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Lipid Extraction of Microalga *Chlorella* sp. Cultivated in Palm Oil Mill Effluent (POME) Medium

Hadiyanto and M.M. Azimatun Nur

Center of Biomass and Renewable Energy (CBIORE), Department of Chemical Engineering, Diponegoro University, Jln. Prof. Sudartho, Tembalang, Semarang, 50239

Abstract: Indonesia is the largest palm oil producer in the world. Increasing of palm oil product generates large amount of palm oil mill effluent (POME). Due to high nutrient contents, palm oil mill effluent has been studied for their potential as medium growth of microalgae. The objective of this research was to investigate the utilization of best POME as medium source of algae growth especially *Chlorella sp*. The research was conducted with variation of POME concentration (20-60%v) in medium and addition of synthetic nutrient (0-100%v) to enhance the algae growth. The growth of microalgae was monitored under constant pH =7 and light intensity 600 W. The lipid extraction was assisted by using ultrasound to accelerate the extraction process. The result showed that *Chlorella sp* cultivated by using 20% POME + 40% synthetic nutrient could optimally grow and gave higher lipid productivity of 34 mg/L/day.

Key words: *Chlorella* sp · POME · Synthetic nutrient · Lipid

INTRODUCTION

Indonesia is the largest producer of oil palm in the world. Since 2008, Indonesia produced 45% of crude palm oil as shared contribution to world's demand. Moreover, the production still increases (5.2% annually) based on the prediction from 2010 to 2014 (Table 1). The large production of CPO not always give a positive impact, but it also gives a threat to the environmental, especially due to large amount liquid waste produced during the production. It was reported that about 1 ton fresh fruit bunch (FFB) can be converted to 0.2 ton CPO while about 0.5-0.66 ton is resulted as palm oil mill effluent (POME) [2]. This large amount of POME was resulted due to large amount of water used in the process [3]. Conventionally, POME is treated by using traditional facultative anaerobic-aerobic ponds to reduce COD and BOD contents. This system has disadvantages of requiring large area and long hydraulic resident times (HRT). Some inventions of modifying reactor have been developed for COD removal with high efficiency (>85%) such as UASFF (Up-flow Anaerobic Sludge Fixed Film) Bioreactor[3], anaerobic baffled reactor (ABR) [4], single up-flow anaerobic sludge blanket (UASB) reactor [5], two-stage UASB system [6] and membrane anaerobic system (MAS) [7]. However, the research of reuse nutrients in POME wastewater such as nitrogen and phosphor were still limited.

The characteristic of POME before and after treated using anaerobic pond is listed in Table 2. From the composition, it is shown that POME still contains high nutrients such nitrogen and phosphorus, which eventually useful for algae medium. This wastewater could generate eutrophication at river or lake. However, nutrient in POME is highly potential fertilizer for plant growth including microalgae for their photosynthetic reaction [8,9]. This medium could reduce nutrient cost for microalgae cultivation, since nitrogen and phosphorus fertilizer prices have almost doubled every year [10].

Microalgae have been considered as new sources of renewable energy including oil, hydrogen and ethanol. The oil productivity of microalgae is higher as compared to other terrestrial plant [11]. For growth, microalgae require nutrients: Nitrogen and Phosphor for photosynthetic reaction to produce biomass. For this purpose, some attempts showed possibilities to use waste

Corresponding Author: Hadiyanto, Center of Biomass and Renewable Energy (CBIORE), Department of Chemical Engineering, Diponegoro University, Jln. Prof. Sudartho, Tembalang, Semarang, 50239.
Tel/Fax: (024)7460058.

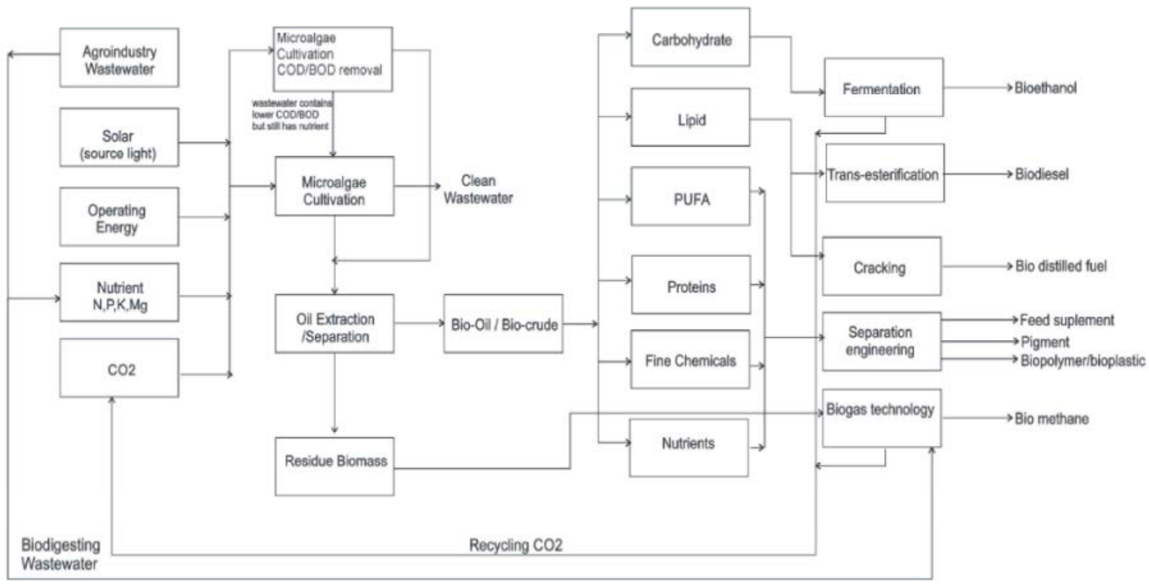


Fig. 1: Schematic diagram of biofuel based microalgae production using wastewater as growth medium [12]

Table 1: Commodities of Indonesia agriculture 2010-2014

		Growth /annum		
Commodities	Year	2011	2012	2013
Oil Palm	24.429	25.046	27.046	5.22%
Rubber	2.711	2.741	2.771	1.10%
Coconut	3.290	3.317	3.348	0.86%

Table 2: POME and Digested POME Characteristic

Parameter	POME	Digested POME	Standard Indonesian
pH	3.91-4.9	4-8	6-9
COD	83356	21227.5	500
TSS	49233.57	4798.5	300
Total N	1494.66	456	20
NH_3-N	50.42	34.2	-
PO_4-P	315.36	68.4	-

water as nutrient sources for microalgae [12]. These researches reported that waste water from agro industry could be used as bioenergy production through microalgae cultivation [12] (Fig. 1).

As the growth of algae is determined by its photosynthetic reaction, therefore the study of C:N:P ratio is also important. Our previous research [9] showed that variation of C:N:P ratio of medium had significant impact to the growth rate of algae at high COD concentration of waste. The objective of this research was to investigate the use of palm oil mill effluent (POME) as modified medium for *Chlorella* sp and to investigate the lipid production during cultivation.

MATERIALS AND METHODS

Palm Oil Mill Effluent (POME): POME was collected from PTPN VII Lampung and before its use as medium of algae cultivation, POME was pretreated to reduce its COD and BOD contents. The conventional method was normally facultative anaerobic ponds where the POME was fed to four ponds of stabilization, acidification and anaerobic ponds. The digested POME from 4th pond still contains COD of 1400 ppm, BOD of 700 ppm, total nitrogen of 280 ppm and total phosphorous of 75 ppm. POME was then filtered by using filter cloth 40µm to reduce its suspended solid and impurities. For preservation, samples were refrigerated at about 4°C in order to any contamination and limit the activity of biodegradation process [13].

***Chlorella* sp Culture:** Microalgae *Chlorella* sp was collected from BBPAP Jepara and cultivated in a modified medium consisted of 875 ppm NaHCO3 as carbon source, 45 ppm urea as nitrogen source, 5 ppm TSP (triple super phosphate) as phosphate source, 1ppm FeCl3 and 25 µg/l B12 as micronutrient source.

Cultivation Condition: *Chlorella* sp was cultivated in different POME concentration (20,40,60% v/v) and different synthetic nutrient (SN) concentration of (0,20,40,60,80,100% w/w). Synthetic nutrient (SN) was composed from 45 ppm urea and 5 ppm TSP. It was noted that $NaHCO_3$ was not added to POME medium.

The COD from POME was used as a solely carbon source. This SN addition will give variation of C: N: P ratio in the culture medium.

Chlorella sp with 10% v/v (OD_{680}=0.7) was used as inoculum. About 1 ppm of FeCl3 and 25 µg/l vitamin B12 were added for every trial to enhance micronutrient demand. Light intensity was maintained at 4000-6000 Lux using flourescent lamp as light source, pH was adjused by using NaOH and HCl in the range of 6.8-7.2, temperature was maintained at 26-28°C. Medium was aerated using air pump to mix the medium.

Measurement: The concentration of biomass was measured by using spectrophotometer Optima sp-300, at wavelength of 680 nm during its cultivation. Growth rate (µ) of microalgae was calculated according to Equation 1.

$$\mu = \frac{\ln(OD_1) - \ln(OD_0)}{t - t_0} \quad (1)$$

OD_1 is the optical density at end of cultivation, OD_0 is the optical density at the first day of cultivation, t is end time of cultivation (day) and t_0 is the beginning time of cultivation (day).

Biomass Harvesting: The biomass of algae was harvested by using flocculation method[14]. In this flocculation, NaOH 0.5 M was added continuously to increase the pH until it reached 10.5. The microalgae suspension was then intensively mixed (1000 rpm) for 10 min followed by gentled mixing (250 rpm) for an additional 20 min. Subsequently, the suspension was settled for 30 min. Biomass was dried at 70°C in a tray dryer for 3 hours and the biomass product was weighed as W_1.

Lipid Extraction: Dry Biomass of *Chlorella* sp was extracted by using n-hexane (1:8 w/v). Mix of dry biomass and n-hexane was placed in a ultrasound unit (Branson 2510-DTH 40KHz) for 1 hour and heated at temperature 50°C. The suspension was filtered to separate residual dry biomass from supernatant using Whatman filter paper No.41. Solvent was recovered from supernatant by distillation method until no more solvent remains. Solvent was used again to extract dry residue biomass for three replications [15]. Total lipid was collected and weighed as W_2. Lipid content was then calculated by Equation 2.

$$L = \frac{W_2}{W_1}.100\% \quad (2)$$

L is lipid content (%), W_1 is total biomas product in dry basis (mg/L), W_2 is total lipid content(mg/L).

Lipid productivity (Y, mg/L/day) was calculated using Equation 3.

$$Y = \frac{L.W_1}{t} \quad (3)$$

where t is total duration of cultivation in day, Y is lipid productivity in mg/L/day, L is lipid content (%) and W_1 is total biomass content (gr/l). Lipid profile was analyzed using GC-MS method according to previous research [16].

RESULT AND DISCUSSION

Dry Biomass vs OD Callibration: Relationship between DW (dry weight) and OD (optical density) in this research was described in Figure 2.

The result was described in linear equation of y=1.2281x-0.0191 (R^2=0.9924) by using autoflocculation method from Vandamme et al. [14]. This result is slightly different with the correlation obtained by Puangbut & Leesing [17]) with y = 1.5343x, (R^2= 0.977). These differences may be due to separation method used in both works. Puang and Leesing [17] used centrifugation for harvesting the biomass.

Algae growth in POME Medium: *Algae Chlorella* sp cultivated in different mediums was described in Fig. 3a. A higher optimum phase was shown by algae cultivated in a control (fresh) medium as compared to POME medium. This phenomena was also found in Hadiyanto and Nur [9] and Habib *et al*. [10], higher POME concetration in medium could inhibit growth rate and influence lag phase due to dark color of waste that lowering light intensity penetrated to the culture.. Wang *et al*. [18] found that dark color of manure wastewater influence the growth rate of *Chlorella* sp. The impurities of waste including clay, silt, inorganic-organic matter, or soluble colored organic compounds may retard the absorption of light by microalgae [19]. However, interesting phenomena of algae growth has been shown when synthetic nutrient (SN) was introduced to the medium (Figure 2). *Chlorella* sp cultivated in mixed medium of POME and synthetic nutrient showed higher growth rate than the growth in POME only. This shows that carbon, nitrogen and phosphorus contents in medium influence growth rate of *Chlorella* sp through photosynthetic reaction. An optimum C:N:P ratio for microalgae is 106:16:1 molar ratio or 56:9:1 C:N:P in weight ratio. Table 3 shows that medium have a required ratio for algae growth.

Table 3: Summary of data growth rate, content of carbon nitrogen and phosphorus ratio in different medium POME and SN

				C	N	P	C/N
POME (%v/v)	SN (%w/w)	μ (/days)	part C* (mg-atom/l)	weight ratio			
0	100	0.757	125	56	9	1	9,47
20	0	0.653	180	78,26	8,26	1	7,04
	20	0.698		62,04	8,82	1	6,36
	40	0.749		56,60	8,90	1	5,24
	60	0.675		46,96	8,97	1	4,64
	80	0.643		41,87	9,02	1	4,17
	100	0,566		37,78	9,06	1	9,47
40	0	0.449	340	75,56	9,33	1	8,10
	20	0.464		68,83	9,34	1	7,37
	40	0.498		63,20	9,35	1	6,76
	60	0.531		58,42	9,36	1	6,24
	80	0.459		54,31	9,36	1	5,80
	100	0.445		50,75	9,37	1	5,42
60	0	0,220	460	73,02	9,37	1	7,80
	20	0,238		68,25	9,37	1	7,28
	40	0,242		64,07	9,37	1	6,83
	60	0,244		60,37	9,38	1	6,44
	80	0,269		57,07	9,38	1	6,08
	100	0,239		54,12	9,38	1	5,77

*C is carbon content converted from COD of POME x $\frac{12}{32}$ [4]. SN=syntheric nutrient

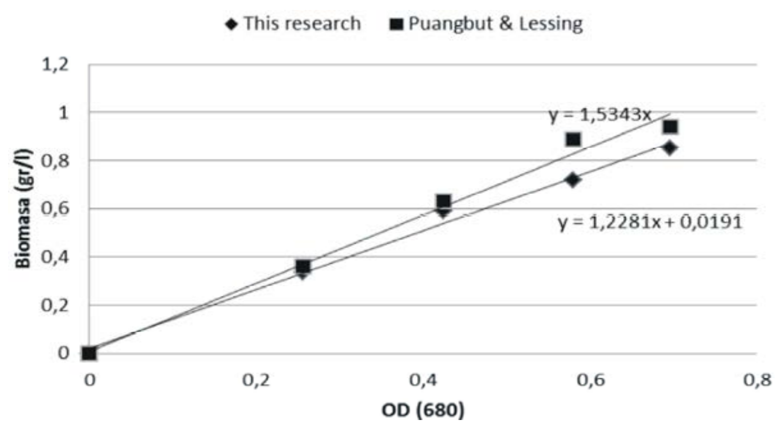
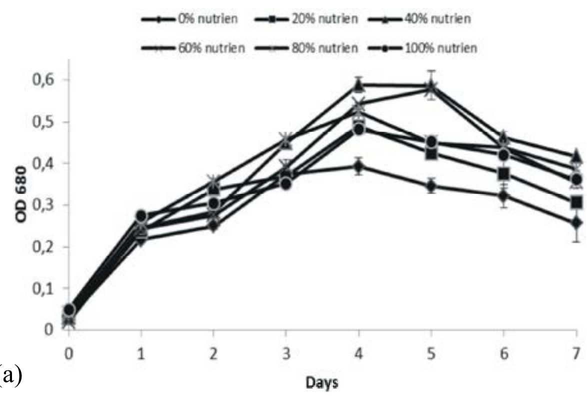


Fig. 2: Correlation between OD and Dry biomass of *Chlorella* sp cultivated in synthetic medium at 680nm

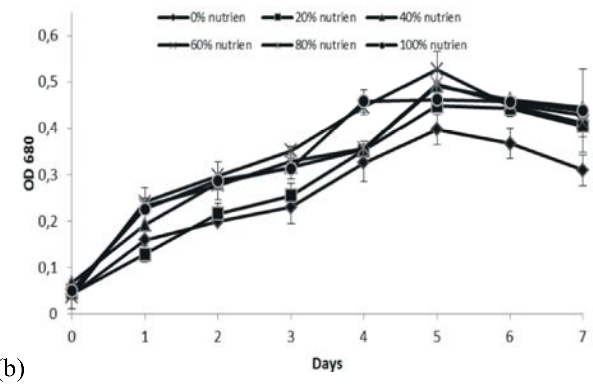
Synthetic ratio has an effect in the C:N:P ratio due to its content of COD, phosphorous and Nitrogen (Table 3). The carbon sources were obtained from COD available in palm oil mill effluent [10, 13]. It is noted that most of unicellular algae and benthic plants such seaweed use light energy to fix carbon (C), nitrogen (N) and phosphorus (P) at relatively constant stoichiometric ratios [21, 22, 23]. Goldman [23] further reported that C:N:P ratio will also light intensity dependent.

Biomass Production: Figure 4. describes biomass production from algae *Chlorella* sp under different medium composition. A higher dry biomass was recorded in culture medium of 0%POME+100%SN, followed by 20% POME+40%SN, 40%POME+60%SN and 60% POME+80%SN. This phenomena was also supporting the growth rate described in section 3.2. However the control medium still provide the highest biomass production and the growth rate. This brought to the fact that C:N:P ratio, light intensity, micronutrient, pH and other factors could limit biomass production [20, 24, 25].

Moreover, algae *Chlorella* sp showed low growth rate when cultivated in higher POME concentration medium and the cells stayed in longer adaptation phase (Fig. 3). The algae cultured in these highly concentrated wastewater took a long time to reach the stationary phase



(a)



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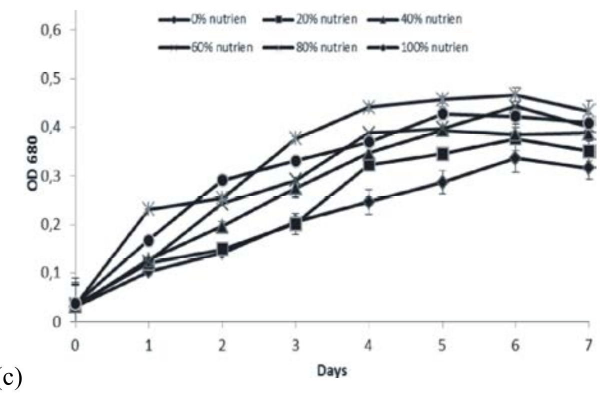

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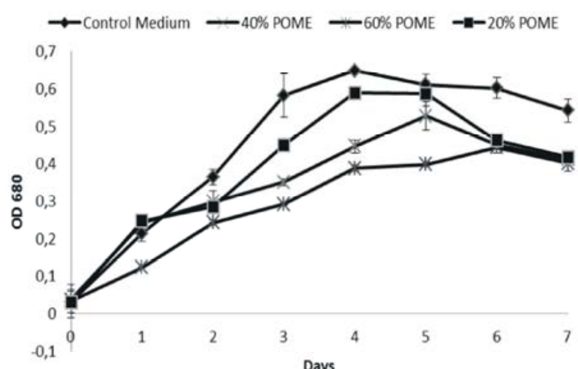

(d)

Fig. 3: Growth phase of *Chlorella* Sp in different synthetic nutrient & POME addition (a) 20% POME, (b) 40% POME, (c) 60% POME, (d) optimum growth phase in different medium

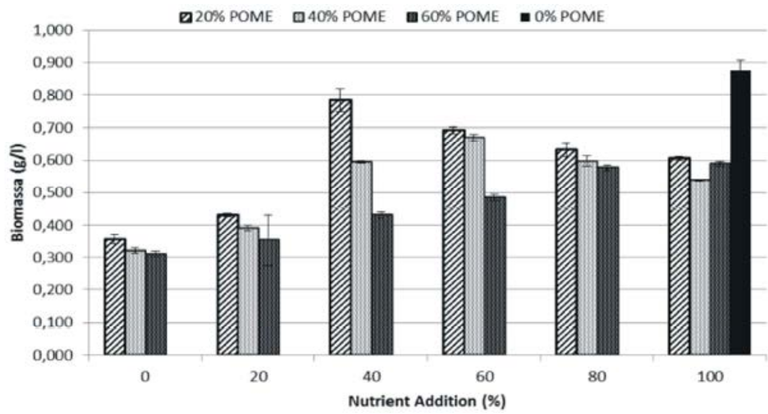

Fig. 4: Biomass production of *Chlorella* sp in different mediums

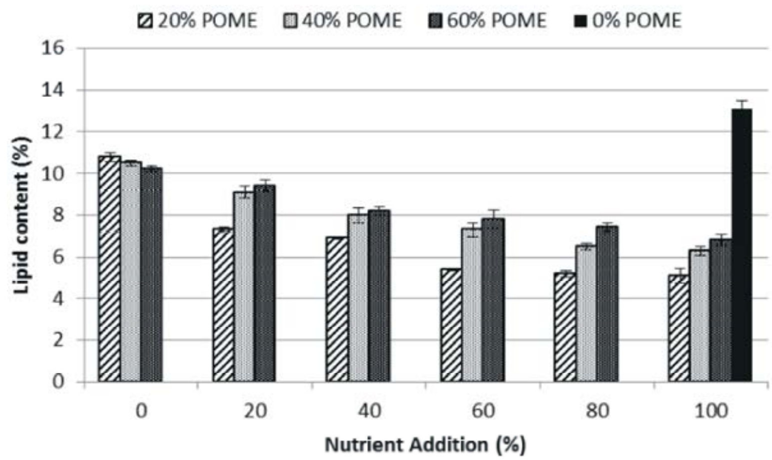

Fig. 5: Lipid accumulation of *Chlorella* sp in different mediums

and generated lower biomass due to longer lag phase. It seems that light shading also influences biomass production due to colored POME from tannic acid [18, 25, 26, 27]. This phenomena was also shown by Habib *et al*. [10] when cultivated green microalgae under different POME concentration. Ji *et al*. [27] cultivated *Chlorella vulgaris* under different diluted piggery wastewater added by synthetic medium and showed the biomass could be produced. Furthermore, Ji *et al*. [27] also indicated that higher concentration of POME in medium gave lower biomass production due to nutrients inhibition. This was probably due to high concentration of ammonium ions and nitrate that will have strong influences even at low concentration [28, 19, 30].

Lipid Content: The high lipid content wasfound in the medium with 20-60% vol POME (Fig. 5). This lipid production was also suppored by the work of Habib *et al*. [10], Wang *et al*. [18], Ji *et al*. [27], Jiang *et al*. [31] and Mandal & Mallick [32] by using POME, piggery, manure and municipal wastewater, respectively.

Furthermore, Wang *et al*. [18] described that lipid production was affected by C/N ratio in wastewater. According to Table 3, higher C/N ratio was found in 20,40,and 60% POME without synthetic nutrient addition, respectively (Figure 5).

When synthetic nutrient was added to the POME the medium, the lipid contents slightly decreased. Adding synthetic nutrients will reduce affect to C/N ratio. At a higher C/N ratio or less nitrogen available in the medium, could lower the growth rate of algae as well as its biomass [33, 34, 35, 36]. This condition generally occurs when microalgae grow in a stress condition with low nitrogen concentration. When nitrogen content in medium is lower, microalgae require more energy to proceed the photosynthetic reaction and consequently to the grow becomes slower.

Lipid Productivity: Several researcher reported that mixotrophic condition gave higher lipid accumulation rather than autotrophic or heterotrophic condition even in different C/N ratio [35, 37, 38]. The variation of waste

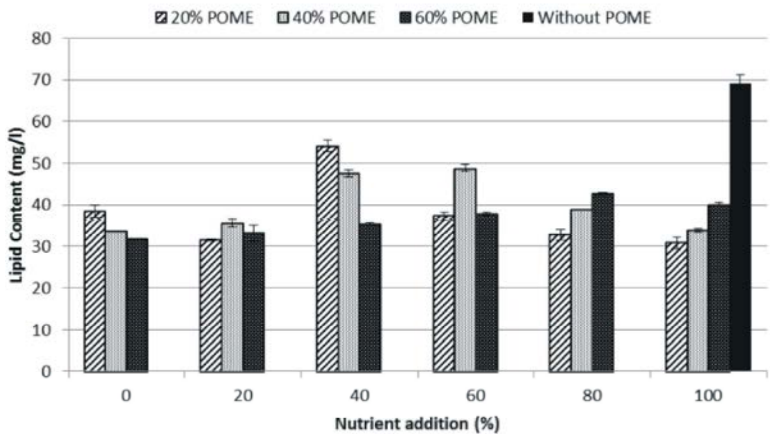

Fig. 6: Lipid content of *Chlorella* sp in different mediums

Table 4: Summary report of microalgae cultivation in various wastewater

Medium	Time (day)	Growth rate (/day)	Biomass content (g/L)	Lipid content (%)	Lipid productivity (mg/L/d)	Source
Digested Dairy Manure	21	0.474	1.7	14	11.3	Wang *et al*. [18]
MSG Wastewater	5	1.803	1.6	14	44	Xue *et al*. [39]
Artificial Wastewater	14	0.663	1.7	33	40	Yujie *et al*. [40]
2nd Municipal	9	0.458	0.67	31	22.9	Cho *et al*. [41]
20%POME +40% SN	7	0.749	0.73	6.9	37.4	This study
40%POME+60% SN	7	0.531	0.69	7.3	25.9	This study
60%POME+80%SN	7	0.269	0.59	7.6	11.8	This study

Table 5: Lipid profile from 20%POME + 40%SN

Component	Content (%)
C12: 0	0,997
C14: 0	1,178
C16: 0	65,356
C16: 1	3,439
C17: 0	1,642
C18: 0	0,091
C18: 1	14,719
C18: 2	7,734
C18: 3	0,831
C20: 0	1,398
C20: 3	0,548
C20: 4	1,117
C22: 1 (n-9)	0,027
C24: 0	0,180
C24: 1 (n-9)	0,743

water used for algae growth medium is shown by Table 4. It was reported that higher lipid productivity was found in MSG wastewater, followed by artificial wastewater and municipal wastewater (Table 4).

Our research showed that medium with 20% POME and 40% synthetic nutrients gave higher lipid productivity as compared to municipal wastewater and digested dairy wastewater. This also supports that POME has high potential as nutrients source for algae growth.

Lipid profile resulted from GCMS is shown in Table 5. In this research, lipid content produced by microalgae cultivated in 20% POME + 40% SN was dominated by saturated fatty acid such palmitic acid (65,34%), oleic acid (14,71%) and linoleic acid (7,73%). This result was similar to Wang *et al*. [18] and Cho *et al*. [41] who used *Chlorella* sp cultivated in wastewater medium. Biodiesel requires high saturated fatty acids such as palmitic to increase its its heating value and cetane number [42, 43]. Therefore, we concluded that lipid obtained from Chlorella was suitable for biodiesel sources.

CONCLUSION

Research was done by cultivating *Chlorella* sp in different SN and POME addition. It was found that CNP ratio, concentration of POME and addition of synthetic nutrient influenced growth rate, biomass production and lipid accumulation. Medium of *Chlorella* sp by using 20%POME and 40% of synthetic nutrient gave 34 mg/L/day. POME is a potential wastewater for *Chlorella* sp growth as replacement for synthetic nutrient. Lipid product from *Chlorella* sp was dominated by saturated fatty acid which lead algae biomass was suitable for biodiesel source.

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