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Abstract. The marine algae S*pirulina platensis* was cultured in a medium of palm oil mill effluent (POME) and the production of lipid in the biomass was optimized. Among 7 species *Spirulina platensis* was isolated from the marine sample which have greater lipid potential was collected from Pantai Teluk Cempedak, Kuantan which is located at East Coast region of Pahang state. At 28±2 °C, the maximum lipid content in the biomass harvested at the end of a 15 days batch culture was quantified with the following values of the experimental factors: POME concentration with (10%, 20% and 30%) (v/v) and light intensity (3,000 lux, 4,000 lux, 5,000 lux). Under the optimized conditions with 20% of POME, the maximum dry mass concentration of the was 754.5 mg L^{-1} with lipid 34.5% production on day 13 of a batch culture, declining to ~687 mg L^{-1} on day 15. At 5,000 lux light intensities, the maximum yield obtained was 854.6 mg L^{-1} with lipid 35.8% production on day 14, it declined to 732.8 mg L^{-1} on day 15.

1. Introduction

Microalgae are single-cell photosynthetic organisms which use light and carbon dioxide for growth and production of biomass [1,2]. Microalgae can grow in a variety of environments, including rivers, lakes, pounds, oceans, wastewaters and even deserts. Its biodiversity is extensive; there are species that grow in fresh water, such as *Chlorella vulgaris*, and others in saltwater [2]. Microalgae can play an important role in treating wastewater, particularly at the level of nutrient removal and wastewater treatment plants' operating costs can be reduced. Microalgae or microalgae – symbiosis of bacteria has been shown to provide a good quality of treated water by extracting organic matter and nutrients including nitrogen, phosphorus, harmful contaminants, heavy metals and even eliminating specific pollutants [3].

Agricultural wastewater is characterized by high concentrations of nutrients which could contribute to the growth of algae [4]. Especially palm oil mill effluents (POME) is extremely contaminant-rich and 100 times more harmful than industrial wastewater, with high biochemical oxygen (BOD) and chemical oxygen (COD) requirements. The effluent also includes higher amounts of organic nitrogen, phosphorus, and other substitute substances [5,6]. POME does not harmful waste but, due to the natural

and supplementary material, it may pose an environmental problem due to the massive oxygen draining potential to oceanic system. This is also considered to provide a healthy nutrient source [7,6].

Production of microalgal biofuels has two main challenges, such as rising production costs and defining the harvesting cycle [8]. The combination of biomass production and wastewater treatment reduces the nutrient and freshwater requirements. Studies on wastewater carried out to determine the efficiencies in nutrient removal (efficiency in the treatment of wastewater) and the productivity of biomass (possible growth inhibition) [9]. The capacity for producing various biofuels (biodiesel, biogas, bioethanol, among others) with the biomass produced from the waste water [9]. Microalgae release oxygen which, in effect, can be used by other microorganisms to improve the overall efficiency of aerobic degradation which will further which the POMEs BOD and COD and thus achieve the desired wastewater treatment target [10-12].

According to [5] study, the pollutants which present in POME can be reduced up to 70% with biological treatment of microalgae. Phytoremediation is an economical way to reduce the POME by using for the growth of microalgae. Many studies indicate a major dilution need (50%) to increase the alkalinity of POME which is potential for microalgae growth medium [4,13]. Microalgae can make use of the POME nutrient and organic carbon. It also develops lipids, protein, carbohydrates and other bioactive compounds as the accumulation of energy stock in stress state [14]. Therefore, selection of microalgae strain is also significant for the purpose of reducing pollutant and also producing valuable product. *Spirulina platensis* is one of the major commercial microalgae grown in POME medium [5]. The biorefinery is a strategy that combines cell culture, biomass conversion and separation, with the emphasis on obtaining bioactive compound products [15]. In this study *Spirulina platensis* were cultured by feeding palm oil mill effluent (POME) as nutrient and determine the growth at different POME concentration (v/v) and light intensities (lux). Then determined the lipid production from harvested biomass by extracting lipid by using ultrasonication assisted method.

2. Materials and methods

2.1. Sample collection.

Palm oil mill effluent: POME was collected form Dominion Square Palm Mill, Gambang, Kuantan, Pahang. Filter net were used eliminate the solid particles and measured the pH before collect 10 L of POME. The POME stored at dark place to avoid organic compound degradation. The pH of POME is increased to 8.0±0.5 by adding 1 M of NaOH buffer solution in POME. and sterilized at 121 ℃ for 20 min.

The microalgae water samples were collected from Pantai Teluk Cempedak, Kuantan which located is at East Coast region of Pahang state. The Information on the temperature of water (27 °C), pH (8.0±0.5), salinity (3.5%) were reported to ensure consistency in the laboratory scale. The samples were centrifuged at 5,000 rpm, 15 ℃ for 10 min and discarded the supernatant. The samples are enriched by mixing the sample (90%) and cultural medium (10%) in 100 ml of Erlenmeyer flask with Bold basal medium and BG-11 medium. Those nutrient media were chosen to pre-select fast-growing microalgae.

2.2. Isolation of microalgae strains

After an enrichment stage, samples from the best medium were used in the dilution process, chosen based on the predominance of cells with similar morphological characteristics. In 30 ml solid medium with 1.5% agar in (100×15 mm), 500 µL of the diluted samples were streaked under aseptic condition in laminar flow. The inoculated plates were incubated under controlled temperature 25±2 °C and average continuous illumination of 3,000 lux for a period of 14 days. After the incubation period, individual colonies were replaced and re-cultivated for 14 days, to ensure the isolation of a single species of microalgae. Periodic testing on an optical microscope confirmed the confirmation of the microalgae colony's purity and compare the strains with algae manual to confirm and identified the species [16].

2.3. Preparation of inoculums

The isolated species which is *Spirulina platensis* strains were cultured in 100 ml of BG-11 medium in 500 ml of Erlenmeyer flask under controlled temperature 27±2 °C and average continuous illumination of 3,000 lux for 15 days. The volume of culture keeps multiplied by adding sterilized BG-11 medium and prepared as stock culture for further experiments [17].

2.4. Cultivation of Spirulina platensis

2.4.1. Effect of POME in growth of Spirulina platensis. The sterilized POME medium was feed to *Spirulina platensis* culture in different concentration of POME (10%, 20% and 30% v/v) in 2 L of Erlenmeyer flask. The culture was aerated with filtered air by using pump and provide average continuous illumination of 3,000 lux for 24 h continuously. The culture was grown for 15 days and recorded the absorbance reading via GENESYS 10S UV-Vis spectrophotometer to measure the growth rate. For every concentration of POME, the flasks were taken in triplet [6].

2.4.2. Effect of light intensities in growth of Spirulina platensis. In 20% of POME medium, the *Spirulina platensis* was cultured and determined the growth in different light intensities which was another parameter to enhanced the biomass production. As light is essential for the *Spirulina platensis* photosynthesis, different light intensities such as 3,000 lux, 4,000 lux, and 5,000 lux were set for the light intensity test (the culture was prepared in flasks as stated before). For every light intensity the flasks were taken in triplet.

2.5. Measurement of growth

The growth rate was measured by the density of culture by measuring the optical density value at 680nm by using Spectrophotometer. Typically, 680nm is associated with chlorophyll absorption. The algal biomass was separated by centrifugation for 5 min at 10,000 rpm, 4 °C. Then the algal pellets were washed with distilled water and dried it in oven at 90 ℃ for 36 h. Then measured the net weight of dried *Spirulina platensis* biomass which were collected from all the experimental flasks [18-20].

2.6. Lipid extraction of Spirulina platensis

The dried biomass was collected and then grinded to form fine powder by using mortar and pestle. It was then tightly sealed in an empty jar for subsequent extraction of the lipids. Ultrasonicated assisted technique was used for extraction of lipid, 5.0 g of the dried biomass of *Spirulina platensis* was placed in 50 ml of falcon tube. 45 mL of a methanol-chloroform solution with a volume ratio of 2:1 was poured into tube, and the mixture was vortexed for 1 min at room temperature. Later, the tube was placed on the ultrasonicated assisted water bath at 70 ℃ for 2 h. After that, the mixture was centrifuged at 8,000 rpm for 5 min at 15 ℃ and collected the supernatant by separating from biomass residue. Again, 45 mL of a methanol-chloroform solution with a volume ratio of 2:1 was poured into tube with biomass residue, and the mixture was vortexed for 5 min at room temperature. Later, the tube was placed on the Ultrasonicated assisted water bath at 70 ℃ for 2 h. These steps continued until the biomass mixture turn to colorless. The collected supernatant was poured in round bottom flasks and fixed in water bath at 70 ℃, 90 rpm in the rotary evaporator to separate the Lipid from mixture by evaporating the organic solvent. The lipid collected from the evaporation was weighed and the results were recorded [21,22].

2.7. Fatty acid composition analysis

The extracted lipid was trans-esterified by adding methanol KOH and vortexed for 30 to 60 seconds and placed it in water bath at 80 ℃ [23]. The extracted fatty acid methyl ester kept at inert environment (N_2) in a freezer at -18 ℃. In Agilent 7820A Gas chromatography equipped with capillary column of MEGA WAX MS (0.50 µm × 0.32 mm × 30 m) and a flame ionization detector with temperature of the injector and detector set at 190 ℃ and 230 ℃ respectively. The following thermal ease of 5 ℃/min until it reached 230 ℃. Carrier gas was Helium have been used at 4.41 ml/min. the hydrogen gas and purified

air were flowed at 30 and 450 ml/min, respectively. The quantification of FAME was analyzed in triplicate by adding an internal standard sample (C_{17} methyl ester) [24].

3. Results and discussion

3.1. Isolation of potential strain

There were total seven species of marine microalgae were identified. *Spirulina platensis* were isolated as shown in Figure 1 due to their high potential of lipid content in their biomass. Moreover, the survivability of *Spirulina platensis* was greater as compared to other identified species. *Spirulina platensis* easily adapts and ease to culture to laboratory environment.

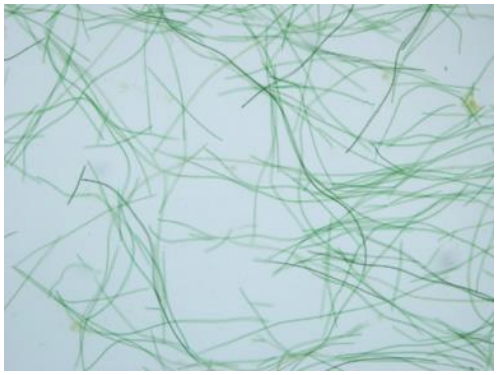

Figure 1. Image of microalgae *Spirulina platensis* under fluorescence microscope (10x).

3.2. The effect of POME concentration on Spirulina platensis.

Culturing *Spirulina platensis* in conical flask has its limitation in providing complete information concerning growth, progress and production of value-added compounds. Various factor contributing for enhancing bioactive compounds in microalgae especially *Spirulina platensis*. The compounds which present in raw palm oil mill effluent highly playing lead role in the growth of *Spirulina platensis* [25]. Based on Table 1 the characteristics of raw wastewater of POME, these substances are consumed by the strains and increase cultivation rate. As result, it increases the production of biomass per liter of culture with POME medium. As shown in Figure 2 the highest biomass produced in 20% of POME concentration are 754.5 mg/L with 260.3 mg of lipid yield on day 13. In 10% and 30% of POME medium, the biomass obtained are 554.7 mg/L and 443.3 mg/L and in turn lipid of 147.5 mg (26.6%) and 87.3 mg (19.7%) respectively. in 30% of POME found lower growth due to lower light penetration into the culture. The dark color from high concentration of POME resist the culture from obtaining light source as a result, culture unable to grow well.

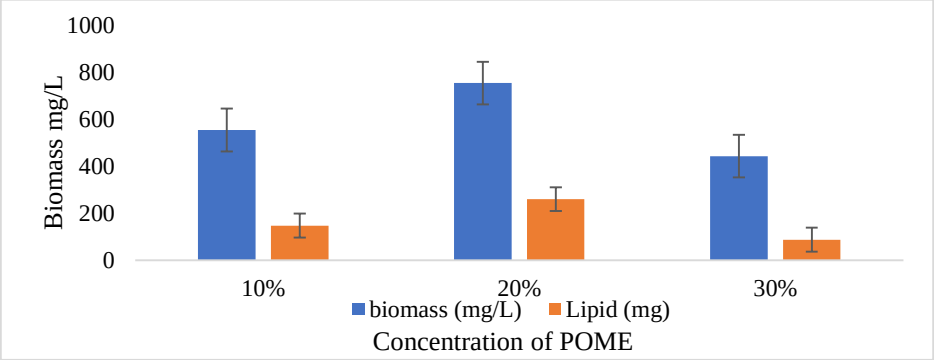

Figure 2. The yield of dry biomass and lipid content.

According to study of [26], in 5% of POME in a flat panel PBR exhibited the highest specific growth with 0.5 per day and biomass productivity as 137.5 mg liter per day. According to [27], a largescale growth of microalgae for biofuel production is based on the cultivation rate and oil content (in % dw). High COD, BOD, N, and P present in POME. It used in growing different strain of microalgae [25,26]. Generally, cultivation of microalgae required high nitrogen and other related chemical fertilizers, moving the process towards a non-environmentally friendly approach [19]. At the other hand, cultivating *Spirulina platensis* may in fact play an important role as a natural wastewater self-purification process [28]. Due to technically low cost and high impact, microalgae culture as a tertiary treatment before POME is released. As a result, the vast majority of nutrients such as nitrate and ortho-phosphates not removed during anaerobic digestion should be contained in addition in a microalgae bath. The cultivated microalgae would thus be used as a food source for live feed crops [29,30].

Spirulina platensis cultured in different concentration POME have produced higher yield and lipid extraction (243 mg/l to 553 mg/l) and the lipid content from 15.3% to 35.03% was obtained. From the study [31], POME concentration which gave the highest growth rate of 0.32 per day for chlorella UMAC 283 was 12.5% followed by POME at a laboratory scale of 6.25%. Since POME contain the high inorganic components such as nitrate (NO_3) and phosphate (PO_4) that are easily consumable nutrients for the microalgae multiplication [32]. While the biomass of strains increased, it indirectly increases the production of lipid. Due to that, strain selectivity is very important in order to produce more lipid from microalgae. In high concentration of POME, the growth rate decreased in *Spirulina platensis* cultivation. This is because in high concentration of POME, light penetration is insufficient due to the darker color of POME. The presents of tannic acid in POME cause the color appearance [26].

Table 1. Characteristic of palm oil mill effluent [33].

Parameter	Value
pH	7.1
Chemical Oxygen demand (COD, mg L^{-1})	1560±2
Total suspended solids (TSS, mg L^{-1})	77±6
Ammonium (mg L^{-1})	9.9±0.4
Phosphate (mg L^{-1})	5.9±0.3
Nitrite (mg L^{-1})	1.5±0.2
Nitrate (mg L^{-1})	3.5±0.6

3.3. Effect of light intensity on the growth of Spirulina platensis

The greater growth found in 20% of POME and in order to enhance the biomass production, additional different light intensities were given to optimized the light. The biomass production was increased while providing light intensities at different lux. The duration, intensity and quality of lighting are key factors for growth of photosynthetic species. Light intensity is known to affect the synthesis of the different cell components. Based on Table 2, At intermediate light intensities (5,000±200 lux), at 28±2 ℃, the maximum biomass 854.6 mg/L obtained with 35.8% of lipid content. The lowest yield was obtained at (3,000±200 lux) light intensity with 539.1 mg/L and extracted 23.7% of lipid. This indicating that, possibly in the light intensity of (5,000±200 lux), the light intensity was adequate to promote maximum cell growth and higher light intensity values under the assayed conditions did not likely to increase cell growth due to a shadow effect that could cause growth interruption. [34] Reported intermediate light exposure in *Spirulina platensis* cultivation, indicating initially low light intensity to prevent photolysis [35]. It is also noticed that they were subjected to photolysis of high light intensity. Based on Pandey report [36], Result indicates 5,000 lux is optimal light for the growth of *Spirulina platensis* in Madhya

Pradesh subtropical area. Chlorophyll *a* and protein content at 5,000 lux light intensity is 9.8 mg/g and 64.3%. Chlorophyll *a* maximum concentration is 14.2 mg/g at 3,000 lux light intensity.

The effect of illumination on cell growth was apparent, showing a strong relationship between concentration of the cell and intensity of light. Nevertheless, once the nutrient and temperature requirements have been met, such that growth is not constrained by these, the light intensity and its length determine the growth rate and the yield of production. In report [37] stated that an increase in light intensity initially favored cell division, and then a further increase in light intensity prevented cell division after optimum light intensity had been achieved.

Table 2. Effect of different light intensity on *Spirulina platensis* biomass.

Light intensity (Lux)	Dry biomass (mg/L)	Lipid content (%)
3,000±200	539.1	23.7
4,000±200	732.7	29.5
5,000±200	854.6	35.8

3.4. Analysis of polyunsaturated fatty acid profile

Samples with maximum lipid yield from each of the previous experiments were analyzed using gas chromatography to identify the composition types of fatty acids that each sample consisted of. A total of five samples were analyzed. A total of nine types of fatty acids compositions were reported from the results analyzed from the GC-MS and can be classified as saturated and unsaturated fatty acids [38]. Table 3 lists certain fatty acids. Algal fatty acids are the fundamental components of algae biosynthesis Polyunsaturated fatty acids (PUFA), responsive to changes in the aquatic climate, are responsible for structural function, whereas monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) constitute the lipid fraction for storage [22]. The ability to alter the lipid metabolism of algae in response to variations in the environment is therefore crucial to their growth and survival [2,39,40].

Table 3. Fatty acids profile of *Spirulina platensis.*

Name of compounds	Molecular formula	Molecular weight (g/mol)	Compositions (%)
Linoleic acid	$C_{18}H_{32}O_2$	280.40	14.10
Octadecenoic acid	$C_{18}H_{34}O_2$	282.50	8.24
Cyclopropaneoctanoic acid	$C_{22}H_{38}O_2$	334.28	10.52
Eicosapentanoic acid	$C_{20}H_{30}O_2$	302.45	7.24
Palmitic acid	$C_{16}H_{32}O_2$	256.43	18.04
Heneicosanoic acid	$C_{22}H_{44}O_2$	340.33	12.70
Palmitoleic acid	$C_{16}H_{30}O_2$	254.41	7.08
Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	8.74
Eicosanoic acid	$C_{20}H_{40}O_2$	312.50	13.34

4. Conclusion

The marine algae S*pirulina plate* was successfully cultured in POME medium for the production of lipid in the biomass. The optimized experimental factors were the incident light and the concentrations of nitrate, phosphate and silicate. POME concentration with 30% (v/v) was found to be the best concentration for the maximum growth of *Spirulina platensis* at 754.5 mg L^{-1} with 34.5% lipid production on day 13. In light intensity, under 5,000 lux maximum biomass produced was 845.6 mg L^{-1} with 35.8% of lipid yield on day 14. Palmitic acid and eicosanoic acid were the dominant fatty acids among a total of nine fatty acids presence in the extracted lipid. It is evident from the results that the cultivation medium POME, a waste effluent from palm oil mill, found to be a suitable nutrient source that is rich in ammonium, phosphate, nitrile and nitrate for the cultivation of this species. Utilization of such waste effluent for lipid production is one of the efforts for producing useful materials and economical way in a sustainable manner and should be given a serious attention.

5. References

[1] Mata T M, Martins A A and Caetano N S 2010 *Renew. Sust. Energ. Rev.* **14** 217
[2] Metsoviti M N, Papapolymerou G, Karapanagiotids L T and Katsoulas N 2019 *Plants*. **9** 31
[3] Ayre J M, Moheimani N R, and Borowitzka M A 2017 *Alga. Res.* **24** 218
[4] Zerrouki D and Henni A 2019 *Spring*. Switzerland
[5] Hadiyanto S D and Christwardana M 2013 *Env. Sci. Tech.* **6** 79
[6] Kamyab C H, Chelliapan S, Din M F, Rezania S, Khademic T and Kumar A 2018 *Intechopen*. 13
[7] Kamyab H, Fadhil M, Lee C, Ponraj M, Soltani M and Eva S 2014 *J. Tek.* **5** 1
[8] Tan C H, Nagarajan D, Show P L and Chang J S 2019 *A. Pres.* 601.
[9] Lopes A P C, Santos F M L F and Pires V J P V 2018 *Spring.* 35
[10] Manaf I S A, Embong N H, Khazaai, S N M, Rahim M H A, Yusoff M M, Lee K T and Maniam G P 2019 *Energ. Convers. Manage.* **185** 508
[11] Jayakumar S, Yusoff M M, Rahim M HA, Maniam G P, and Govindan N 2017 *Sust. Energ. Rev.* **72** 33
[12] Hindryawati N and Maniam G P 2015 *Ultrason. Sonochem.* **22** 454
[13] Sudhakar M P, Kumar B R, Mathimani T and Arunkumar K 2019 *J. Clean. Prod.* **228** 1320
[14] Mubarak M, Shaija A and Suchithra T V 2015 *Alga. Res*. **7** 117
[15] Yaakob Z, Ali E, Zainal A, Mohamad M and Takriff M S 2014 *J. Biol. Res.* **21** 10
[16] Schuelter A R, Kroumov A D, Hinterholz C L, Fiorini A, Trigueros D E G, Vendruscolo E G Zaharieva M M and Modenes A N 2019 *Biochem. Eng. J.* **144** 28
[17] Raposo M F J, Morais R M S C and Morais A M M B 2013 *Lif. Sci.* **93** 479
[18] Molino A, Mehariya S, Sanzo G D, Larocca V, Martino M, Leone G P, Marino T, Chianese S, Balducchi R, and Musmarra D 2020 *J. CO_2 Util.* **36** 196
[19] Chen Z, Wang L, Qiu S and Ge S 2018 *Biomed. Res. Int.*
[20] G, Premalatha M and Anantharaman N 2008 *Alga. Res*. **35** 471
[21] Ratledge C 2004 *Biochimie*, **86** 807
[22] Mauro D P 2008 *J. Diet. Suppl.* **6** 143
[23] Ramprasath V R, Eyal I, Zchut S, Shafat I and Jones P J 2015 *Lip. Health. Dis.* **14** 142
[24] Sayeda M A, Gamila H A and Farouk K El-Baz 2015 *Int. J. Pharm. Sci. Rev. Res.* **34** 210
[25] Kamyab H, Din M F M, Ghoshal S-K, Lee C T, Keyvanfar A, Bavafa A A 2016 *Waste. Biom. Val.* **7** 779
[26] Idris N, Loh S K, Lau H L N, Yau T C, Mustafa E M, Vello V and Moi P S 2018 *J. Oil. Palm. Res*. **30** 141
[27] Griffiths M J, and Harrison S T 2009 *J. Appl. Phycol.* **21** 493
[28] Neto P M, Souza R A S, Leon-Nino A D, Costa J D, Tiburcio R S, Nunes T A, Mello T C, Kanemoto F T, Saldanha-correa F M P and Gianesella S M F 2013 *Renew. Energ.* **55** 525

[29] Borowitzka M A, and Moheimani N R 2013 *Alga.Biof. Energ.* 1
[30] Kalsum U, Kusuma H, Roesyadi A and Mahfud M 2018 *Korean. Chem. Eng. Res.* **57** 301
[31] Nwuche C O, Ekpo D N, Eze N E, Aoyagi H and Ogbonna J C 2014 *British. Biotech. J.* **4** 305
[32] Zhu L D, Li Z H and Hiltunen E 2016 *Biomed Res. Int.* 1
[33] Govindan M, Maniam G P, Yusoff M M, Rahim M H A, Chatsungnoen T, Ramaraj R, and Chisti Y 2020 *J. Appl. Phyco.* **32** 375
[34] Dubey R C 2006 Chand S& Co New Delhi 732.
[35] Vonshak A and Richmond A 1988 *Biomas.* **15** 233
[36] Pandey J P, Mishra R M Tiwari A 2010 *J. Alga. Biom. Uti.* **1** 70
[37] Sorokin C and Krauss R W 1965 *American. J. Bot*. **52** 331
[38] Mutanda T, Ramesh D, Karthikeyan S, Kumari S, Ananraj A and Bux F 2011 *Biores. Tech.* **102** 57
[39] Spolaore P, Joannis-Cassan C, Duran E and Isambert A 2006 *J. Biosci. Bioeng.* **101** 87
[40] Singh S K, Kaur R, Bansal A, Kapur S and Sundaram S 2020 *Elsev*. 221

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