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ARTICLE

Biodiesel production by microalgae *Nannochloropsis* sp. grown in palm oil mill effluent

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ABSTRACT

A marine microalga, *Nannochloropsis* sp., was discovered in a water body in Teluk Cempedak, Kuantan, Pahang. In *Nannochloropsis* sp., for biomass and lipid synthesis, POME was used as a substitute medium. The isolated monoculture was grown in various concentrations of POME (5%, 10%, 15%, and 20%), as well as a standard control medium. *Nannochloropsis* sp. showed greater cell growth at 10% POME, with a maximum dry biomass of 1.504 g L^{-1} and extracted 35.9% lipid after 14 days of flask cultivation. Fatty acids namely oleic acid, linoleic acid, palmitic acid, and stearic acid were shown to be prominent in GC-MS analysis. The fatty acid oleic acid has been discovered to be the most abundant (73.40%). POME has the prospective to be used as a growth media for the cultivation of microalgae *Nannochloropsis* sp.

1. Introduction

Extensive use of fossil reserves has resulted in rapid depletion of fossil fuel reserves (Bhuyar et al., 2020a; Abd Malek et al., 2020; Ramaraj et al., 2016), which combined with the negative impacts of CO_2 emissions on the atmosphere, has prompted a search for more environmentally sustainable and renewable energy sources (Rameshprabu et al., 2015). Biofuel is a renewable energy source generated from biomass that aims to reduce not just reliance on fossil fuels (Kumar et al., 2020), but also greenhouse gas emissions. Biofuel made from agricultural commodities, on the other hand, raises a number of issues, including the possibility of rising global food prices and a disruption in the soil nitrogen cycle (Unpaprom et al., 2015). As a result of its excellent photosynthetic potential, biofuel production from microalgae *Nannochloropsis* sp. is recognized as the leading alternative raw resource (Ma et al., 2016) and able to produce a lipid content that ranges from 37% to 60% (of DW), which is greater than most other microalgal strains, indicating its superiority (Bhuyar et al., 2018; Saengsawang et al., 2020). Nonetheless, the cultivation of microalgae is expensive as they demand nutrients and water in a massive amount (Bhuyar et al., 2020b).

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Palm oil processing, on the other hand, results in the production of polluting effluent known as Palm Oil Mill Effluent (POME) (Omar, 2019; Nithin et al., 2020). Direct discharge of effluents will contaminate the environment and may impair the ecosystem (Rupani et al., 2010). POME is the most suitable raw material for microalgae development since it contains a high concentration of carbohydrates, proteins, nutrients (nitrogen and phosphorus), lipids, and minerals (Bhuyar et al., 2019a; Ahmad et al., 2020). *Nannochloropsis* sp. cultivation in POME can reduce cultivation costs by providing an inexpensive source of nutrients for *Nannochloropsis* sp. to grow and accumulate lipid for biodiesel production, while also treating the POME by reducing the organic load of effluent due to metabolism and uptake of wastewater components by *Nannochloropsis* sp (Alqadi et al., 2017). The idea of this research is to explore the usage of POME in the culturing of *Nannochloropsis* sp. microalgae in order to assess biomass productivity for fatty acids. After studying the optimal culture concentration, the experiments were scaled up to a larger volume to proceed with the lipid extraction before producing and characterizing methyl esters (biodiesel) via transesterification reaction using potassium hydroxide (KOH) as a catalyst.

2. Materials and Methods

2.1. Microalgae sample collection, isolation, and identification

Microalgae specimens were obtained at Pantai Teluk Cempedak (3°55’33’’N, 103°22’23”E), Kuantan, Pahang, Malaysia. The microalgae sample was collected with a 0.5 µm plankton net made of bolting silk fabric. The collection of microalgae sample was performed as described by (Govindan et al., 2020) with slight modification. The collected water sample was spun at 2,000 rpm for 5 minutes at 15°C to condense the algal cells and remove the solid waste materials. A 90 mL sediment-free sample of the residual water was supplemented with 10 mL sterile Conway media and cultivated on a laboratory rack under continuous fluorescence light at 26 ±2°C. For 24 hours, the sample was continuously aerated with filtered air using an air pump. The sample was cultivated until apparent symptoms of algal development appeared. The growing culture was serially diluted from 10-1 to 10-5 with distilled water that is sterile. This procedure's goal is to dilute the numbered cell in water. Standard plating techniques were employed to separate algal populations from the study area in order to isolate single microalgal species. These diluted samples were plated in sterile plastic petri dishes holding about 35 mL of solidified Conway agar medium. 1 mL of the diluted sample was put onto a media plate and fairly distributed across the surface. All inoculated petri dishes were secured with parafilm wax strips, inverted, and incubated for 14 days at a regulated temperature (25°C) with a light intensity of 2,000 lux. After multiple streaks, the inoculums are cultured in a pre-sterilized test tube with 10 mL liquid media.

Isolated colonies' morphological structures were studied with a fluorescence microscope (OLYMPUS, BX 53) and a Field Emission Scanning Electron (FESEM) (Joel, USA JSM-7008 FESEM) and verified using the algal standard manual and previous research. Then proceeded to scale up to Erlenmeyer flasks of 500, 1000, and 2000 mL.

Nomenclature and abbreviation

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

2.2. Chemical properties of palm oil mill effluent (POME)

A palm mill near Gambang, Pahang, Malaysia provided samples of palm oil mill effluent (POME) from anaerobic treatment ponds' final discharge phase (Pond 7). To remove the solid suspended material, the POME sample was filtered through a 0.45 µm pore size membrane and centrifuged (Refrigerated Centrifuge 5810R). The sample was sterilized at 121°C for 20 minutes after being diluted with an equivalent volume of distilled water. Using 1M NaOH, the pH of the diluted sterile POME was altered to 7.5 ±0.5. These procedures were taken in the same order as in past research (Shah et al., 2016). Analysis of COD, TN, and TP in wastewater were evaluated utilizing the American Public Health Association's approved procedures (Table 1).

Table 1 Chemical properties of POME.

Parameters	Concentration (mg/L)
pH	6.5
COD	1704
NH3-N	21.8
TN	150.4
TP	29.1

2.3. Nannochloropsis sp. cultivation in different POME concentrations

Performance in terms of *Nannochloropsis* sp. growth was examined in varying concentrations of POME. In the Erlenmeyer flask, POME medium was added to a stock culture of *Nannochloropsis* sp. in proportions of

5, 10, 15, and 20% (v/v), resulting in a total volume of 1L. Conway medium with 20% (v/v) culture has been utilized as a control. For 24 hours, the flasks were exposed to 2500 lux light, and the culture was aerated with filtered air at 28 ±2 ℃ (Khammee et al., 2021).

2.4. Measurement of Nannochloropsis sp. growth

The *Nannochloropsis* sp. growth was investigated in terms of optical density (OD) and dry biomass weight for 14 days and 15 days respectively. Using a spectrophotometer, the optical density of *Nannochloropsis* sp. culture was measured at a wavelength of 680nm. Every three days, the dry cell weight was evaluated

gravimetrically. Microalgae culture aliquots were routinely collected and for 10 minutes, the sample was centrifuged at 6,000 rpm. The pellet was collected and placed in a glass petri dish that had been dried and weighed beforehand. The samples were dried in a 60°C oven overnight before being weighed. Dry weights were expressed as g L^{-1} (Zhao et al., 2019).

2.5. Lipid extraction

The lipid deposited in the biomass of *Nannochloropsis* sp. was retrieved using the Soxhlet method (Khammee et al., 2020). Approximately 5 g of dry biomass was carefully weighed using an analytical scale and as a pre-treatment, the cells were immersed in 50 mL of hexane overnight for cell disruption, in which the cell walls rupture and the lipid is promptly released from intracellular. The soaked biomass sample was then placed in a cellulose thimble with 3-5 tiny rocks to help with drainage and placed in the Soxhlet extractor. The extraction solvent, 300 mL of pure n-hexane, was transferred to a 500 mL round bottom flask and positioned on the heating mantle. To extract the lipid, the n-hexane was subjected to boil for 7.5 hours at 60°C. The sample was left to cool after the extraction process was completed before removing the solvent flask containing the lipid extracts. The mixture was then passed through a rotary evaporator to separate the solvents. Formula was used to compute the extracted lipid.

2.6. Transesterification of Nannochloropsis sp. lipid

5 g of microalgae oil was measured and combined with a 1% potassium hydroxide (KOH) catalyst with the volume ratio of 1.5:1 of MeOH/oil. The solution was then vortexed for 30 seconds before being immersed in a water bath for two hours at 58 ±2°C in three quadrants of the tube. The solution separated into two different solvent layers, with the higher layer solvent (methyl esters) being carefully retrieved and put into a 2 mL centrifuge tube that had been pre-weighed. In the fume hood, the residual methanol in the layer was evaporated for 30 minutes. The methyl esters were then measured and placed into an amber vial, where they were kept chilled until needed (Wu et al., 2017).

2.7. Composition analysis of fatty acid methyl ester (FAME)

Gas chromatography-mass spectroscopy (GC-MS) was employed to evaluate for FAME in the transesterified lipids from the preceding step (Severes et al., 2017). 0.2 mL of the transesterifies oil were mixed in 0.8 mL of hexane and injected into GC-MS. The total sample injection volume was 1 µL, with helium as the carrier gas. The injection temperature was fixed at 250°C. Following 1 minute at 70°C, the column temperature was raised up to 270°C at a pace of 5°C/min. It took 54 minutes to complete the analysis in total.

3. Results and Discussion

3.1. Nannochloropsis sp. isolation and identification

After many re-streakings on the plate, six species of microalgae namely *Chlorella* sp., *Amphora* sp., *Gyrosigma* sp., *Tetraselmis* sp., *Spirulina* sp., and *Nannochloropsis* sp. were found and tracked down from the fresh water acquired obtained in Teluk Cempedak, Kuantan, Pahang, Malaysia. When compared to other microalgae, *Nannochloropsis* sp. exhibited the best visibility and growth throughout the preliminary cultivation. *Nannochloropsis* sp. was selected for this research as a potential oleaginous model microalga because it has a higher lipid composition than other microalgal strains. Under a fluorescent microscope, Figure 1 depicts the identified *Nannochloropsis* sp. microalgae strain.

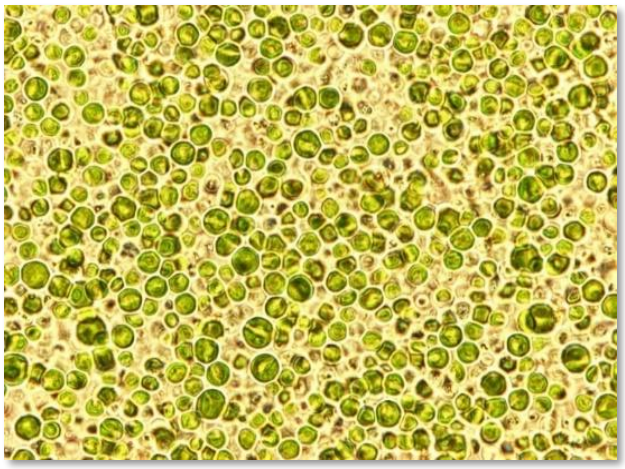

Fig. 1 Identified *Nannochloropsis* sp. microalgae strain

3.2. Cultivation of Nannochloropsis sp.

The *Nannochloropsis* sp. growth was investigated at various POME concentrations (%, v/v). Figure 2 shows the absorbance of *Nannochloropsis* sp. grown in control, 5, 10, 15, and 20% POME media at 680 nm from day 0 to 14. The cell concentration of *Nannochloropsis* sp. generated an OD value in the range of 0.556-0.565 at inoculation (day 0). The cultures progressed through four stages of development during cultivation: lag, exponential, stationary, and death. During the lag phase, *Nannochloropsis* sp., particularly those growing in varied POME concentrations, went through an adaptation process in the new medium (Palanisamy et al., 2019). According to Figure 2, the lag phase occurred between days 0 and 1 for all cultures cultivated in medium with POME concentrations of 10%, 15%, and 20%, with the exception of the culture grown in media with POME concentration of 5%, which had a 2-day lag phase. This is because the lag time of the growth curve varies based on the nutrient content of each media. Because the quantity of essential nutrients for photosynthetic activity, notably nitrate and phosphate, was low at 5% POME, *Nannochloropsis* sp. required a prolonged lag time before attaining exponential level. At 20% POME, however, the required amount of nutrients for photosynthesis was sufficient, resulting in a faster lag time (Hadiyanto et al., 2017).

For cultures cultivated in media with POME concentrations of 20% and 5%, the exponential phase appeared between day 1 and day 5 and day 2 to day 8 correspondingly. When compared to the other cultures, the exponential phase of culture produced in 20% was shorter because a higher concentration of POME resulted in a

darker POME solution, which reduced the amount of light that penetrated the medium. Despite this, the lower concentration of 5% POME offers enough nutrients for the growth of algae and allows more light to penetrate the medium. These aspects will drive growth until all of the nutrients present in POME has used up and light penetration has greatly reduced owing to the biomass concentration (Palanisamy et al., 2019). Cultures grown in media with 10% and 15% POME concentrations, as well as control cultures, demonstrated higher growth results, as they continued to proliferate from day 1 to day 9 before entering stationary phase. It is due to the nutrient availability in the medium that resulted in a sustainable growth *Nannochloropsis* sp.

The stationary period appeared between days 5 and 11 and days 8 and 12 for cultures cultivated in media with POME concentrations of 20% and 5%, respectively. For cultures grown in 20% POME, a longer stationary phase may have contributed to reduced cell concentration. The death process began after day 12 for cultures grown in 20%, but death began after day 13 for cultures cultivated in 5%, 10%, and 15%. During this phase, nutrients are depleted, preventing cells from continuing to develop. As a result, the cell concentration drops geometrically because cells die quicker than they expand (Putri et al., 2019).

Overall, *Nannochloropsis* sp. grown in a 10% POME concentration had a high culture density of 1.121, whereas *Nannochloropsis* sp. grown in a 20% POME concentration had a lower culture density of 0.528, owing to the presence of tannin acid, which gave the POME a dark colour, resulting in a high turbidity content in the medium, inhibiting photosynthesis and biomass production (Hadiyanto et al., 2017). Despite the fact that the cell concentration of *Nannochloropsis* sp. grown in 10% POME was equivalent to that of the control, the cell concentration of *Nannochloropsis* sp. grown in POME medium was much greater. Furthermore, previous study reported that the greatest cell density of 66.2x106 cells mL^{-1} and dry weight of 0.84 g L^{-1} for N. oculata was attained under 10% POME (Shah et al., 2016). In contrast to the findings of this experiment, a previous study revealed that the maximum biomass concentration of 3.46 g L^{-1} and 3.30 g L^{-1} were achieved when cultured Chlorella vulgaris and Chlorella sorokiniana were grown at POME concentrations of 5% and 20% (v/v), respectively (Cheah et al., 2018).

The dry cell weight (DCW) of *Nannochloropsis* sp. was measured every three days for 15 days to monitor growth. Figure 3 illustrates the dry weight of *Nannochloropsis* sp. at the optimal concentration of 10% POME medium. *Nannochloropsis* sp. dry cell weight gradually increased from day to day. On day 12, the maximum dry biomass of *Nannochloropsis* sp. in late stationary phase was 1.503 g/L. After day 12, however, the dry cell weight tends to decrease. This could be due to a lack of nutrients or because the cells are approaching the end of their life cycle due to restrictions in nutrient supply and light source. This disrupted the photosynthetic cycle and halted the accumulation of biomass in *Nannochloropsis* sp., resulting in a decline in microalgal dry cell weight. The dry cell weight was 0.934 ±0.005g L^{-1} on average.

Fig. 2 *Nannochloropsis* sp. absorbance readings at various POME concentrations.

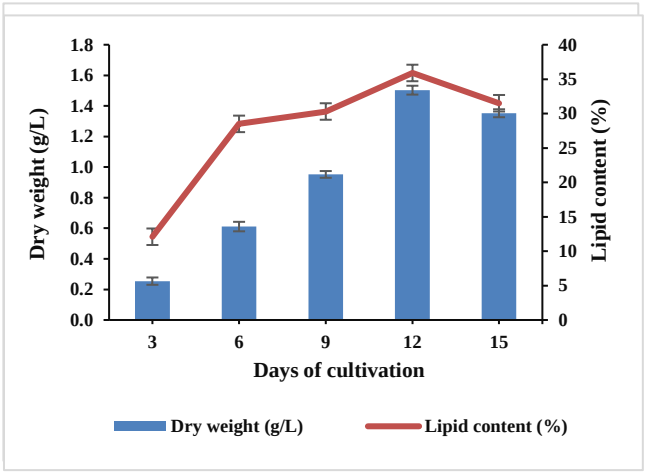

Fig. 3 Dry cell weight (g/L) and content of *Nannochloropsis* sp. lipid (%) in 10% POME culture.

3.3. Nannochloropsis sp. lipid extraction

The Soxhlet method was used in this work because it has been shown to be a reliable, effective, efficient, and user-friendly method for extracting lipids from algal biomass (Bhuyar et al., 2019c). In the lipid extraction from *Nannochloropsis* sp., the Soxhlet technique has a greater effect (Palanisamy et al., 2019). The lipid content recovered from *Nannochloropsis* sp. biomass harvested from a 10% (v/v) culture yielded a maximum yield of 35.9%. Throughout each cycle of solvent evaporation and condensation, Soxhlet replenishes *Nannochloropsis* sp. biomass with fresh solvents, minimising solvent usage. As a result, it improves extraction efficiency in order to achieve the maximum lipid yield. The requirement relatively low temperature (65ºC) makes the Soxhlet method of extraction cell penetration better compared to other extraction methods. Using Soxhlet extraction of dry biomass employing hexane as the solvent, past researches attained the highest lipid yield content (Kanda et al., 2020).

3.4. Analysis of fatty acid methyl ester (FAME)

The chemical features of fatty acids, such as the carbon chain's length and the level of unsaturation, govern the attributes of biodiesel. The fatty acid content of *Nannochloropsis* sp. was evaluated using GC-MS from a lipid extract derived from biomass of 10% POME. The most important fatty acids found in *Nannochloropsis* sp. were oleic acid (C18:1), linoleic acid (C18:3), palmitic acid (C16:0), and stearic acid (C18:3), as shown in Table 2. These findings are in line with past findings that oleic acid, linoleic acid, and palmitic acid are the most frequent fatty acids detected in microalgal lipid acid (Shin et al., 2014). In *Nannochloropsis* sp., monounsaturated fatty acid (MUFA) is the most prevalent component, which can give oxidative stability. However, in *Nannochloropsis* sp., polyunsaturated fatty acid (PUFA) and saturated fatty acid (SFA) levels were substantially

lower. According to previous researchers, it is not only desirable to produce high lipids, but it is also necessary to have a suitable fatty acid composition in order to be regarded a suitable candidate for the production of biodiesel. Additionally, because of their ability, microalgal oils with high quantities of C16 and C18, particularly monounsaturated fatty acids like palmitic acid (16:0) and oleic acid (18:1), have been reported to have a decent balance of biodiesel quality. *Nannochloropsis* sp. appears to be the most promising choice for biodiesel production. This is due to the fact that among other fatty acids, oleic acid was discovered to be the most abundant (73.40%) among the remainder of the MUFA composition, with 9.11% PUFA.

Table 2 Lipid composition of *Nannochloropsis* sp.

Fatty acids	Structure	Composition (%)
Oleic acid	C 18:1	73.40
Palmitic acid	C 16:0	12.40
Linoleic acid	C 18:3	8.86
Stearic acid	C 18:0	3.10
Palmitoleic acid	C 16:1	1.21
Eicosanoic acid	C 20:0	0.49
Gadoleic acid	C 20:1	0.30
∑SFAs		15.99
∑MUFAs		74.90
∑PUFAs		9.11

4. Conclusion

The focus of this research was to determine whether POME could be used as a microalgae growing medium. As per the results of this study, POME seems to have the prospective to be used as a medium for the growth of microalgae *Nannochloropsis* sp. Microalgae growing in wastewater provide a cost-effective effluent treatment approach, reduces nutrient costs while increasing microalgae biomass output by providing the nutrients and organic matter needed for microalgae metabolism. In 10% (v/v) of POME, a significant rate of growth of 1.121, maximum dry biomass of 1.504 g L^{-1} and extracted lipid of 35.9% were discovered. However, the growth rate is slowed upon increasing content of POME medium due to restriction in light intensity penetrations which results in photosynthesis disruption. Monounsaturated fatty acids are discovered in larger amounts (74.90%) in *Nannochloropsis* sp. cultivated in 10% POME medium, according to the results.

Declaration of competing interest

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

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