] which is due to its ability to contain nutrient (nitrogen and phosphorus), lipid, phenolic compounds, heavy metals and carbohydrate. One of these biotechnological areas involved the utilization of POME as suitable growth medium for the cultivation of microalgae for biofuel production [5]. This is of economic important because, using wastewater as medium for culturing microalgae will directly eradicate the need of synthetic medium and therefore this may reduce the cost of production.

Microalgae are micro plant capable of growing in wastewater containing nitrogen and phosphorus [6, 7]. Various researches have been shown that, the biomass of wastewater grown algae has enormous important. For instance, A report by [6] indicated that, microalgae biomass can use for the production of animal feed. The cultivation of Chlorella sp. in anaerobic digested dairy manure effluent for the production of lipid was conducted by [7]. The result of the study showed that, the biomass of microalgae contains total lipid content of 13.7%. Kang and his co-workers [8] also reported the cultivation of *Haematococcus pluvialis* in piggery wastewater, and the result showed that, large amonut of astaxanthin was produce from the microalgal biomass after treament. Similarly, [9] reported the succesfull cultivation of Nannochloropsis in POME coupled together with recovery of high amount polyunsaturated fatty acid.

Considering the above mention important of algal biomass, the cultivation of algae will benefit immensely from the economic point of view. Despite the existence of report on utilization of POME for microalgae production, Literature have indicated that different species of microalgae varies in terms of their capacity to growth in stress environment like wastewater. Our paper emphasized specifically on *C. sorokiniana* strain because literature reported that this species have strong cell wall making it the most robust among the families of *Chlorella* and most exploitation has not been given to it in relation to its growth in POME. Since POME is dark brown in color which can potentially affect photosynthesis by algae [10], then we came up with dilutions of the POME as an adaptive mechanism in

order to study the effect of POME turbidity on the increase in biomass and speicific growth rate of *Chlorella sorokiniana*. Various dilutions of 80. 60, 40, and 20% (v/v) was used inorder to determined the optimal condition for enhance biomass production in POME.

Materials and Methods

Sample collection

POME was collected at the facultative anaerobic pond (FAP) from local palm oil mill industry (Kilang Sawit Bukit Besar, Johor Bahru Malaysia). Due to its turbid characteristic, it was left to settle for four hours before being filtered using Whatman filter paper. POME was sterilized by autoclaving at 121^{o}C for 20 minutes and later placed in a laminar flow under the effect of UV sterilization for 15 minutes in order to obtained sterile filtrate. For the purpose of preservation, POME was refrigerated at 4^{o}C to prevent biodegradation.

Algae and Culture Condition

A pure strain of *C. sorokiniana* was obtained from algae culture collection center at University of Texas, Austin Texas USA. The culture was maintained on Proteose media at 29^{o}C. The composition of Proteose media consist of $NaNO_3$ (25 g/L), $CaCl_2.2H_2O$ (2.5 g/L), $MgSO_4.7H_2O$ (7.5 g/L), K_2HPO_4 (7.5 g/L), KH_2PO_4 (17.5 g/L), NaCl (2.5 g/L), Urea (1.5 g/L). pH of the media was kept at 7. The experiments were conducted in a batch system. 1 L flasks were used for the algae cultivation. Before the commencement of the experiments, about 20% volume of algae was inoculated in 250 mL Proteose media (composition as shown above) for 21 days. This is to ensure that, *C. sorokiniana* acclimatize well before inoculation in POME. The justification of using *C. sorokiniana* for these experiments was due to its robust nature and ability to grow under limited photoheterotrophic making it one of the most tolerant microalgae to grow in a strange environment.

Characterization of POME

Various wastewater parameters such as nitrate, phosphate, phenols and ammonium were determined following the Hatch DR 6000^{TM} Spectrophotometric Manual (DR/6000, Hatch Co. Ltd. Tokyo 2008)**.**

Determination of wastewater parameters

Nitrate and phosphate were specifically determined using cadmium reduction method and an acid hydrolysable method respectively while ammonium and phenols were determined using Nessler and 4-Aminoantipyrine methods [11, 12]. Total carbohydrate was determined using phenol sulphuric acid assay [13]. Suitable dilutions were made for the high concentration and the final results were computed by multiplying the dilution factor. 5 mL microalgae suspension was taken from each Erlenmeyer flask to measure the nutrient removal throughout the experiments. The samples were centrifuged at 4,000 rpm for 15 min and afterward, the supernatants were analyzed for nitrate, phosphate, ammonium and phenols based on the HACH DR 5000 spectrophotometer manual [12].

Determination of Turbidity

A volume of 10 mL sample was taken and pours in a turbidity vial, afterward, it was then analyzed in turbidity using turbidity meter (model; 2100Q).

Determination of pH

pH was determined using the portable pH meter according to the standard methods of APHA, [14].

Determination of COD

Ten mL of POME was taken and then centrifuged at 4000 rpm for 15 minutes. The supernatant was mixed thoroughly using vortex and was placed in a preheated digester block at 1,500°C. Following the next 2 h, the sample was withdrawn and measured using spectrophotometer.

Determination of Heavy Metals Ions

To determine the concentration of heavy metals in POME, 10 mL of POME was acid digested using 2.5 mL concentrated nitric acid (65% HNO_3) and 0.8 mL of hydrochloric acid (HCL) at 180^{o}C for 20 min in a microwave oven (model; Bergh of *Speed wave 4*). Afterward, the digested samples were taken out, allowed to cool, filtered and diluted with distilled water. The sample was then analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES) according to the method portrayed by [15]. All the data were analyzed using Microsoft excel 2010.

Experimental Setup

The experiments were conducted in a batch system using 1 L Erlenmeyer flask. At the inception of the experiment, 20% (v/v) of pre-cultured microalgae cell was cultivated in sterilized POME initially diluted to 80, 60, 40, and 20% (v/v) of sterilized and unsterilized POME with distilled water. Cultivation of algae in proteose medium was used as the control experiment. This initial inoculum concentration was kept constant throughout the experiment. The experiment was repeated three times and the average values of the replicate results were used for analysis. Various level of turbidly was monitored during the growth of the microalgae and the specific growth rate was determined for the individual dilutions. The cultivation condition was maintained by a continuous supply of carbon dioxide (CO_2) for mixing purpose using an air pump. The pH was maintained at 7 by the addition of 1 M sodium hydroxide (NaOH) and 2 M HCL. Fluorescence lamps (3000 lux) were used as a source of illumination for 12 h (Day: Night) interval. The experiment was conducted at temperature 25-30^{o}C for 15 days.

Determination of Biomass Concentration

Algal biomass was determined using cell dry weight (CDW) based on the method described by [4]. 20 mL of algal culture was centrifuged at 4000 rpm for 15 min. The supernatant was discarded and the pellet was washed three times with distilled water. The pellets were dried at 70^{o}C in a hot air oven until constant weight was achieved. Biomass ~~was~~ determined in terms of cell dry weight per volume of culture (g/L).

Determination of Specific Growth Rate

The specific growth rate was calculated by the equation;

$$\mu = \frac{1}{t} In\left(\frac{Xm}{Xo}\right) \qquad \textbf{eq. (1)}$$

Where X_o and X_m are the initial and final concentration of biomass respectively, and t is the duration of the batch run.

Result and Discussion

The anaerobic digested POME was characterized and the initial values of various parameters were summarized in Table 1. It can be seen that, POME despite it treatment via anaerobic digestion, still contain high amount of nutrients. This nutrient has the potential to

support the successful growth of microalgae as this study demonstrates. Sample inoculation set and POME dilution can be found in Figure 1.

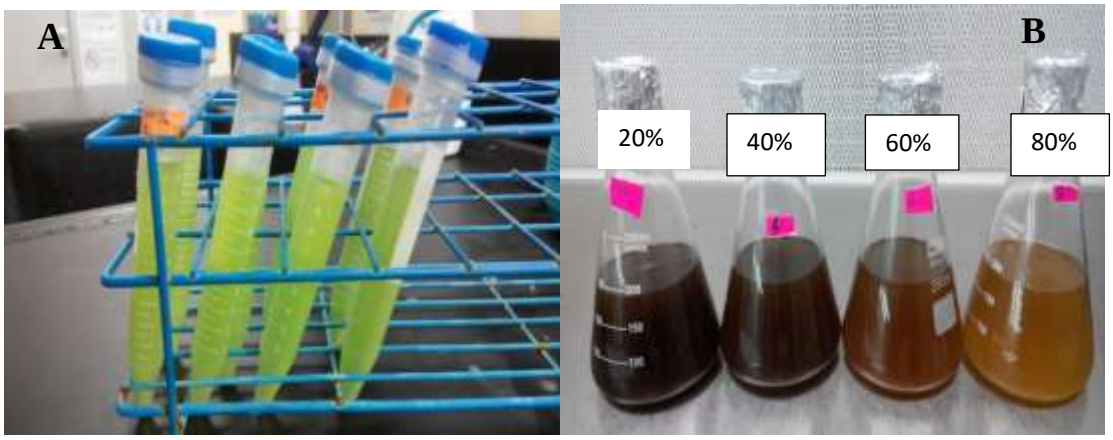

Fig 1 Pictures of sample inoculation (**A**) and POME dilutions (**B**)

Table 1 Characteristic of anaerobic digested POME

General Characteristics	**POME[a]**	**Malaysian discharge limits[b]**
pH	6.5	5-9
COD	2100	-
TSS	3200	400
Nitrate	181	-
Phosphate	131	-
Ammonium	245	-
Phenols	295	0.5
Total carbohydrate	211	
Turbidity	420	
Heavy metals		
Zn^{2+}	0.79	1
Fe^{2+}	118.1	5
Mn^{2+}	9.2	1

[a] All parameters unit is in mg/L except pH and turbidity (NTU). [b] Reference [16]

Algal growth is affected by several factors, which include nutrients, light, temperature, and inoculum size. Among these, inoculum size was identified as the most important factor affecting the growth of microalgae and its nutrient removal ability. For instance, [7] conducted an experiment using *Chlorella* sp. in dairy wastewater and reported that an optimal initial cell density of 1.2×10^7 cells mL^{-1} is sufficient to overcome shading effect factor thereby allowing better growth and nutrients removal. In this study, an optimal initial size of the inoculum was reported to be 1.2×10^6. While the inoculum size reported from this study was lower than that reported by [7], still satisfactory treatment was achieved. This could be due to the difference of the substrate used for the cultivation and the variation in the substrate dilution factor.

The correlation between 9 day specific growth rate of *C. sorokiniana* and turbidity strength of the four dilutions under both sterilized and unsterilized POME was plotted in Figure 1 and 2**.** The aim of this analysis was to investigate whether turbidity could be an important factor affecting the growth of algae in POME. The low specific growth rate relates well with high turbidity in both the two conditions. The results showed that the increase in POME dilution decreases the specific growth rate of microalgae and vice versa. The lowest turbidity of 45 NTU at 80% dilution produces the maximum specific growth rate of 0.14 d^{-1} in sterilized POME, whereas the highest turbidity of 98 NTU at 20% dilution produces the lowest specific growth rate of 0.07 d^{-1} (Fig 2).

It was however noted that, the control experiment gave specific growth of 0.19 d^{-1} which is relatively higher than the rate produced by POME dilution.

In the case of unsterilized condition; the minimum turbidity of 45 NTU at 80% dilution produces a maximum specific growth rate of 0.2 d^{-1} whereas the maximum turbidity of 98 NTU at 20% dilution produces the lowest specific growth rate of 0.07 d^{-1} (Fig 3). The R^2 values for sterilized and unsterilized POME were found to be above 0.90 indicating the accuracy of the graphs. Moreover, the specific growth rate of control (0.19) was found to produce almost similar growth rate of 2 in 80% dilutions.

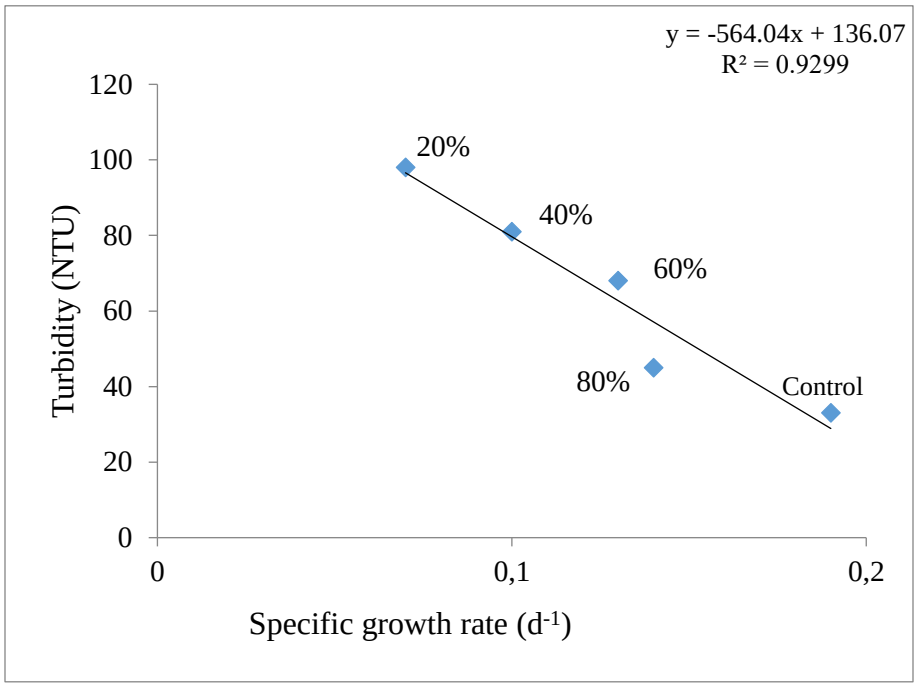

Fig 2 Relationship between sample turbidity and specific growth rate in the first 9 days under various dilutions of sterilized POME

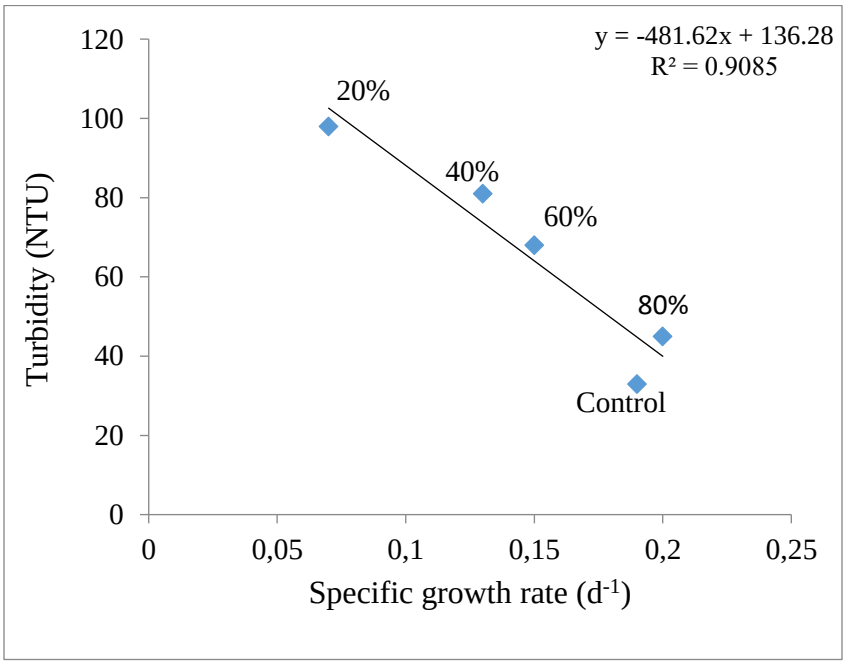

Fig 3 Relationship between sample turbidity and specific growth rate in the first 9 days under various dilution of unsterilized POME

It was noted that, unsterilized POME support maximum specific growth rate of microalgae than sterilized which could be due to the presence of bacteria that could assist the microalgae growth during aerobic degradation [4]. During the growth of microalgae in unsterilized POME, aerobic bacteria degrade the organic substrate leading to the production of carbon dioxide which will then be utilized by microalgae for photosynthesis. The bacteria utilized the oxygen produce by microalgae as by-product of photosynthesis for their growth [4].

High turbidity in POME is usually caused by the presence of freely suspended material such as fine particles, colour organic compounds and impurities [11]. Also, report indicated that occurrence of certain chemical reaction between tannic acid and other substrates at the point of refluxing (steam sterilization) have been confirmed to be responsible for the darkening of POME. These materials brought darkening of POME which prevent sufficient light penetration thereby creating a shading effect and further affect chlorophylls formation. Since POME was diluted and filtered in the case of sterilized POME, the high turbidity in 20% and 40% was as result of the presence of disintegrating organic matter remains. For unsterilized POME, the turbidity could result from the presence of a microorganism in the medium.

The Dark colour of POME can also cause *C. sorokiniana* to switch its growth mode from autotrophic to the heterotrophic or mixotrophic condition [4]. This mode of growth occurs especially when growing in a medium of minimal dark coloration (such as 40% or 20%). *C. sorokiniana* can utilize organic carbon substrates for both heterotrophic and mixotrophic growth [17]. Mixotrophic growth mode occur when microalgae utilized light and organic carbon as a source of carbon while heterotrophic growth means that, organic carbon is the only source of carbon for the growth of microalgae. A report by [18] reported that microalgae produce a high amount of biomass when growing under mixotrophic condition than other mode of growth.

The quantity of the biomass accumulation under different concentrations of sterilized and unsterilized POME was showed in Table 3. The rate of biomass accumulation in 80, 60, 40 and 20% of sterilized POME was found to be 4.06, 2.66, 2.65 and 2.36 g/L respectively. Furthermore, when compared with control, biomass accumulation of 5.88 g/L was

obtained. While under unsterilized condition the various biomass productions in all the dilutions was found to be 5.94, 5.43, 3.59 and 3.16 g/L respectively with the control experiment providing the highest amount of biomass. Overall the dilutions, 80% stimulate the highest biomass production and therefore can be designated as the optimal concentration of POME dilution for maximum biomass production using *C. sorokiniana*. One of the factors responsible for the variation in the biomass concentration is the nutrient strength since POME was diluted to different percentages. The high amount of biomass production under unsterilized POME was due to the symbiotic activities of algae with bacteria which favoured high biomass production [19]. Low biomass production in low diluted POME could be due to the presence of some toxic substance such as phenols and organic acid [20, 21].

Table 2 Effect of different POME dilutions on biomass production

POME dilutions	**Biomass production (g/L)**	
	Sterilized POME	**Unsterilized POME**
80	4.06±0.16	5.94±0.12
60	2.66±0.13	5.43±0.16
40	2.65±0.28	3.59±0.22
20	2.36±0.21	3.16±0.22
Control	5.88±0.31	5.88±0.31

The growth of *C. sorokiniana* was poor in 20% POME as indicated by low specific growth rate of $0.07d^{-1}$ (sterilized and unsterilized POME) and low biomass production of 2.36±0.21 (sterilized) and 3.16±0.22 (unsterilized). This could be due to the low photosynthetic activities of algae resulted from limited light penetration. The high turbidity value of 20% dilution presented in Fig 1 and 2 further confirmed the assertion that efficient photosynthetic activities did not take place. Algae growth in high dilution (60 and 80%)

were reported to better because high dilution cause reduction in the concentration of toxic substances and also increases the clarity of the medium given the chance for maximum light penetration as well as efficient photosynthetic activities to occur. This could be the reason why *C. sorokiniana* were able to produce high biomass of 4.06±0.16 (sterilized) and 5.94±0.12 (unsterilized) as indicated by high specific growth of 0.14 (sterilized) and 0.2 (unsterilized) POME. Efficient photosynthesis was observed in control experiment as the medium is not cloudy giving it the ability to induce better algae growth. Moreover, the amount of biomass produces in unsterilized POME was relatively close with biomass produce under control experiment. This could be due the presence bacteria growing in unsterilized which concomitantly increase the biomass concentration. Results of this study suggested that dilution of high strength wastewater could be employed as an adaptive mechanism toward reduce wastewater pollutant and allowing better light penetration for the application of algae. This research can contribute in finding an optimal dilution of POME for enhance cultivation of *C. sorokiniana* for the production of value added substances as the darkening characteristics of POME affect algae growth.

Conclusion

C. sorokiniana was successfully cultivated in different dilutions of sterilized and unsterilized POME and 80% (v/v) was reported to produce higher specific growth rate and biomass production. Dilutions strategy was observed to support the growth of algae in POME. Moreover, advance pre-treatment techniques can be incorporated in order to reduce the effect of other inhibitory substances in POME.

Acknowledgments
The Author wishes to acknowledge and thank the financial support received from Universiti Teknologi Malaysia (08H27) for the funding of this research

References

1. Jamaludin, N. F., Z. Ab Muis, and H. Hashim, An integrated carbon footprint accounting and sustainability index for palm oil mills. Journal of Cleaner Production, 2019. 225: p. 496-509.
2. Wu, T.Y., et al., Pollution control technologies for the treatment of palm oil mill effluent (POME) through end-of-pipe processes. Journal of Environmental Management, 2010. 91(7): p. 1467-1490. doi:10.1016/j.jenvman.2010.02.008.

3. Abdullah, N., Z. Ujang, and A. Yahya, Aerobic granular sludge formation for high strength agro-based wastewater treatment. Bioresource Technology, 2011. 102: p. 6778-81. doi:10.1016/j.biortech.2011.04.009.
4. Saidu, H., et al., Low-cust biodiesel production. Asian Journal of Applied Sciences, 2017. 10(2): p. 57-65. DOI: 10.3923/ajaps.2017.
5. Mohsenpour, S. F., et al., Integrating micro-algae into wastewater treatment: A review. Science of The Total Environment, 2020. p. 142168.
6. Kamyab, H., et al., Micro-macro algal mixture as a promising agent for treating POME discharge and its potential use as animal feed stock enhancer. Jurnal Teknologi, 2014. p. 5:1-4.
7. Wang, L., et al., Anaerobic digested dairy manure as a nutrient supplement for the cultivation of oil-rich green microalgae *Chlorella* sp. Bioresource Technology, 2010. 101: p. 2623-2628.
8. Kang, C.D., et al., Astaxanthin biosynthesis from simultaneous N and P uptake by the green alga *Haematococcus pluvialis* in primary-treated wastewater. Biochemical Engineering Journal, 2006. 31(3): p. 234-238.
9. Resdi, R., J.S. Lim, and A. Idris, Batch kinetics of nutrients removal for palm oil mill effluent and recovery of lipid by *Nannochloropsis* sp. Journal of Water Process Engineering, 2020. p.101767.
10. Chaijak, P., M. Lertworapreecha, and C. Sukkasem, Phenol removal from palm oil mill effluent using *Galactomyces reessii* termite-associated yeast. Polish Journal of Environmental Studies, 2018. 27(1): p. 39-44.
11. Saidu, H., H. Jamaluddin, and S.E. Mohamad, Nutrient removal and biokinetic study of freshwater microalgae in palm oil mill effluent (POME). Indian Journal of Science and Technology, 2017. 10(24): p. 1-10.
12. Idi, A., et al., Majid, Biokinetics of nitrogen removal at high concentrations by *Rhodobacter sphaeroides* ADZ101. International Biodeterioration and Biodegradation, 2015. 105: p. 245-251.
13. Kamala, S.A., et al., Pre-Treatment Effect of Palm Oil Mill Effluent (POME) during Hydrogen Production by a Local Isolate *Clostridium butyricum*. International Journal on Advanced Science Engineering Information Technology, 2012. 2(4): p. 54-60.
14. American P.H.A., Association, Standard methods for the examination of water and wastewater. American Public Health Association (APHA)" *Washington, DC, USA.* 2005.
15. Meche, A., et al., Determination of heavy metals by inductively coupled plasma-optical emission spectrometry in fish from the Piracicaba River in Southern Brazil. Microchemistry Journal, 2010. 94: 171-74. doi:10.1016/j.microc.2009.10.018.
16. Lam, M. K., et al., Malaysian palm oil: Surviving the food versus fuel dispute for a sustainable future. Renew and Sustainable Energy Review, 2009. 13: p. 1456-1464.
17. Ramanna, L., et al., The optimization of biomass and lipid yields of *Chlorella sorokiniana* when using wastewater supplemented with different nitrogen sources. Bioresource Technology, 2014. 168: p. 127-135.
18. Bhatnagar, A., S. Chinnasamy, M. Singh and K.C. Das, Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. Applied Energy, 2011. 88(10): p. 3425-3431.
19. Su, Y., A. Mennerich, and B. Urban, Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: influence of algae and sludge inoculation ratios. Bioresource Technology, 2012. 105: p. 67-73.
20. Nwuche C.O., et al., Use of Palm Oil Mill Effluent as Medium for Cultivation of *Chlorella sorokiniana* British Biotechnology Journal, 2014. 4(3): p. 305-316.
21. Saidu, H., Kinetic Studies for Phycoremediation and Nutrient by *Chlorella sorokiniana* of Palm Oil Mill Effluent. PhD thesis, Universiti Teknologi Malaysia Repositor, 2017.