# Evaluation of the Potential of Palm Oil Mill Effluent as a Growth Media for *Chlorella vulgaris* and Its Effect on Its Lipid Production

**Mariadaniela Bolaños Madriz**

**Patricio E. Paz Castillo**

Escuela Agrícola Panamericana, Zamorano
Apartado Postal 93, Km 30 carretera de Tegucigalpa a Danlí, Valle del Yeguare
Municipio de San Antonio de Oriente, Francisco Morazan, Honduras, C.A.

## Abstract

*Dependency on fossil fuels has generated an interest in searching for alternatives to supply its demand and reduce the environmental impact. Since the seventies, renewable sources of energy have been developed, contributing to the net production of energy. Of the sources of renewable energy with high potential is the generation of third generation biofuels from algae. Chlorella vulgaris has the potential to produce high amounts of lipids with an accelerated growth rate in residual waters such as palm oil meal effluent (POME). The objective of this study was the evaluation of 25%, 50% and 75% POME as growth media forChlorella vulgaris. The specific growth* $\mu(d^{-1})$ *was determined as well as extraction percentage, fatty acid profile and productivity. Chlorella vulgaris in 25% POME presented the best results, with an extraction percentage of 12.9%, a lipid production of 55.05 mg/L/d and a predominance of oleic acid in its lipid profile (40.95%).*

**Keywords:** Algae, biofuel, *Chlorellavulgaris*, lipids, POME.

## *1. Introduction*

The rapid population growth has provoked an increase in the dependency and use of fossil fuels in response to an ever growing demand for energy, which is supposed to reach a plateau of 37% in 2035 (BP, 2015). The sources of primary energy are those that have not suffered any chemical or physical modification for its use. In 2013, 78% of the production of primary energy corresponded to sources of fossil fuels (EIA, 2015). These sources are directly linked to the atmospheric concentration of carbon dioxide. In the period between 1970 and 2004, of the 70% of anthropogenic greenhouse gas emissions 56.6% was generated by fossil fuels (Pachauri and Reisinger, 2007). In the next 20 years, and increase of 35% is expected in the emission of carbon dioxide due to a 12% increase in the use of energy resources per capita (BP, 2015).

According to the United Nations Environmental Program (UNEP), the investment on renewable energy resources has grown 17% in 2014 since the previous year. This represents 48% of the world net power of 2014, excluding hydroelectric power (FS-UNEP, 2015). Today, renewable energy sources supply 3% of global energy consumption, with an expected increase of 5% for 2035 (FS-UNEP, 2015). Of this increase, almost two thirds will be provided through wind and solar energy, while the generation of energy based on biomass production will grow at an annual rate of 3.1% (BP, 2015)

First generation and second generation biofuels have been an option to supply the demand for energy, but their disadvantages include competition for arable land and food supply (Medina, 2013). The tendency now is toward third generation biofuels from algae and microorganisms, due to its capacity of survival in environments that include salt and freshwater, its relative small size, abundance, rapid growth rate and lipid profile (Chisti, 2007; Griffiths et al., 2011; Mata et al., 2010). In terms of lipid profile, algae are able to produce between 9 and 23 times more lipids per hectare than the African oil palm (Chisti, 2007). Algae can also play an important role in bioremediation of effluents due to its use of ionized and unionized ammonium and phosphates (Mata et al., 2010). Both types of ammonium are the principal forms of available nitrogen in the effluent of African palm oil, making it a good culture medium for algae (Fakir and Yakoob, 2011).

The culture of algae can also make use of the inorganic carbon being produced by palm oil extraction, with an uptake of up to 18% for algae such as *Chlorella vulgaris* (Wan-Loy, 2012). The production of 100 tons of dry biomass of algae would require 183 tons of carbon dioxide, making the production of algae attractive for the assimilation of carbon dioxide that would otherwise go to the environment (Chisti, 2007).

## *2. Materials and Methods*

**2.1 Location.** The experiment was done at the Biology Lab of Escuela Agrícola Panamericana, Zamorano (EAP), located in the Yeguare Valley, 32 km from Tegucigalpa, Honduras. The EAP is 800 meters above sea level with an average annual temperature of 26 °C and a mean annual precipitation of 1,110 mm distributed from May to November.

**2.2 Growth Media.** The growth media constituted palm oil mill effluent (POME) collected from Palmas Centro Americanas S.A., located in Aldea El Castaño, Honduras. The POME was moved to EAP in plastic containers where it was later refrigerated to 4 °C to halt the biodegradation process of its components and further contamination of the media.

**2.3 Algae and synthetic culture media.** The alga Chlorella vulgar is(UTEX 2714)was purchased through UTEX´s Culture Collection of Algae in Austin, Texas. The alga was shipped in 15 ml of agar in a sealed test tube. Proteose medium was also used to culture the algae and reproduce it before its use in the experiment.

**2.4 Culture conditions.** The algae were transferred to the proteose medium for a period of 34 days under constant light conditions. On day 35, the algae were transferred to 1 L Erlenmeyer tubes with 500 mL of the experimental culture media with the different proportions of POME and distilled water: 25:75, 50:50 and 75:25. The POME was filtered through a Buchner funnel with a 12.5 cm Whatman filter, using a vacuum pump. Each treatment, with its duplicate, was inoculated with a concentration of $2.1 \times 10^9$ cells of the algae (each treatment received a 20 mL homogenized solution with 105,156.25 cells/µL). The initial concentration was calculated with a Neubauer chamber.

The 1 L Erlenmeyer's were placed into a BW-20H heating bath at 26 ± 2 °C with a 40 Watt fluorescent light source with a 16:8 light/dark regimen during 23 days. Aeration was provided to each culture tube through a LifegardQuietOne® 1200 air pump connected to aquarium air stones in order to maintain the cells in suspension.

**2.5 Data Recollection.** Treatment pH was measured with a Hanna HI98107 tester from day eight of culture. The specific growth rate was measured every 4 days by obtaining a 3 mL sample per treatment and using a Cary 8454 UV-Vis spectrophotometer calibrated to 505 nm and 650 nm. The absorbance data was entered into the formula developed by Sacasa Castellanos (2013) and Kamyab et al., (2015):

Specific Growth Rate, $\mu d^{-1}$: $\frac{lnOD_1 - lnOD_0}{t_1 - t_0}$

Where:

$OD_1$ = Optical Density at the end of the sample period
$OD_0$ = Optical Density at the beginning of the sample period
$t_1$ = Initial day of sample period
$t_0$ = Final day of sample period
Biomass productivity (lipid, biomass, and extraction percentage) was determined with the following formulas (Belotti et al., 2013; Kamyab et al., 2015):

Biomass Productivity: $P_B$ (mg/Ld) = $\frac{(W_{BF} - W_{BO})}{t}$

Where:

$W_{BF}$ = Weight of Dry Biomass at the end of the sample period
$W_{BO}$ = Weight of Dry Biomass at the beginning of the sample period
t = Overall culture time

Lipid Extraction $= \frac{W_{EL}}{W_B}$

Where:

$W_{EL}$ = Weight of Extracted Lipids
$W_B$ = Weight of dry Biomass
Lipid Productivity, $P_L = P_B \times L(\%)$
Where:
PB = Biomass Productivity
L = Lipid Content

**2.6 Biomass Harvest and Determination of Dry Weight**

All treatments were subjected to flocculation in order to harvest the cells more easily. The harvest of cells was adapted from the procedure stipulated by Hadyianto and Nur (2014): a 0.5M of NaOH was added slowly to the sample to increase pH to 11.5. Each sample container was mixed at 1,000 rpm during 10 minutes and later the speed was reduced 250 rpm during 20 minutes and left to settle during 30 minutes. Three-3 mL samples were taken for each treatment before and after flocculation for analysis with the spectrophotometer to evaluate flocculation efficiency.

After flocculation, all treatments were subjected to centrifugation via a Symphony™ 4417R centrifuge at 4,500 rpm during 10 minutes. The supernatant was removed and the biomass collected was placed in 25 mL beakers and heated using a Fisher Scientific 750F furnace at 105 °C during 12 hours. The beakers with the dry biomass were later placed at -20 °C to allow for lipid extraction the following day.

**2.7. Extraction**

Lipid extraction was done with a modified Bligh and Dyer method (Bligh and Dyer, 1959; Ghasemi et al., 2014). For every gram of dry biomass, 5 mL of chloroform and 10 mL of ethanol were added and the mixture was agitated with a Fisher Vortex Genie 2™ during 5 minutes. After the first agitation, 5 mL of chloroform were added and the mixture was agitated for a second time for 5 minutes and 9 mL of distilled water was added once agitation was finished. The resulting product was centrifuged at 6,000 rpm during 20 minutes.

**2.8 Lipid Profile**

All treatments were analyzed for fatty acid profile predominant in the samples, thus evaluating its potential as biofuel. The procedure for the preparation of fatty acid methyl esters was done according to the AOCS Ce 2-66 method and the prepared samples were injected in an Agilent 7890A gas chromatograph following the AOCS Ce 1j-07 method for their identification (AOCS, 2013).

**2.9 Statistical Analysis**

The General Linear Model (GLM) was used with a completely randomized design for the evaluation of specific growth of the algae of each treatment for days 8, 10, 15, 19 and 23 at two wavelengths, 505 nm and 650 nm. Means separation was done through Fisher´s Least Significance Difference (LSD) with a probability of $P \leq 0.05$. For the variables dry weight, extraction percentage, dry weight productivity and lipid productivity, a completely randomized design with mean separation by LSD and a probability of $P \leq 0.05$.

## *3. Results and Discussion*

**3.1 Specific Growth**

Table 1 shows specific growth per day for day 8, 10, 15, 19 and 23. The maximum specific growth rates were observed for treatments 25:75 (1.29 $d^{-1}$) and 50:50 (1.08 $d^{-1}$), these results were higher than those reported by Hadiyanto and Nur (2012) who obtained a mean of 0.066 $d^{-1}$ when evaluating *Chlorella* sp. in 50% POME + 1 g/L urea in 15 days of culture. Putri et al. (2011) and Kamyab et al. (2015) also had lower specific growth rates (0.084 $d^{-1}$ and 0.168 $d^{-1}$, respectively) with *Chlorella vulgaris* in Bold Basal Medium with POME as a carbon source and *Chlorella pyrenoidosa* in POME diluted 10 times with a hybrid photo-bioreactor system. According to Serrano Bermudez (2011), iron availability as a trace element for algae growth is vital for metabolism function, due to its key role in electron transport, nitrite and sulfate reduction, molecular nitrogen fixation and the elimination of free radicals and peroxides.

The lowest specific growth rate was obtained with 75% POME and 25% water and was attributed to the dark coloration of the media due to the high POME concentration and high tannic acid concentration (Hadiyantoand Nur, 2012; Selmani et al., 2013). The presence of tannic acids as antinutritionalfactors inhibiting mineral absorption, such as iron, could have affected growth in this treatment (Sukumaran et al., 2014). This growth rate (0.76 $d^{-1}$) can also be explained due to the reduced photosynthetic rate and lowered carbon dioxide assimilation with high concentrations of POME which had a shading effect, limiting light penetration to the cells (Sukumaran et al.,2014).

**3.2 Productivity Parameters**

Table 2 shows the results of extraction percent, dry weight productivity (mg/L/d) and lipid productivity (m/L/d).

**3.3 Dry Weight Productivity**

Treatments consisting of 50:50 and 25:75 POME and water presented higher dry weight productivity and with a significant difference with the 75:25 treatments. The maximum productivity (4.43 mg/L/day) for 50:50 may be considered low in comparison to the values reported by Putri et al. (2011) and Kamyab et al. (2015) (5.90 and 100 mg/L/day, respectively).

The low dry weight productivity results obtained in this study may be due to the reduced amount of carbon dioxide in the system; this is supported by Mejia Rendón et al. (2013), who increased productivity of *Chlorella vulgaris* from 0.296 g/L to 1.592 g/L by increasing the carbon dioxide content in the system from 0.037% to 8.5% through injection.

Chinnasamy et al. (2009) concluded that productivity of *C. vulgaris* in a 6% carbon dioxide concentration is 99% higher than that of the same algae grown under an environmental concentration of carbon dioxide after 10 days of incubation. Hadiyanto and Nur (2014) explain the low productivity of *C. vulgaris* grown with 60% POME to the prolonged latency phase that the algae presented while adapting to the media.

**3.4 Lipid Productivity**

The best lipid productivity of 55.05 mg/L/day is higher than the previous values (11.2 to 40.0 mg/L/day) reported by Mata (2010), but no significant difference was observed for all treatments for lipid productivity. The 55.05 mg/L/day was obtained using the 25:75 treatment, indicating a higher efficiency of transforming dry biomass to lipids, but more research is necessary to prove this point.

**3.5 Extraction Percentage**

The extraction percentage obtained for all treatments in this study are similar to those reported by Hadiyanto and Nur (2014), where an extraction percentage of 14% was obtained using dairy residues and municipal residual water with *Chlorella* sp. The extraction percentage obtained for all treatments in this study are higher than those reported by Hadiyanto and Nur (2014) using POME and synthetic nutrients at different concentrations. The comparison for the results of this study and that of Hadiyanto and Nur (2014) are compiled in Table 3.

**3.6 Lipid Profile**

Tables 4, 5 and 6 show the fatty acid profile obtained for each treatment. These results differ from those obtained by Hadiyanto and Nur (2014) working with *Chlorella* sp. and 20% POME and 40% SN who obtained a profile characterized by 65.34% palmitic acid, 14.71% oleic acid and 7.73% linoleic acid, showing the impact of genetics on the fatty acids that are produced by the algae.

Likewise, the slight variations in the fatty acid percentages between the treatments are due to the nutritional differences of the growth media, supported by the findings of Sharma et al. (2012), who proposed that algae are able to modify their lipid metabolism according to changes in the conditions in which they are produced, resulting in varying patterns of the fatty acids produced. As an example of this finding, under stress the algae are able to modify their lipid biosynthesis, accumulating neutral lipids. For the elaboration of biofuels a high percentage of saturated fatty acids is better.

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**Table 1. Specific growth, μ($d^{-1}$) of *Chlorella vulgaris* cultured in POME**

| Treatment | Specific Growth rate per Day ($d^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Day 8 | Day 10 | Day 15 | Day 19 | Day 23 |
| 25:75 | 0.85[a] | 0.63[a] | 0.43[a] | 0.35[a] | 1.29[a] |
| 50:50 | 0.49[b] | 0.55[a] | 0.52[a] | 0.32[a] | 1.08[a] |
| 75:25 | 0.55[b] | 0.53[a] | 0.53[a] | 0.44[a] | 0.76[b] |
| Probability | P≤0.0445 | P≥0.5056 | P≥0.4942 | P≥0.4780 | P≤0.049 |

$R^2$= 0.74
CV% = 32.56

**Table 2. Production parameters of *Chlorella vulgaris* cultures in Palm Oil Mill Effluent (POME)**

| Treatment | DryWeight (g) | Extraction % | $P_{DW}$ (mg/L/day) | LP (mg/L/day) |
|---|---|---|---|---|
| 25:75 | 0.13 | 12.90 | 4.25[a] | 55.05 |
| 50:50 | 0.11 | 10.28 | 4.43[a] | 45.72 |
| 75:25 | 0.10 | 12.61 | 3.44[b] | 42.96 |
| Probabilidad | P≥0.5733 | P≥0.78 | P≤0.01 | P≥0.57 |
| $R^2$ | 0.57 | 0.36 | 0.99 | 0.57 |
| CV% | 21.52 | 23.77 | 1.67 | 21.53 |

$P_{DW}$: Productivity (DryWeight)

LP: LipidProductivity

[ab]: Data with the same letter within columns do not present significant differences according to LSD test with 5% significance

**Table 3: Comparison of the production parameters of *Chlorella vulgaris* and *Chlorella* sp. cultivated in media with different POME concentrations**

| Growth Media | Culture days | SpecificGrowth μ($d^{-1}$) | Extraction Percentage | Lipid Productivity |
|---|---|---|---|---|
| 20%POME +40% SN [δ] | 7 | 0.749 | 6.9 | 37.4 |
| 40%POME+60% SN [δ] | 7 | 0.531 | 7.3 | 25.9 |
| 60%POME+80%SN [δ] | 7 | 0.269 | 7.6 | 11.8 |
| 25% POME [β] | 23 | 1.29 | 12.9 | 55.05 |
| 50% POME [β] | 23 | 1.08 | 10.28 | 45.72 |
| 75% POME [β] | 23 | 0.76 | 12.61 | 42.96 |

[δ] Treatment applied to*Chlorella* sp.(Hadiyanto and Nur, 2014); SN = Urea
[β] Treatment applied to*Chlorella vulgaris*

**Table 4. Fatty acid profile for *Chlorella vulgaris* grown in 25% POME and 75% water.**

| Analysis | S1 | S2 | %R1 | %R2 | Average | CV % | Reference Method |
|---|---|---|---|---|---|---|---|
| Total SaturatedLipids | 4.9 | 2.4 | 40.2 | 40.6 | 40.4 | 0.7 | AOAC 996.06 |
| HEXADECANOIC ACID (PALMITIC) | 4.0 | 1.8 | 32.4 | 30.5 | 31.5 | 4.4 | AOAC 996.06 |
| OCTADECANOIC ACID (ESTEARIC) | 0.9 | 0.6 | 7.8 | 10.2 | 8.9 | 18.7 | AOAC 996.06 |
| Total MonounsaturatedLipids | 5.4 | 2.2 | 43.6 | 38.4 | 40.9 | 8.9 | AOAC 996.06 |
| OCTADECENOIC ACID (OLEIC) | 5.4 | 2.2 | 43.6 | 38.4 | 40.9 | 8.9 | AOAC 996.06 |
| Total PoliunsaturatedLipids | 2.0 | 1.2 | 16.2 | 21.0 | 18.6 | 18.2 | AOAC 996.06 |
| OCTADECADIENOIC ACID (LINOLEIC) | 2.0 | 1.2 | 16.2 | 21.0 | 18.6 | 18.2 | AOAC 996.06 |
| Total TransLipids | 0 | 0 | 0 | 0 | 0 | 0 | AOAC 996.06 |
| Total Area | 12.3 | 5.8 | 100 | 100 | 100 | | |

**Table 5. Lipid acid profile for *Chlorella vulgaris* grown in 55% POME and 50% water.**

| Analysis | S1 | S2 | %S1 | %S2 | Average | CV % | Reference Method |
|---|---|---|---|---|---|---|---|
| Total SaturatedLipids | 3.9 | 2.2 | 62.3 | 53.4 | 57.8 | 10.9 | AOAC 996.06 |
| HEXADECANOIC ACID (PALMITIC) | 1.5 | 0.7 | 24.4 | 17.8 | 21.1 | 22.3 | AOAC 996.06 |
| OCTADECANOIC ACID (ESTEARIC) | 2.4 | 1.5 | 37.9 | 35.6 | 36.7 | 4.4 | AOAC 996.06 |
| Total MonounsaturatedLipids | 1.0 | 1.1 | 16.0 | 27.2 | 21.6 | 36.7 | AOAC 996.06 |
| OCTADECENOIC ACID (OLEIC) | 1.0 | 1.1 | 16.0 | 27.2 | 21.6 | 36.7 | AOAC 996.06 |
| Total PoliunsaturatedLipids | 1.4 | 0.8 | 21.7 | 19.5 | 20.6 | 7.7 | AOAC 996.06 |
| OCTADECADIENOIC ACID (LINOLEIC) | 1.4 | 0.8 | 21.7 | 19.5 | 20.6 | 7.7 | AOAC 996.06 |
| Total TransLipids | 0 | 0 | 0 | 0 | 0 | | AOAC 996.06 |
| Total Area | 8.3 | 4.9 | 116.4 | 110.0 | 113.2 | | |

T**able 6. Lipid acid profile for *Chlorella vulgaris* grown in 75% POME and 25% water.**

| Analysis | S1 | S2 | %S1 | %S2 | Average | CV % | Reference Method |
|---|---|---|---|---|---|---|---|
| Total SaturatedLipids | 3.1 | 1.9 | 39.4 | 41.4 | 40.4 | 3.5 | AOAC 996.06 |
| HEXADECANOIC ACID (PALMITIC) | 2.1 | 1.1 | 26.4 | 22.2 | 24.3 | 12.4 | AOAC 996.06 |
| OCTADECANOIC ACID (ESTEARIC) | 1.0 | 0.9 | 13.0 | 19.2 | 16.1 | 27.5 | AOAC 996.06 |
| Total MonounsaturatedLipids | 2.9 | 1.8 | 37.1 | 38.6 | 37.9 | 2.8 | AOAC 996.06 |
| OCTADECENOIC ACID (OLEIC) | 2.9 | 1.8 | 37.1 | 38.6 | 37.9 | 2.8 | AOAC 996.06 |
| Total PoliunsaturatedLipids | 1.9 | 0.9 | 23.5 | 20.0 | 21.8 | 11.3 | AOAC 996.06 |
| OCTADECADIENOIC ACID (LINOLEIC) | 1.9 | 0.9 | 23.5 | 20.0 | 21.8 | 11.3 | AOAC 996.06 |
| Total TransLipids | 0 | 0 | 0 | 0 | 0 | 0 | AOAC 996.06 |
| Total Area | 7.9 | 4.7 | 100 | 100 | 100 | | |