Energy Nexus 9 (2023) 100169

Contents lists available at ScienceDirect

Energy Nexus

journal homepage: www.elsevier.com/locate/nexus

Agriculture of microalgae *Chlorella vulgaris* for polyunsaturated fatty acids (PUFAs) production employing palm oil mill effluents (POME) for future food, wastewater, and energy nexus

Monaambighai Kumaran[a], Karthick Murugan Palanisamy[a], Prakash Bhuyar[b], Gaanty Pragas Maniam[a], Mohd Hasbi Ab. Rahim[a], Natanamurugaraj Govindan[a,*]

[a] Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang, Malaysia
[b] Organic Agriculture Management, International College, Maejo University, Nong Han, San Sai, Chiang Mai 50290, Thailand

ARTICLE INFO

Keywords:
Chlorella vulgaris
Effluent waste
Mass cultivation
Wastewater bioremediation

ABSTRACT

Malaysia is one of the second largest world palm oil exporters; correspondingly, the massive production of palm oil mill effluent (POME) is a significant concern. The present investigation aimed to isolate and characterize the potential microalgae and maximize the growth and biomass productivity of *Chlorella* sp. using POME. *Chlorella vulgaris* was cultured in BG-11 (control) and POME with 1:1, 1:2, 1:3, and 1:4 dilution ratios. Binary solvent extraction was applied to extract lipids, and polyunsaturated fatty acids (PUFAs) in the fatty acid methyl ethers were determined using gas chromatography coupled with mass spectrometry (GC-MS). Results suggest the lipid level of 21 wt.% was the highest amount obtained from biomass *Chlorella vulgaris* cultivated in 1:4 diluted POME. *Chlorella* sp. in POME with a 1:4 dilution ratio gives the highest biomass yield. It also yields the maximum dry cell weight of 0.42 g/L. Therefore, this study indicates that diluted POME with different dilutions is a suitable growth medium for *Chlorella vulgaris* biomass cultivation. Pairing wastewater with algae culture reduces biomass production and water treatment costs. Further studies demand more attention for future food, wastewater, and energy nexus.

1. Introduction

Southeast Asia is the world's largest palm oil producer, generating substantial effluents [1–3]. Palm oil mill effluent (POME) wastewater is created during typical wet processing, posing significant environmental problems. Wastewater treatment using microalgae was recently initiated due to the benefits of substantially lowering nutrient composition, while biomass may be employed as large biomass or value-added products [4–6]. Because of its low capital cost, more than 85% of Malaysian palm oil mills have used the ponding method for POME treatment [7–9]. Microalgal biotechnologies have emerged with a high potential for removing various organic pollutants, such as pharmaceutical and personal care products (PPCPs), from waste streams [10–12].

Polyunsaturated fats are healthy fats, commonly known as polyunsaturated fatty acids (PUFA). Aquatic animals such as fishes are the potential sources of PUFAs. Undeniably, the fish could not synthesize PUFAs on its own. They acquire it from primary producers like microalgae that can amass PUFAs straight away or implicitly through the food chain [13]. Microalgae have sufficient potential to substitute the role of FO as they are viable producers of essential PUFAs [14]. Furthermore, they also form LC-PUFAs with more than 20 carbon atoms at high levels [15]. Microalgae demonstrate numerous beneficial characteristics, including more lipid content in biomass, rapid growth rate within a shorter time [16], high tolerance towards environmental conditions, excellent photosynthetic rates, and better carbon dioxide (CO_2) capture capacity [17] and also can convert solar energy to electrical energy via biological pathways [11]. The flexibility of microalgae enables its cultivation almost everywhere [18].

Out of 40,000 species of microalga, *Chlorella vulgaris* is an auspicious PUFA supply. They encompass long-chain PUFAs, more than half of the total fatty acids [19]. This long chain PUFAs reveals therapeutic actions against diabetes, atherogenesis and hypertensive diseases [20]. Light, carbon source, and nutrients at desired quantities are mandatory requisites to support *Chlorella vulgaris* growth [21]. *Chlorella vulgaris* is water-blooming species that acclimatizes to wastewater [22]. *Chlorella vulgaris* is economical when incorporated with wastewater treatment owing to its ability to simultaneously accomplish CO_2 bio-fixation, lipid generation, and nutrient or pollutant removal as an alternative nutrient source

Abbreviations: PUFA, Polyunsaturated Fatty Acids; POME, Palm Oil Mill Effluent.

* Corresponding author.
E-mail address: natanam@ump.edu.my (N. Govindan).

https://doi.org/10.1016/j.nexus.2022.100169
Received 18 October 2022; Received in revised form 23 November 2022; Accepted 28 December 2022
Available online 29 December 2022

for microalgae cultivation of *Chlorella vulgaris* [23,24]. The synergetic effects can be seen while water, food and energy nexus as the microalgae employed for wastewater treatment can be converted into biofuels.

Since Malaysia is one of the world-ranked palm oil contributors, palm oil mill effluent was also found massively [25,26]. POME contains a profusion of macro and micronutrients, organic compounds, total suspended solids, water, oil and grease [27]. The abundant presence of these nutrients, such as N, P, and organic compounds in wastewater, are undesirables, especially in POME. Conventionally, they are removed from the POME by chemical methods. Those methods are considered inefficient for commercialization because they require plenty of strong acids, which indirectly leads to high secondary pollution, including greenhouse gas emissions [28]. Likewise, the discharge of POME effluents from the convention pond system does not comply with the stringent. Treating all those pollutants intensify operating costs again [29,30].

Cultivating *Chlorella vulgaris* supports their metabolism pathway to increase lipid production by converting the assimilated pollutants from the effluent. Oxidation of the organic matter by *Chlorella vulgaris* facilitates reducing chemical oxygen demand (COD) and biological oxygen (BOD) levels of POME. So, the water quality is enhanced for repeated usage [31]. Thereupon, POME of various dilution ratios was used to nourish *Chlorella vulgaris* in this study. They were cultivated at optimum conditions in order to produce the maximum amount of PUFA-rich biomass. So, microalgae cultivation using POME is inspired as sustainable technology to treat and diminish pollutants in the final discharge below the standards. At the same time, it also acts as a food supply that contains adequate nutrients. It enhances the sustainability of food availability in sufficient quantity with a zero-waste biorefinery approach for sustainability [32–34]. Therefore, the present investigation aimed to isolate and characterize the potential microalgae and maximize the growth and biomass productivity of *Chlorella* sp. using POME. Different dilutions concentration was applied to study the growth yield. Finally, extracted lipids from the biomass and subjected to determine the fatty acids methyl ester by using GC-MS analysis.

2. Materials and methods

2.1. Microalgae isolation and identification

The microalgae sample was collected from Pantai Balok, Kuantan Coast region (Latitude: 3°56.194′ N, Longitude: 103°22. 608′ E) in Pahang State, Malaysia. The 5 μm plankton net was used for the water surface collection. All collected water samples were stored in 50 mL falcon tubes and refrigerated while transferred to the FIST laboratory of the Universiti Malaysia Pahang, Malaysia, for further testing. Collected water samples were autoclaved to overcome unwanted microbes without removing the minerals, allowing them to grow on a specific medium for 14 days [26]. Standard plating methods were applied to separate the microalgae population on BG-11 nutrient agar. The microalgae were allowed to grow on Petri dishes filled with BG-11 medium with pH 7.5 ± 0.1, considered the most suitable condition for microalgae to thrive at optimum level. The nitrogen stress condition was formed by adding 0.025 g/L urea to the sample media. All microalgae were allowed to be cultured for two weeks. The cultures were examined using the microscopic method to identify microalgae strains. Identifying the species requires a data combination of morphology, microscopic and genetic characterization.

2.2. Identification of microalgae using fluorescent and scanning electron microscope

A small drop of culture was placed onto a microscopic slide, and a coverslip was carefully put onto it. The decline was observed from lower magnification up to higher magnification for morphological identification. The species identification was made by observing the morphology under a microscope with referred microalgae library. Cells were further examined using Scanning Electron Microscope (SEM) for morphological features. SEM sample preparations were started by fixing it with buffered aldehyde, followed by osmium tetroxide. It was dehydrated, dried, mounted on a specimen stub, coated with platinum, and examined under the electron beam using SEM brand FEI QUANTA 450, USA.

2.3. Isolation and characterization of Chlorella vulgaris

The 200 mL microalgae culture obtained from UMP Algae Laboratory was grown in 50 mL of BG-11 medium in a conical flask equipped with an air bubble pump [35]. After colonization, a 13 mL culture was centrifuged at 4000 rpm for four minutes using a benchtop centrifuge (Labnet Hermle, Japan). Then, the resulting pellet was taken using a sterile loop and streaked on each Petri plate containing solid BG-11 medium to obtain isolated colonies of *Chlorella vulgaris*. Those Petri plates were stored under continuous illumination with white fluorescent lamps at room temperature [33]. To characterize *Chlorella vulgaris*, the single colony was isolated and placed on a microscope slide to observe the morphology of the grown microalgae. The morphological features of potential *Chlorella vulgaris* were detected and recorded photographically at 100 x magnification using a fluorescence microscope. The submicroscopic cellular components also were observed with a high degree of specificity [36,37].

2.4. POME medium preparation

POME was obtained from Felda Global Ventures Plantations (Kilang Sawit Panching, Kuantan, Pahang, Malaysia) and stored in a plastic container at 4 °C to avoid contamination or biodegradation [38]. The suspended solids of the POME were eliminated by filtering them using a filter cloth [39] and then centrifuged at 8000 rpm for 10 min. Removing suspended solids increases the light penetration that aids the photosynthetic metabolism of microalgae [40]. The supernatant, which contained nutrients for microalgae culture, was obtained. Later, the POME was diluted using distilled water [41] to the ratios of 1:1 (500 mL: 500 mL), 1:2 (333 mL: 667 mL), 1:3 (250 mL: 750 mL), and 1:4 (200 mL: 800 mL) as shown in Fig. 1. The dilution of POME improves the light penetration into the medium. The pH of the diluted medium was adjusted to set about 7.0 - 7.5 and autoclaved for 20 min at 121 °C to kill indigenous contaminants. After autoclaving, the pH of the POME medium was readjusted to the range of 7.0 - 7.5 and re-filtered through filter paper Grade 1 Whatman filter papers (diameter = 150 mm) before using it [41]. The diluted POME medium with different ratios was sent to the Central Laboratory of Universiti Malaysia Pahang, Malaysia, for characterization of POME before microalgae cultivation.

2.5. Cultivation and culture conditions

2.5.1. Cultivation in BG -11 medium

The single colonies of *Chlorella vulgaris* were picked aseptically from the pre-cultured agar plates and inoculated into a 500 mL conical flask containing autoclaved BG-11. They were grown under continuous illumination (approximately 400 μ mol m^{-2} s^{-1}) with white fluorescent lamps and shaken manually thrice daily [42]. The culture was supplied with atmospheric air aeration as the carbon source and for agitation of *Chlorella vulgaris* cells [43]. The air supply was filtered using a 0.45 µm pore size Whatman nylon syringe filter [44]. The operation of the culture was maintained at 25 ± 1 °C [40]. After the growth of *Chlorella vulgaris* was confirmed when the culture turned from light green to dark green within one week, 480 mL of culture was transferred to a 1 L conical flask containing 120 mL BG-11 medium and upscaled further until it reached up to 2 L. The cultivation period was about 14 days.

2.5.2. Cultivation in palm oil mill effluent

800 mL of the pure culture of *Chlorella vulgaris* was transferred to a 2 L conical flask. 200 mL of diluted POME medium with a ratio of

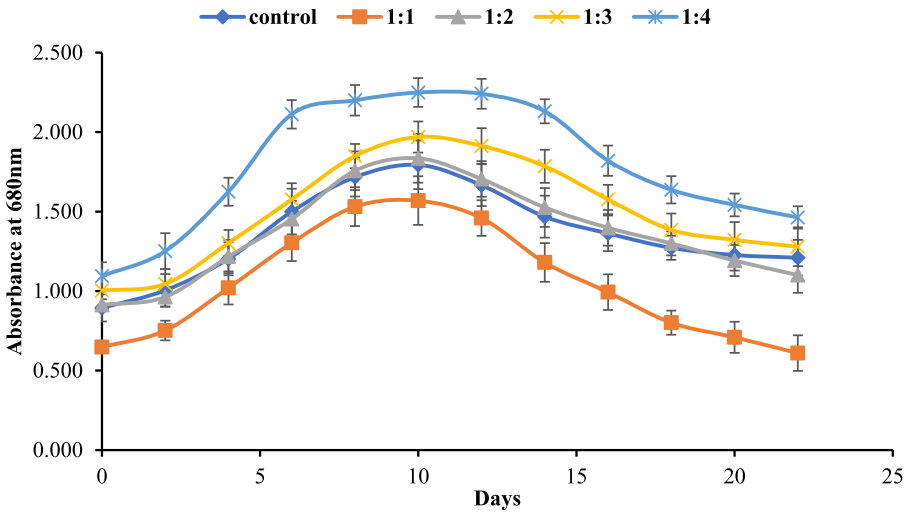

Fig. 1. The growth rate of *Chlorella vulgaris* in BG-11 medium (control) and POME medium of different dilution ratios.

1:1 was added to the same flask as the growth medium. The steps were repeated using diluted POME medium with the ratio of 1:2, 1:3, and 1:4 in three different 2 L conical flasks. The inoculum-to-medium ratio was maintained at 1:4, and the cultivation period plus conditions were kept similar to cultivation using BG-11 medium. The culture was upscaled until the total culture volume reached 2 L [25].

2.6. Biomass concentration determination

The biomass concentration was determined daily throughout the cultivation period. 1 mL of culture was obtained from each of the culture flasks and pipetted into a clean cuvette. The BG-11 medium and diluted POME mediums with respective ratios were used as blank. The cultures' optical density (OD) was determined at 680 nm using Thermo Scientific GENESYS UV-Vis Spectrophotometer. The biomass dry cell weight (DCW) was calculated based on OD reading at 680 nm as one OD at 680 nm equals 0.19 g/L dry cell weight [41]. The standard curve of dry cell weight versus the number of days was constructed.

2.7. Recovery of biomass

All the cultures of *Chlorella vulgaris* were grown for seven days before harvest [44] as they achieved the stationary phase on approximately day eight. The culture broth was transferred to 250 mL centrifuge tubes and centrifuged at 5000 rpm for 15 min using a refrigerated high-speed refrigerated centrifuge (CR21G III, HITACHI, Japan) [36]. The pellets were washed twice with distilled water. The supernatant was decanted for cultures grown in BG-11; meanwhile, for the cultures grown in POME, the supernatant was collected and sent to Central Laboratory UMP for characterization of POME after cultivation. The harvested wet biomass (pellet) was placed in pre-weighed Petri dishes and oven-dried overnight at 60 °C [16,45]. After drying, the weight of petri dishes with dried biomass was obtained again. The dry weight of biomass was calculated by subtracting the importance of the empty petri dish from the weight of the petri dish containing dried biomass. The recovered biomass was grounded using mortar and pestle before lipid extraction [46,47].

2.8. Extraction of lipid

Bligh and Dyer's method was applied to extract crude oil from dried biomass. About 1.25 grams were placed into a centrifuge tube (ISOLAB, Germany). Then, 12.5 mL of chloroform: methanol (2:1 v/v) mixture was added into the biomass and left soaked in the organic solvents for four hours under continuous shaking at 150 rpm using IKA® KS 260 basic rotary shaker at room temperature. After that, the mixture was centrifuged for five minutes at 4000 rpm, and the lipid with solvent was transferred into a separating funnel. The oil was separated from the solvent once five millilitres of distilled water was added to the mixture. The oil containing PUFAs found in the organic phase (bottom layers) was collected in a rotary flask. The chloroform layer was aspirated from the separated biomass and the oil by using a rotary evaporator. Finally, the oil weight was calculated by subtracting the weight of the empty vial from that of the vial containing the oil; meanwhile, the oil percentage (w/w, %) was calculated using the following formula [48].

$$\text{Oil content (\%)} = \frac{Extracted\ oil\ weight\ (mg)}{Sample\ weight\ (mg)} \times 100 \quad (1)$$

2.9. Transesterification

The lipid was trans-esterified into FAME by taking 10 mg of *Chlorella vulgaris* lipid and mixing it with 2 mL of hexane and 200 µL of 2 M methanolic KOH as the catalyst. Vigorous agitation was given to the mixture for five minutes using a vortex (8031102000 MX-S, DLAB, USA). For the FAME analysis by gas chromatograph, the upper clear supernatant with hexane (organic layer) was collected [16].

2.10. Gas chromatography-mass spectrometry (GC-MS) analysis

The FAME sample was injected at a volume of 1 μL in splitless mode by an Agilent 7693 autosampler into an Agilent 7890A gas chromatograph (Agilent, Santa Clara, California, USA) equipped with a 60 m × 250 µm × 0.25 µm DB-Wax column with a chemically bonded FAME stationary phase. The injector temperature was 250 °C. The initial purge flow rate was 50 mL/min, and after 2 min changed to 20 mL/min. The column temperature was held at 50 °C for 1 min, increased by 25 °C/min to 2000 °C for 0 min, increased by 3 °C/min to 230 °C for 18 min, and the run time was 35 min. The column effluent was introduced into the MS Source and MS Quad. The transfer line and the source temperatures were 230 °C and 1500 °C, respectively. All GC–MS data were analyzed [44].

3. Results and discussion

3.1. Isolation of microalgae Chlorella vulgaris

The microalgae samples obtained from UMP Algae Laboratory were grown under controlled conditions. After colonization, they were isolated using the streak plate technique. The consortium of microalgae

Table 1
General characteristics of palm oil mill effluent (POME).

Parameters	Range of values for raw POME	Values obtained for raw POME	Regulatory discharge limits	References
COD (mg/L)	2845 ± 159	2900	1000	[38]
BOD (mg/L)	25000 ± 1000	25000	100	[48]
pH	8.0 ± 0.5	8.3	5.0 - 9.0	[38]
Total Nitrogen (mg/L)	753 ± 15	750	200	[50]
Phosphate (mg/L)	102 ± 2	100	-	[50]

species was grown on the solidified BG-11 media. Pure microalgae cultures such as blue-green and green algae were gained by picking up single colonies from the plate based on their colour and morphology. The picked colonies were then again cultured on fresh media plates.

3.2. Identification of microalgae

The morphological identifications of microalgae were made using a fluorescence microscope (Olympus, Japan) based on their morphology, colour, shape and physical microalgae. They were the single strain of *Chlorella vulgaris* was isolated and identified. *Chlorella vulgaris* have spherical or slightly oval-shaped cells, about 2 - 15 μm in diameter, without flagella. The green colour of the microalgae shows that they belong to the family Chlorellaceae of class Trebouxiophyeae and division of Chlorophyta. It appears as single cells or in colonies. The matured vegetative cell has multiple parietal cup-shaped chloroplasts; younger cells contain only one chloroplast and nucleus. Since *Chlorella vulgaris* are non-motile microalgae, auto sporulation was the only mode of reproduction where the mother cells could divide up to 32 autospores via mitosis. The newly formed cell wall remains fragile during its early formation in its auto sporangia. Still, cell wall thickness and composition are not constant in the mature stage because they can change according to different growth and environmental conditions [49].

Adding on, *Chlorella vulgaris* can accumulate lipids, especially polyunsaturated fatty acids. Chlorella's rapid growth rate is ideal for production because it is remarkably resistant to harsh conditions and invaders. On the one hand, they are a food source, raw material for biofuel production and also applied for wastewater treatment as they efficiently remove Nitrogen, phosphorus, COD, plus BOD concurrently. *Chlorella vulgaris* produce oxygen via photosynthesis, which will be consumed by bacteria in wastewater. Symbiotically, the bacteria exhale CO_2 to convert the nutrients available in the wastewater for their growth and metabolism [8].

3.3. Characterization of POME

The characterization of POME may vary according to the performance of the palm oil mill's production flow [50]. The overall characteristics of raw POME obtained from the Felda Global Ventures Plantations (Kilang Sawit Panching, Kuantan, Pahang) plant and the regulatory discharge limits are presented in Table 1. Undeniably, the discharged effluents from palm oil mills often do not fulfil the regulatory discharge limits. Therefore, converting POME into an environmentally friendly waste requires an efficient treatment like microalgae cultivation [51].

The microalgae were cultivated based on various dilution ratios. The raw or partially treated POME has an extremely high content of degradable organic matter. The more organic content in POME, the higher the oxygen demand required to decompose organic matter, thus leading to relatively more elevated BOD [52]. This is proven in the 1:1 dilution ratio of POME compared to 1:4 because the portion of POME is higher concurrently high BOD (Table 1). Moreover, *Chlorella vulgaris* also achieves almost 50 % reduction in COD concentration, correspondingly to Al-Amshawee et al. [53].

Furthermore, the POME medium showed a better carbon source for *Chlorella vulgaris* growth than the BG-11 medium. The photosynthetic CO_2 fixation by microalgae allowed the equilibrium to shift to replace the exploitation of CO_2 by the microalgae. The efficient photosynthetic metabolism of microalgae enhanced O_2 evolution that reacts with free hydrogen ions to form free OH^- radicals and contribute to pH increase. Therefore, CO_2 was supplied continuously in this study to ensure that the pH values did not drastically increase. POME contains sufficient Nitrogen and phosphate, which supports microalgal growth. They substituted the role of BG-11 medium and performed much better. These nutrients promote the photosynthesis of *Chlorella vulgaris* to yield lipid-rich biomass. As the microalgae grow, it facilitates nutrient reduction in the POME medium and improves the quality of POME [54]. The comparative differences in the characteristics of POME before and after *Chlorella vulgaris* cultivation are presented in Table 2.

Table 2
Changes in the characteristics of POME before and after *Chlorella vulgaris* cultivation.

Parameters	Values obtained for diluted POME							
	Before cultivation				After cultivation			
Dilution ratios	**1:1**	**1:2**	**1:3**	**1:4**	**1:1**	**1:2**	**1:3**	**1:4**
COD (mg/L)	706	404	294	287	487	287	147	144
BOD (mg/L)	64	46	32	26	41	26	22	20
pH	7.3	7.5	7.3	7.2	6.8	7.1	7.4	7.3
Total Nitrogen (mg/L)	3.3	14	7	14	1.8	10	5	5
Phosphate (mg/L)	14	8	6	10	10	6	4	3

3.4. Growth analysis of Chlorella vulgaris

The biomass concentration of *Chlorella vulgaris* was measured using a UV-Vis spectrophotometer at 680 nm [8,41,55]. The optical density (OD) for control and experiments was plotted against the number of days, as shown in Fig. 1. The growth of the *Chlorella vulgaris* (control, ratio 1:2 and 1:3) had increased from day 1 to day 10 and began to decrease from day 11 to day 22. Notably, the growth of *Chlorella vulgaris* in ratio 1:4 has shown a shorter lag phase and reached the stable stationary phase quickly on the sixth day than others (day eight). Acclimatization of microalgae strains in POME can enhance the adaptability and shorten the lag phase of microalgae growth [56] since the growth phase of the microalgae has a strong connection with lipid accumulation in microalgae cells, the stable plus lengthier stationary phase in ratio 1:4 aids the generation of high lipid-rich biomass. The overall growth in ratio 1:4 was vigorous, while in ratio 1:1 was poor compared to control or other dilution ratios. This proves that raw POME should be diluted to enhance microalgae growth. Likewise, the weak growth of *Chlorella vulgaris* in the 1:1 dilution ratio indicates that the higher portion of POME is not suitable, and the darker colour of POME leads to inadequate light penetration [22,57].

Besides, the specific growth profile of *Chlorella vulgaris* in terms of dry cell weight was calculated based on mean OD readings at 680 nm (1 OD at 680 nm = 0.19 g/L dry cell weight) [41]. Fig. 2 demonstrated the dry cell weight of *Chlorella vulgaris* in a 1:3 dilution ratio was slightly higher at day 10 (0.3741 g/L) than in BG-11 medium (0.3409 g/L), 1:1(0.2981 g/L) and 1:2 (0.3487 g/L) dilution ratios. But the dry cell weight of *Chlorella vulgaris* in a 1:4 dilution ratio reached up to 0.4273 g/L. This was due to sufficient nutrient supply in the POME medium with a 1:4 dilution ratio. This means that *Chlorella vulgaris* ex-

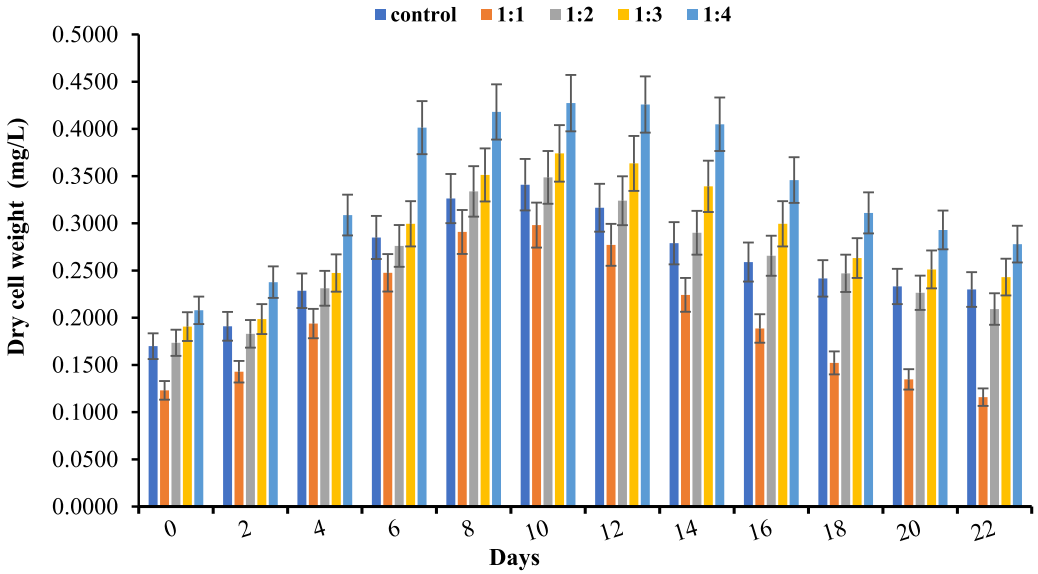

Fig. 2. The dry cell weight of *Chlorella vulgaris* in BG-11 medium (control) and POME medium with a 1:4 dilution ratio.

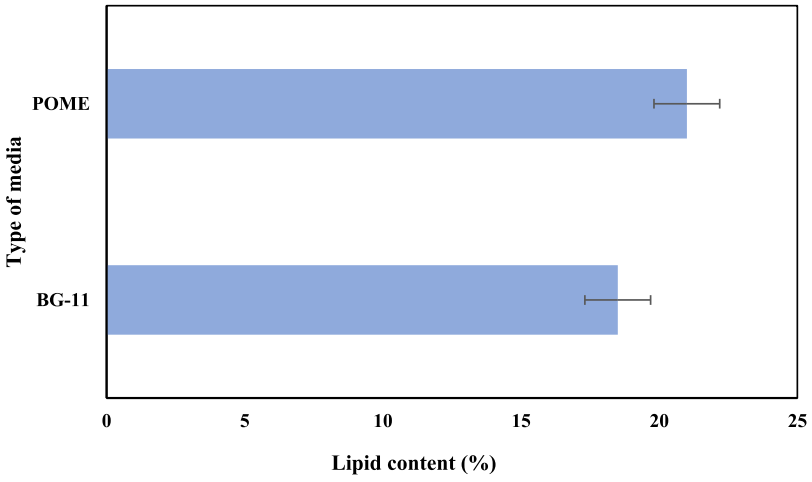

Fig. 3. The lipid content of *Chlorella vulgaris* in BG-11 medium (control) and POME medium with a 1:4 dilution ratio.

hibits higher feasibility to grow in lower POME concentration than in BG-11 medium. In a cultivation process, organic compounds in palm oil mill effluent faced a degradation that led to increased inorganic nutrients that contributed to greater microalgae cell density. At the same time, nutrients will be depleted as microalgae consumption successfully removes pollutants at the end of the culture [8].

3.5. Extraction of microalgal lipids

The effectiveness of using POME for microalgae cultivation was determined by biomass concentration and lipid content. Fig. 3 shows the lipid obtained from the biomass of *Chlorella vulgaris*. Lipid content increased along the cultivation cycle until the stationary phase. The *Chlorella vulgaris* cultivation with POME using a 1:4 dilution ratio yielded 21 % lipid (0.42 g/L) (Fig. 3) from two grams of dried biomass. Meanwhile, the percentage is not up to the lipid produced by *Chlorella vulgaris* when grown in BG-11 medium (18.5 % = 0.37 g/L). During the log phase, the microalgal cells grew exponentially and quickly consumed nutrients in the POME media. So, the biomass concentration is higher than lipid accumulation [31,36]. Despite this, overall slow biomass growth along the stationary phase, particularly after the 10th day, has brought about relatively higher lipid yield [55]. Furthermore, centrifugation also supports the production of a high amount of lipids and FAMEs [36].

3.6. Fatty acid methyl ester analysis by gas chromatography-mass spectrometry

A few techniques can be used to detect fatty acids or derivatives (FAME), especially GC-MS. This technique has been used broadly in detecting fatty acids in animals, plants and other organisms that have 12 to 24 carbon atoms in their carbon chains. The methyl esters were obtained by converting the fatty acids in the lipid of *Chlorella vulgaris*. This aids them to be volatile enough for gas chromatography analysis. Table 3 shows GCMS compounds that were detected in the FAME of Chlorella vulgaris cultivated in BG-11 and 1:4 POME medium. Nirmala & Dawn [58] have studied the growth of *Chlorella vulgaris* in various cultivation mediums for biomass and lipid productivity. Among all of the medium, *Chlorella vulgaris* perform better concerning biomass and lipid in the BG-11 medium. There were many other PUFAs present when they altered the cultivation conditions. According to Chi et al. [59], *Chlorella vulgaris* was able to yield higher biomass and lipid. Its fatty acid profile is more suitable for nutritional uses because it is more concentrated in polyunsaturated fatty acids such as eicosapentaenoic acid (EPA). The present

Table 3
Comparison of fatty acids composition of *Chlorella vulgaris* between BG-11 and POME media biomass.

Name	Fatty acids	Yield (%, w/w) under different media	
		BG-11	POME
Palmitic acid	C16:0	40.39	40.41
Palmitoleic acid	C16:1	1.87	1.77
Stearic acid	C18:0	3.21	3.25
Oleic acid	C18:1	28.14	28.53
Linoleic acid	C18:2	24.43	24.22
Linolenic acid	C18:3	1.85	1.86
Cis-11 eicosenoic acid	C20:1	0.11	0.15
Saturated fatty acid		*43.6*	*42.28*
Monosaturated fatty acid		*30.12*	*30.55*
Polyunsaturated fatty acid		*26.28*	*26.18*
Total		100	100.19

results agree with the previous study that reported that *Chlorella vulgaris* could survive easily in wastewater and improve polyunsaturated fatty acids like eicosenoic acid and docosahexaenoic acid (DHA) [36].

After lipid extraction, esterification was carried out with methanol under potassium hydroxide catalysis to determine the fatty acids. The fatty acid content of the lipids recovered from *Chlorella vulgaris* cultivated under different media conditions is shown in Table 3. Algal lipid quality classified in biotechnological applications depends on the fatty acid profile. Fatty acid chains varied from C16:0 to C20:1 in length. The predominant saturated, monosaturated fatty and polyunsaturated fatty were C16:0 (palmitic acid), C18:1 (oleic acid) and 18:2 (linoleic acid). There were no significant differences in the fatty acid accumulation between BG-11 and POME media (p-value < 0.05). Moradi-Kheibari et al. [60] reported that the overall fatty acids concentrations stabilized as longer chain fatty acids were produced by consuming the shorter chain fatty acids from the 20th day onwards of cultivation. Therefore, harvesting after an optimal time of cultivation can yield more fatty acids. Biosynthesis of fatty acid occurs when shorter chain fatty acids act as a precursor for the longer chain of fatty acid synthesis. These fatty acids are omega-3 fatty acids associated with fetal development, cardiovascular disease, inflammation, lowering cholesterol levels and prevention of Alzheimer's disease upon regular consumption, thus being a valuable food supplement [61].

4. Conclusions

This research has demonstrated that growing the *Chlorella* sp. in POME had correspondingly improved the biomass amount and PUFA content. The growth of *Chlorella* sp. in POME with a 1:4 dilution ratio gives the highest biomass yield (0.42 g/L); meanwhile, it also contains additional PUFA content in the microalgae's lipid (0.42 g). Although POME contains abundant nutrients that aid the growth of PUFA-rich *Chlorella* sp., it is always the most significant barrier to meeting standard discharge limits by the industries. Moreover, raw POME is not favourable for growing microalgae as its darker colour can affect the light intensity and high acidity level. Nevertheless, diluting POME is preferable to make it worthwhile for microalgae growth. Hence, the 1:4 dilution ratio was the best formulation for the *Chlorella* sp. growth in this study because it supports the growth and lipid accumulation at a time. Furthermore, cultivation of *Chlorella* sp. efficiently treats the POME by consuming the organics and nutrients for its growth since the BOD, COD, total Nitrogen and phosphate were reduced after the cultivation in POME with a dilution ratio of 1:4. Eventually, POME also stands a high chance to meet standard discharge limits. GC-MS analysis of FAME in *Chlorella* sp. grown in 1:4 ratio pieces of evidence of the optimization of PUFA content in the lipid. In a nutshell, *Chlorella* sp. can grow well in terms of PUFA-rich lipid and biomass simultaneously when cultivated using POME with a dilution ratio of 1:4.

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

CRediT authorship contribution statement

Monaambighai Kumaran: Investigation, Validation, Writing – original draft, Writing – review & editing. **Karthick Murugan Palanisamy:** Resources, Methodology, Supervision, Writing – review & editing. **Prakash Bhuyar:** Resources, Methodology, Writing – review & editing. **Gaanty Pragas Maniam:** Resources, Methodology, Supervision, Writing – review & editing. **Mohd Hasbi Ab. Rahim:** Formal analysis, Writing – review & editing. **Natanamurugaraj Govindan:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Data Availability

Data will be made available on request.

Acknowledgements

The authors would like to acknowledge Universiti Malaysia Pahang for the funding through Flagship Grant RDU182205.

References

[1] W.Y. Cheah, P.L. Show, Y.J. Yap, H.F. Mohd Zaid, M.K. Lam, J.W. Lim, Y Tao, Enhancing microalga *Chlorella sorokiniana* CY-1 biomass and lipid production in palm oil mill effluent (POME) using novel-designed photobioreactor, Bioengineered 11 (1) (2020) 61–69.
[2] W.H. Leong, K. Kiatkittipong, W. Kiatkittipong, Y.W. Cheng, M.K. Lam, R. Shamsuddin, J.W. Lim, Comparative performances of microalgal-bacterial co-cultivation to bioremediate synthetic and municipal wastewaters whilst producing biodiesel sustainably, Processes 8 (11) (2020) 1427.
[3] A.A. Mohd-Sahib, J.W. Lim, M.K. Lam, Y. Uemura, C.D. Ho, W.D. Oh, W.N. Tan, Mechanistic kinetic models describing impact of early attachment between *Chlorella vulgaris* and polyurethane foam material in fluidized bed bioreactor on lipid for biodiesel production, Algal. Res. 33 (2018) 209–217.
[4] U. Suparmaniam, M.K. Lam, Y. Uemura, S.H. Shuit, J.W. Lim, P.L. Show, P.T.K. Le, Flocculation of *Chlorella vulgaris* by shell waste-derived bioflocculants for biodiesel production: process optimization, characterization and kinetic studies, Sci. Total Environ. 702 (2020) 134995.
[5] W.H. Leong, W. Kiatkittipong, M.K. Lam, K.S. Khoo, P.L. Show, M. Mohamad, J.W. Lim, Dual nutrient heterogeneity modes in a continuous flow photobioreactor for optimum nitrogen assimilation to produce microalgal biodiesel, Renew. Energy 184 (2022) 443–451.
[6] N.S.M. Aron, K.S. Khoo, K.W. Chew, A. Veeramuthu, J.S. Chang, P.L. Show, Microalgae cultivation in wastewater and potential processing strategies using solvent and membrane separation technologies, J. Water Process Eng. 39 (2021) 101701.
[7] P. Bhuyar, D.D. Hong, E. Mandia, M.H.A. Rahim, G.P. Maniam, N. Govindan, Desalination of polymer and chemical industrial wastewater by using green photosynthetic microalgae, *Chlorella* sp, Maejo Int. J. Energy Environ. Commun. 1 (3) (2019) 9–19.
[8] B.R. Nithin, P. Bhuyar, M. Trejo, M.H.A. Rahim, G.P. Maniam, N. Govindan, Culturing of green photosynthetic microalgae (*Chlorella* sp.) using palm oil mill effluent (POME) for future biodiesel production, Maejo Int. J. Energy Environ. Commun. 2 (1) (2020) 1–8.
[9] N.H. Embong, N. Hindryawati, P. Bhuyar, N. Govindan, M.H.A. Rahim, G.P. Maniam, Enhanced biodiesel production via esterification of palm fatty acid distillate (PFAD) using rice husk ash ($NiSO_4$)/SiO_2 catalyst, Appl. Nanosci. (2021) 1–9.
[10] P. Xie, S.H. Ho, J. Peng, X.J. Xu, C. Chen, Z.F. Zhang, D.J. Lee, N.Q. Ren, Dual purpose microalgae-based biorefinery for treating pharmaceuticals and personal care products (PPCPs) residues and biodiesel production, Sci. Total Environ. 688 (2019) 253–261.
[11] D.J. Lee, J.S. Chang, J.Y. Lai, Microalgae–microbial fuel cell: a mini review, Bioresour. Technol. 198 (2015) 891–895.
[12] P. Bhuyar, M.H.A. Rahim, G.P. Maniam, R. Ramaraj, N. Govindan, Exploration of bioactive compounds and antibacterial activity of marine blue-green microalgae (*Oscillatoria* sp.) isolated from coastal region of west Malaysia, SN Appl. Sci. 2 (11) (2020) 1–10.
[13] M.I. Gladyshev, N.N. Sushchik, O.V. Anishchenko, O.N. Makhutova, V.I. Kolmakov, G.S. Kalachova, O.P. Dubovskaya, Efficiency of transfer of essential polyunsaturated fatty acids versus organic carbon from producers to consumers in a eutrophic reservoir, Oecologia 165 (2) (2011) 521–531.
[14] R.G. Gevorgiz, A.A. Gontcharov, S.N. Zheleznova, L.V. Malakhova, T.E. Alyomova, T. Maoka, M.V. Nekhoroshev, Biotechnological potential of a new strain of *Cylindrotheca fusiformis* producing fatty acids and fucoxanthin, Bioresour. Technol. Rep. (2022) 101098.

[15] X.M. Sun, L.J. Ren, Q.Y. Zhao, X.J. Ji, H. Huang, Enhancement of lipid accumulation in microalgae by metabolic engineering, Biochim. Biophys. Acta. Mol. Cell. Biol. Lipids. 1864 (4) (2019) 552–566, doi:10.1016/j.bbalip.2018.10.004.

[16] P. Nawkarkar, A.K. Singh, M.Z. Abdin, S. Kumar, Life cycle assessment of *Chlorella* species producing biodiesel and remediating wastewater, J. Biosci. 44 (4) (2019) 1–15, doi:10.1007/s12038-019-9896-0.

[17] M. Rehmanji, R. Singh, A.A. Nesamma, N.J. Khan, T. Fatma, A. Narula, P.P. Jutur, Multifaceted applications of microalgal biomass valorization to enriched biorenewables, a review of futuristic biorefinery paradigm, Bioresour. Technol. Rep. (2022) 100972.

[18] M.N. Metsoviti, G. Papapolymerou, I.T. Karapanagiotidis, N. Katsoulas, Effect of light intensity and quality on growth rate and composition of *Chlorella vulgaris*, Plants 9 (1) (2020) 31, doi:10.3390/plants9010031.

[19] G.F. Ferreira, L.F. Ríos Pinto, R. Maciel Filho, L.V. Fregolente, A review on lipid production from microalgae: Association between cultivation using waste streams and fatty acid profiles, Renew. Sust. Energ. Rev. (2019) 448–466, doi:10.1016/j.rser.2019.04.052.

[20] G. López, C. Yate, F.A. Ramos, M.P. Cala, S. Restrepo, S. Baena, Production of Polyunsaturated Fatty Acids and Lipids from Autotrophic, Mixotrophic and Heterotrophic cultivation of *Galdieria* sp. strain USBA-GBX-832, Sci. Rep. 9 (1) (2019), doi:10.1038/s41598-019-46645-3.

[21] P. Bhuyar, S. Sundararaju, M.H.A. Rahim, R. Ramaraj, G.P. Maniam, N. Govindan, Microalgae cultivation using palm oil mill effluent as growth medium for lipid production with the effect of CO_2 supply and light intensity, Biomass. Convers. Biorefin. 11 (5) (2021) 1555–1563.

[22] S. Khalid, M. Abbas, H. Bader-Ul-Ain, H.A.R. Suleria, Pharmacological applications of marine-derived compounds: a preventive approach, in: Technological Processes for Marine Foods, From Water to Fork, Apple Academic Press, 2019, pp. 3–22.

[23] A.K. Patel, Y.Y. Choi, S.J. Sim, Emerging prospects of mixotrophic microalgae: Way forward to sustainable bioprocess for environmental remediation and cost-effective biofuels, Bioresour. Technol. 300 (2020) 122741, doi:10.1016/j.biortech.2020.122741.

[24] N.M. Moed, D.J. Lee, J.S. Chang, Struvite as alternative nutrient source for cultivation of microalgae *Chlorella vulgaris*, J. Taiwan Inst. Chem. Eng. 56 (2015) 73–76.

[25] N. Govindan, P. Chadayam, G.P. Maniam, M.H.A. Rahim, A.Z. Sulaiman, Y. Chisti, Production of lipids by *Tetraselmis* sp. grown in palm oil mill effluent, AIP Conf. Proc. 2454 (1) (2022) 040005.

[26] S. Jayakumar, M.M. Yusoff, M.H.A. Rahim, G.P. Maniam, N. Govindan, The prospect of microalgal biodiesel using agro-industrial and industrial wastes in Malaysia, Renew. Sust. Energ. Rev. 72 (2017) 33–47.

[27] K.M. Palanisamy, G.P. Maniam, A.Z. Sulaiman, M.H. Ab-Rahim, N. Govindan, Y. Chisti, Palm Oil Mill Effluent for Lipid Production by the diatom *Thalassiosira pseudonana*, Fermentation 8 (1) (2022) 23.

[28] Cheah, W. Y., Ibrahim, T. N. B. T., Aron, N. S. M., Chai, W. S., & Show, P. L. Understanding Environmental Biotechnology 4.0. In Microalgae for Environmental Biotechnology (pp. 175-204). CRC Press.

[29] P. Chawla, D. Gola, V. Dalvi, T.R. Sreekrishnan, T.U. Ariyadasa, A. Malik, Design and development of mini-photobioreactor system for strategic high throughput selection of optimum microalgae-wastewater combination, Bioresour. Technol. Rep. 17 (2022) 100967.

[30] N.S.M. Aron, K.W. Chew, W.L. Ang, S. Ratchahat, J. Rinklebe, P.L. Show, Recovery of microalgae biodiesel using liquid biphasic flotation system, Fuel 317 (2022) 123368.

[31] Q. Emparan, Y.S. Jye, M.K. Danquah, R. Harun, Cultivation of *Nannochloropsis* sp. microalgae in palm oil mill effluent (POME) media for phycoremediation and biomass production: Effect of microalgae cells with and without beads, J. Water Process. Eng. 33 (2020) 101043, doi:10.1016/j.jwpe.2019.101043.

[32] B. Cheirsilp, W. Maneechote, Insight on zero waste approach for sustainable microalgae biorefinery: Sequential fractionation, conversion and applications for high-to-low value-added products, Bioresour. Technol. Rep. (2022) 101003.

[33] C.Y. Wong, J.W. Lim, F.K. Chong, M.K. Lam, Y. Uemura, W.N. Tan, S.S. Lam, Valorization of exo-microbial fermented coconut endosperm waste by black soldier fly larvae for simultaneous biodiesel and protein productions, Environ. Res. 185 (2020) 109458.

[34] K.M. Palanisamy, M.H.A. Rahim, N. Govindan, R. Ramaraj, P. Kuppusamy, G.P. Gaanty, Effect of blue light intensity and photoperiods on the growth of diatom *Thalassiosira pseudonana*, Bioresour. Technol. Rep. (2022) In Press.

[35] P. Sarwa, S.K. Verma, Identification and Characterization of Green Microalgae, Scenedesmus sp. MCC26 and *Acutodesmus obliquus* MCC33 Isolated from Industrial Polluted Site Using Morphological and Molecular Markers, Int. J. Appl. Sci. Biotechnol. (2017), doi:10.3126/ijasbt.v5i4.18083.

[36] F. Kabir, M. Gulfraz, G.K. Raja, M. Inam-ul-Haq, M. Awais, M.S. Mustafa, M.S. Shadloo, Screening of native hyper-lipid producing microalgae strains for biomass and lipid production, Renew. Energy. 160 (2020) 1295–1307, doi:10.1016/j.renene.2020.07.004.

[37] A.R. Schuelter, A.D. Kroumov, C.L. Hinterholz, A. Fiorini, D.E.G. Trigueros, E.G. Vendruscolo, A.N. Módenes, Isolation and identification of new microalgae strains with antibacterial activity on food-borne pathogens. Engineering approach to optimize synthesis of desired metabolites, Biochem. Eng. J. 5 (4) (2019) 415–422, doi:10.1016/j.bej.2019.01.007.

[38] H.B. Hariz, M.S. Takriff, N.H. Mohd Yasin, M.M. Ba-Abbad, N.I.N. Mohd Hakimi, Potential of the microalgae-based integrated wastewater treatment and CO_2 fixation system to treat Palm Oil Mill Effluent (POME) by indigenous microalgae; *Scenedesmus* sp. and *Chlorella vulgaris*, J. Water Process. Eng. 32 (2019) 100907, doi:10.1016/j.jwpe.2019.100907.

[39] R. Resdi, J.S. Lim, A. Idris, Batch kinetics of nutrients removal for palm oil mill effluent and recovery of lipid by *Nannochloropsis* sp, J. Water Process. Eng. 40 (2021) 101767.

[40] G.T. Ding, N.H.M. Yasin, M.S. Takriff, K.F. Kamarudin, J. Salihon, Z. Yaakob, N.I.N.M. Hakimi, Phycoremediation of palm oil mill effluent (POME) and CO2 fixation by locally isolated microalgae: *Chlorella sorokiniana* UKM2, *Coelastrella* sp. UKM4 and *Chlorella pyrenoidosa* UKM7, J. Water Process. Eng. 35 (2020) 101202.

[41] W.Y. Cheah, P.L. Show, J.C. Juan, J.S. Chang, T.C. Ling, Microalgae cultivation in palm oil mill effluent (POME) for lipid production and pollutants removal, Energy Convers. Manage. 174 (2018) 430–438.

[42] B. Lin, F. Ahmed, H. Du, Z. Li, Y. Yan, Y. Huang, Z. Gao, Plant growth regulators promote lipid and carotenoid accumulation in *Chlorella vulgaris*, J. Appl. Phycol. 30 (3) (2018) 1549–1561, doi:10.1007/s10811-017-1350-9.

[43] I.D. Adamakis, P.A. Lazaridis, E. Terzopoulou, S. Torofias, M. Valari, P. Kalaitzi, K.S. Triantafyllidis, Cultivation, characterization, and properties of Chlorella vulgaris microalgae with different lipid contents and effect on fast pyrolysis oil composition, Environ. Sci. Pollut. Res. Int. 25 (23) (2018) 23018–23032.

[44] C. Niemi, S. Lage, F.G. Gentili, Comparisons of analysis of fatty acid methyl ester (FAME) of microalgae by chromatographic techniques, Algal. Res. 39 (2019) 101449, doi:10.1016/j.algal.2019.101449.

[45] D.T. Tran, B.H. Le, D.J. Lee, C.L. Chen, H.Y. Wang, J.S. Chang, Microalgae harvesting and subsequent biodiesel conversion, Bioresour. Technol. 140 (2013) 179–186.

[46] N. Govindan, G.P. Maniam, M.H.A. Rahim, A.Z. Sulaiman, A. Ajit, Biological macromolecules from algae and their antimicrobial applications, in: Biological Macromolecules, Academic Press, 2022, pp. 203–217.

[47] P Bhuyar, S Sundararaju, M.H.A. Rahim, G.P. Maniam, N. Govindan, Enhanced productivity of lipid extraction by urea stress conditions on marine microalgae *Coelastrum* sp. for improved biodiesel production, Bioresour. Technol. Rep. 15 (2021 Sep 1) 100696.

[48] K. Santhosh Kumar, S. Prasanthkumar, J.G. Ray, Biomass yield, oil productivity and fatty acid profile of *Chlorella lobophora* cultivated in diverse eutrophic wastewaters, Biocatal. Agric. Biotechnol. 11 (2017) 338–344, doi:10.1016/j.bcab.2017.08.006.

[49] I.T.K. Ru, Y.Y. Sung, M. Jusoh, M.E.A. Wahid, T. Nagappan, *Chlorella vulgaris*: a perspective on its potential for combining high biomass with high value bioproducts, J. Appl. Phycol. 1 (1) (2020) 2–11, doi:10.1080/26388081.2020.1715256.

[50] A. Akhbari, P.K. Kutty, O.C. Chuen, S. Ibrahim, A study of palm oil mill processing and environmental assessment of palm oil mill effluent treatment, Environ. Eng. Res. 25 (2) (2019) 212–221, doi:10.4491/eer.2018.452.

[51] K.M. Palanisamy, P. Paramasivam, S. Jayakumar, G.P. Maniam, M.H.A. Rahim, N. Govindan, Economical cultivation system of microalgae *Spirulina platensis* for lipid production, IOP Conf. Ser. Earth Environ. Sci. 641 (1) (2021) 012022 IOP Publishing.

[52] Idris, M. R., Arifuzzaman, M., Basak, A., Saha, T., & Yasmin, J. Cost effective wastewater treatment by natural adsorbent.

[53] S.K. Al-Amshawee, M.Y. Yunus, A.A. Azoddein, A review study of biofilm bacteria and microalgae bioremediation for palm oil mill effluent: possible approach, IOP Conf. Ser. Mater. Sci. Eng. 736 (2020) 022034, doi:10.1088/1757-899X/736/2/022034.

[54] K.M. Palanisamy, P. Paramasivam, G.P. Maniam, M.H.A. Rahim, R. Ramaraj, N. Govindan, Palm oil mill effluent as a potential medium for microalgae *Chlorella* sp. cultivation for lipid production, MIJEEC 2 (2) (2020) 5–11.

[55] W.Y. Cheah, P.L. Show, Y.J. Yap, H.F. Mohd Zaid, M.K. Lam, J.W. Lim, Y Tao, Enhancing microalga *Chlorella sorokiniana* CY-1 biomass and lipid production in palm oil mill effluent (POME) using novel-designed photobioreactor, Bioengineered 11 (1) (2020) 61–69, doi:10.1080/21655979.2019.1704536.

[56] N. Haris, H. Manan, M. Jusoh, H. Khatoon, T. Katayama, N.A. Kasan, Effect of different salinity on the growth performance and proximate composition of isolated indigenous microalgae species, Aquacul. Rep. 22 (2022) 100925.

[57] P. Feng, Z. Xu, L. Qin, M. Asraful Alam, Z. Wang, S. Zhu, Effects of different nitrogen sources and light paths of flat plate photobioreactors on the growth and lipid accumulation of Chlorella vulgarisGN1 outdoors, Bioresour. Technol. (2020), doi:10.1016/j.biortech.2020.122762.

[58] N. Nirmala, D.S S, Phylogenetic analysis for identification of lipid enriched microalgae and optimization of extraction conditions for biodiesel production using response surface methodology tool, Biocatal. Agric. Biotechnol. (2020), doi:10.1016/j.bcab.2020.101603.

[59] N.T.L. Chi, P.A. Duc, T. Mathimani, A. Pugazhendhi, Evaluating the potential of green alga *Chlorella vulgaris* for high biomass and lipid production in biodiesel viewpoint, Biocatal. Agric. Biotechnol. (2019), doi:10.1016/j.bcab.2018.11.011.

[60] N. Moradi-Kheibari, H. Ahmadzadeh, S.R. Lyon, Correlation of total lipid content of *Chlorella vulgaris* With the dynamics of individual fatty acid growth rates, Front. Mar. Sci. 9 (2022) 837067.

[61] B. Ramesh Kumar, G. Deviram, T. Mathimani, P.A. Duc, A Pugazhendhi, Microalgae as rich source of polyunsaturated fatty acids, Biocatal. Agric. Biotechnol. (2019), doi:10.1016/j.bcab.2019.01.017.