



## Production of Fatty Acid Methyl Esther from Wastewater Grown Algae

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### KEYWORDS

*Biodiesel*  
*Fuel*  
*Biomass*  
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*Lipid*

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### ABSTRACT

Biodiesel is an alternative source of fuel that is non-toxic and biodegradable than conventional fossil-based fuel. In this research, biodiesel was produced by the cultivation of *Chlorella sorokiniana* in Palm Oil Mill Effluent (POME). The lipid content of the algae was obtained using the solvent extraction method. Gas-chromatography mass spectrometer was used for the identification of Fatty Acid Methyl Esther (FAME). Based on the results of biodiesel analysis, the components of FAME confirmed in microalgae biomass are mostly saturated fatty acid and they include tridecyclic acid (C13:0), myristic acid (C14:0), pentadecylic acid (C15:0), palmitic acid (C16:0) and stearic acid (C18:0). These indicated that the biomass of *C. sorokiniana* after POME treatment has the potential to be used for bioenergy production.

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## 1. INTRODUCTION

Biodiesel is an alternative source of fuel, which is non-toxic and biodegradable [1]. It is prepared by the transesterification process of triglyceride oil with monohydric alcohols. Some feedstocks such as canola oil, sunflower, rapeseed, and *Jatropha* is referred to as the first-generation feedstock [2]. The importance of the first-generation feedstock is that they affect the availability and supply of food material [3]. Thus, to improve biodiesel production, an alternative feedstock such as microalgae can be utilized. This will go a long way in reducing the current environmental problem and fuel shortage crisis.

When Microalgae is used for biodiesel production, it automatically reduces environmental pollution by sequestering carbon dioxide (CO<sub>2</sub>) from the atmosphere. Algae biomass has enormous applications, such as bio-fertilizer, astaxanthin,

animal feed and bio-protein, besides the production of non-toxic biodiesel without emitting CO<sub>2</sub> into the atmosphere [4]. Compared with fossil fuel, biodiesel (algal-based fuel) commercialization is very low due to the high production cost in biomass harvesting, lipid extraction and the choice of cultivating media to produce biodiesel.

The concept of increasing the viability of using algal oil for biodiesel production has received greater attention among researchers. One viable option to reduce the high cost of algal-based biofuel is substituting synthetic medium with natural media such as wastewater like palm oil mill effluent (Figure 1).

A handful of the research reported that POME contains a suitable medium necessary for algae growth. POME was characterized as dark-brownish colloidal suspension having a high amount of BOD, COD, color and nutrients [5, 6, 7]. The

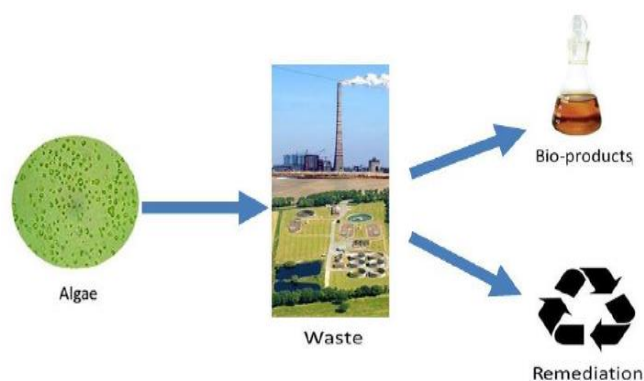
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availability of nutrients qualifies it as a suitable medium that could be used for microalgae cultivation. Usually, Microalgae are cultured in the synthetic growth medium. Since POME contain suitable nutrients, mainly nitrogen and phosphorus, it can be used as a replacement of synthetic medium for microalgae growth.



**Fig. 1.** Win-win strategy of wastewater treatment and biodiesel production

In terms of nutrient strength, industrial wastewater contains higher nutrients than agricultural, followed by the least, which is municipal wastewater. The nitrogen and phosphorus content of wastewater is a vital food source for algae growth [8]. In that case, when microalgae are cultivated in wastewater, they will perform the dual role of nutrients removal and production of biomass for energy generation. This implies that biodiesel production from wastewater-grown algae could be a viable option to alleviate global energy demand.

In one of the articles, Saidu *et al.*, [9] reported that the various experiment regarding kinetic coefficients data had shown that *C. sorokiniana* has a high yield of nutrients removal in POME. This indicates high biomass production [9]. The objective of this paper is to examine the potential of converting the biomass of POME-grown algae for bioenergy production. The significance of the findings of this study is that it could assist in finding a more efficient way of biodiesel production using an alternative feedstock that has not been reported earlier.

Biodiesel production from POME-grown algae could be a viable option for alleviating global energy demand. This paper provides information on the potential of producing Fatty Acid Methyl Ester (FAME) by growing microalgae in POME.

## 2. MATERIALS AND METHODS

All the chemicals used for this experiment were of analytical grade, were used as received without any further purification, and were obtained from Sigma-Aldrich and Fisher Scientific unless stated otherwise. All solutions were prepared with deionized water.

### 2.1. Sample collection

A fresh sample of POME was procured from a facultative anaerobic pond (FAP) from the local palm oil mill industry (Kilang Sawit Bukit Besar, Johor Bahru Malaysia). FAP is a

conventional wastewater treatment system set up by the company. The ponds are four in number and are arranged in parallel. The pure POME coming from the industry is discharged directly into the three ponds. Here, acidification and digestion occur after which the wastewater is moved to the fourth (final) pond. This last pond contains a moderate amount of nutrients. POME sample was collected in a clean plastic bag to avoid loss of moisture due to evaporation during transportation. The area lies between coordinates 1.772670 and 103.702432.

### 2.2. Characterization of POME

Nitrate and phosphate were determined following the Hatch DR 6000TM Spectrophotometric Manual (DR/6000, Hatch Co. Ltd. Tokyo 2008). Nitrate and phosphate were specifically determined using the cadmium reduction method and an acid hydrolyzable method respectively (Wang *et al.*, 2010). Suitable dilutions were made for the high concentration, and the final results were computed by multiplying the dilution factor. pH was determined using the portable pH meter, total suspended solids (TSS) were determined according to the standard methods [10].

### 2.3. Microalgae and Culture Condition

A pure strain of *C. sorokiniana* was obtained from an algae culture collection center at the University of Texas, Austin, Texas, USA and was maintained on Proteose media prepared in the laboratory at a temperature of 29 °C. The Proteose medium contains the basic nutrients element necessary for microalgae growth, such as nitrogen, phosphorus, and ammonium. The selection of *C. sorokiniana* for this experiment was mainly due to its high adaptation to a wide range of substrates. The cultivation condition was maintained under constant aeration. Two fluorescent lamps of 3000 lux were utilized to provide continuous illumination at a 12 h photoperiod interval (Day: Night). The culture pH and temperature were kept at seven and 29 °C, respectively. To avoid false reading, the experiments were undertaken in triplicate and the average readings were used for the analysis. The batch culture consists of a 1-litre flask placed perpendicular to the fluorescent lamp. Each flask was equipped with connecting tubes that convey CO<sub>2</sub> from the aerator to the flask. In all the batches used for the experiment, 0.2 µm syringe filters were fixed to the tubes connecting the aerator and the flask. This step is necessary to filter out potential microbes that might have their way into the culture via the connecting tubes. Axenic techniques were ensured at all times.

### 2.4. Sample preparation

The POME employed for the study was filtered using Whatman's No. 1 filter paper to obtain a clear filtrate. Because of its high turbidity, it was diluted to 80% (v/v), followed by the addition of 1.5 g/L urea before it was autoclave at 121 °C for 15 min. 10% (v/v) inoculum ( $1.2 \times 10^6$  cell density) of *C. sorokiniana* was then transferred to the POME media. The entire cultivation was done photo-autotrophically as described under microalgae and culture condition.

## 2.5 Biomass Harvesting, Cell lysis and Lipid Extraction

Biomass of sterilized POME grown algae was harvested at exponential phase by gravitational settling followed by centrifugation method. A 50 mL of algal culture was taken and allowed to settle under gravitational effect, then centrifuged at 4000 g for 15 min. The supernatant was discarded, and the pellet was dried under the sun on a drying bed at temperatures 25 - 30 °C according to the method described by [11]. Algal biomass (0.2 g) was weighed and placed in a 50 mL centrifuge tube. Four mL of hexane was added to the biomass according to the method described by author *et al.*, (year) [12] with modification, using sonication rather than reflux for cell rupturing. The mixture was then sonicated for 12 min at 20 MHz using a sonicator system (model; Q500, USA). Following the sonication, the mixture was centrifuged at 4000 g for 10 min at 4 °C to separate the biomass from the solution. The supernatant was pooled with 2 mL of distilled water to form two layers: the upper layer of hexane with lipid and the lower layer of water. The solvent was removed from the oil by evaporating the mixture in an incubator at 60 °C for 24 h. The amount of lipid was measured gravimetrically using an analytical balance (Model; ATX224 Shimadzu Corporation). The lipid content was calculated according to the formula in Equation 1.

$$L = W_2/W_1 \quad (1)$$

L is the lipid content (%),  $W_2$  is the weight of the extracted lipid (mg/L).  $W_1$  is the weight of the biomass (mg/L)

## 2.6 Trans-esterification Reaction

Fatty Acid Methyl Ester (FAME) was obtained by acid transesterification reaction according to the method defined by author *et al.*, (year) [13]. One mL of lipid was mixed with chloroform-methanol (2:1) and was evaporated. Two mL of 5% acetyl chloride in 1 mL hexane was added, and the mixture was kept at 80 °C in a water bath (Cole-Parmer, USA) for 2 h. After the reaction was completed, two phases appeared: methanol with acid at the upper phase and hexane-chloroform with lipid at the lower phase. The hexane phase was evaporated, and the dried FAME was collected in a clean GC vial. To analyze the sample using GC-MS, the sample was dissolved in hexane, vortexed and allowed to rest. The mixture was then filtered using 0.2 µm nylon syringe and was subjected to GC-MS analysis.

## 2.7 FAME Analysis and Quantification

FAME was analyzed using a gas chromatography mass spectrometer (Shimadzu GC-MS QP2010 Ultra, USA) equipped with a flame ionization detector (USA) and BPX-70 capillary column (USA). The column oven temperature was kept at 150 °C for 2 min and then raised at 215 °C at a rate of 6 °C  $m^{-1}$ , and then 235 °C for 5 min. The injector temperature was set at 240 °C, and helium gas was used as a carrier gas. FAME was identified in NIST Mass Spectral Database. The quantity of individual FAME was obtained by comparing the peak area with an external standard. The quantity of FAME was obtained using the equation 2, which was adopted from European Biodiesel Specification (EN) [14]

$$Q = \frac{A_a}{A_d} \times 100 \quad (2)$$

Where 'Q' is the FAME quantity (mg/L), 'A<sub>a</sub>' is the area of the sample and 'A<sub>d</sub>' is the area of the standard increase in the algae biomass was determined using cell dry weight. The quantity of FAME was obtained using equation 2 was adopted from [14].

## 2.8 Determination of FAME properties

### 2.8.1 Determination of Water and Sediment

A volume of 0.5 mL sample was mixed with 0.5 mL toluene in each of the two centrifuge tubes. The samples were inverted six times until the content was uniformly mixed and then placed in a water bath at 60 °C for 10 min. The samples were inverted again and centrifuged at 600 g for 10 min. Following the completion of the centrifugation, the final volume of the water and sediment was read from the bottom of the tubes [15]. The reading was expressed in percentage and computed according to equation 3.

$$V = V_1 + V_2 \quad (3)$$

Where V is the water and sediment of the sample (% v/v).

$V_1$ , Final volume of water and sediment in the first tube.

$V_2$ , Final volume of water and sediment in the second tube.

### 2.8.2 Determination of Kinematic Viscosity

The viscosity and shear stress of the sample was determined according to the method described by Ichihara, 2010 [13]. The viscosity and shear stress of the sample was measured using a Brookfield viscometer (DV2TRVT JO USA, Middleboro, MA, USA). Before the measurement was taken, it was ensured that the viscometer was connected to a Brookfield temperature controller. The viscometer was adjusted to the appropriate spindle and rotational speed (g) to be within the working range of the device. The sample's viscosity was taken immediately after the measuring parameters became stable. At the same condition, the reading of shear stress after taking that of viscosity was obtained by selecting the display key on the instrument.

### 2.8.3 Determination of Heating Value

The heating value fatty acid methyl ester is obtained from the empirical correlation equation 4 adopted from Ichihara, 2010 [13].

$$\delta_i = 46.19 - \frac{1794}{M_i} - 0.21 \times N \quad (4)$$

Where;

- $\delta_i$  is the heating value of FAME in MJ/kg
- $M_i$  represents the molecular weight of the FAME,
- N is the number of double bonds in a given FAME

## 3. RESULTS AND DISCUSSIONS

Palm Oil Mill Effluent (POME) in this study was characterized based on specific parameters, and the results were summarized and presented in Table 1. The ability of microalgae to grow in wastewater has long been linked to their ability to assimilate nutrients actively. The results presented in Table 1 has demonstrated that POME could be used as a suitable

medium for microalgae growth. This assertion was confirmed based on a research published recently on nutrient removal and kinetic study of freshwater microalgae in POME [9].

**Table 1:** Characteristic of POME

S/N	Parameters <sup>a</sup> (this study)	POME values	POME (range) <sup>c</sup>
1	pH	4.1	3.4 - 5.2
2	Chemical oxygen demand	44,233	15,000 - 100,000
3	Biochemical oxygen demand <sup>b</sup>	5,849	10,250 - 43,750
4	Total solid	13,400	11500 - 79000
5	Total volatile suspended solid (TVSS)	11,535	5000 - 54,000
6	Total fixed suspended solid (TFSS)	1865	9000 - 72,000
7	Nitrate	234	-
8	Phosphate	250	-
9	Colour	12,520	-

<sup>a</sup> All parameters unit in mg/L except pH

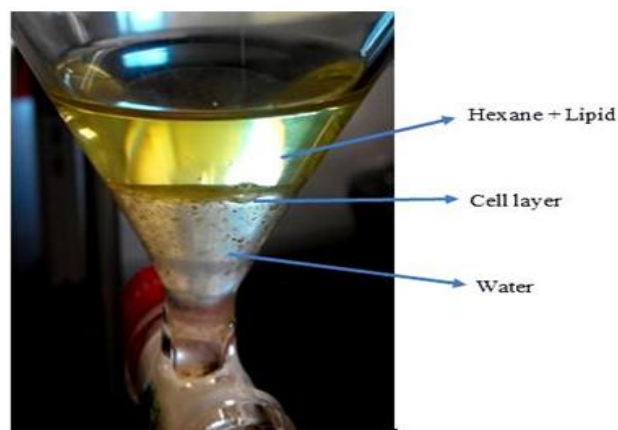
<sup>b</sup> Sample was incubated for 3 days at 30°C.

<sup>c</sup> Reference: Abdullah *et al.*, (2013)

### 3.1 Determination of Fatty Acid Content and Composition

Since *C. sorokiniana* grows well in 80% POME producing important biomass, the potential of extracting lipid from its biomass and converting it to biofuel was examined in this section. The extracting solvent used for the lipid extraction was hexane as it is less toxic in nature. The justification for using hexane other than other solvents is it is less toxicity and ability to extract efficient non-polar lipid. The mixture of the algal biomass, hexane, and water before and after centrifugation resulted in the formation of two layers; hexane with lipid in the upper layer and the lower layer with water. The hexane is then evaporated to leave behind lipid (Figure 2). A Literature reported that the colour of biodiesel can vary from dark brown to a very light yellow [16]. In another study involving microalgae growth in wastewater, it was reported that the colour of the biodiesel produced is light yellow [17]. In the current study, the produced biodiesel was found to be pale brown, suggesting the presence of phenolic compounds. The major phenolic compounds found in raw POME are gallic acid, protoctechinic acid, p-hydroxybenzoic acid, caffeic acid, syringic acid, vanillic acid, p-coumaric acid and phenol [18]. The percentage compositions of the individual fatty acid obtained from this study were compared with others (Figure 3).

The components of FAME confirmed are mostly saturated free fatty acid and they include;



**Fig. 2.** Separation of solvent, lipid and water in a separating funnel after extraction

tridecyclic acid (C13:0), myristic acid (14:0), pentadecylic acid (C15:0), palmitic acid (C16:0), stearic acid (C18:0). The lipid content of POME grown algae was computed using the relationship described in Eq. 2. A total of 52.1% saturated fatty acid composition was determined in this study. Palmitic acid (C16:0) was the most abundant fatty acid in the algae body with a percentage composition of 32.3%, followed by 9% for tridecyclic acid (C13:0), 4% for pentadecylic acid (C15:0) and 2.4% of myristic acid (C14:0).

The percentage composition of tridecyclic acid (9%) obtained from this study was comparatively similar with 8% produced using biomass obtained from *Ulva linza* the other hand, the myristic acid (2.4%) produced from this study is lower than 2.8% obtained using tallow seed oil (TSO) feedstock but higher than 1.23%, 1.22%, 1.89%, and 0.7% produced from *Ulva Linza* biomass, palm oil (PO), lard oil (LO) and cotton seed oil (CSO) biodiesel feedstock. The result from current study produced higher compositions of pentadecylic acid (4%) which is higher than 0.9% reported using biomass of *Ulva Linza* as a biodiesel feedstock. In the case of palmitic acid, the percentage composition of 32.3% reported from the current study was lower than 47.9% obtained from PO (Palm Oil) as biodiesel feedstock but higher than 23.3%, 24.49%, and 20.1% reported using TSO, LO and CSO as a biodiesel feedstock, respectively (Fig. 3). The percentage composition of stearic acid (4.4%) from this study was relatively less than 19.4% and 14.39% obtained using TSO (Tallow seed oil) and LO (Lard oil) feedstock but higher than 4.23% and 2.6% obtained using PO (Palm oil) and CSO (Cotton seed oil) biodiesel feedstock. Based on this comparison, it was observed that pentadecylic acid and tridecyclic acid were only detected in the biomass of algae from this study and that of UL (*Ulva linza*) (UL) biomass but absent in PO, TSO, LO and CSO feedstocks. This indicates that microalgae biomass obtained from POME and UL contains various types and compositions of fatty acid than other feedstock used for biodiesel production [19]. The variations in fatty acid compositions were due to differences in feedstock used for lipid extraction.

This has agreed with the literature that the fatty acid content of biodiesel was strongly influenced by the sources of feedstock used for the production [19]. However, it is noted that

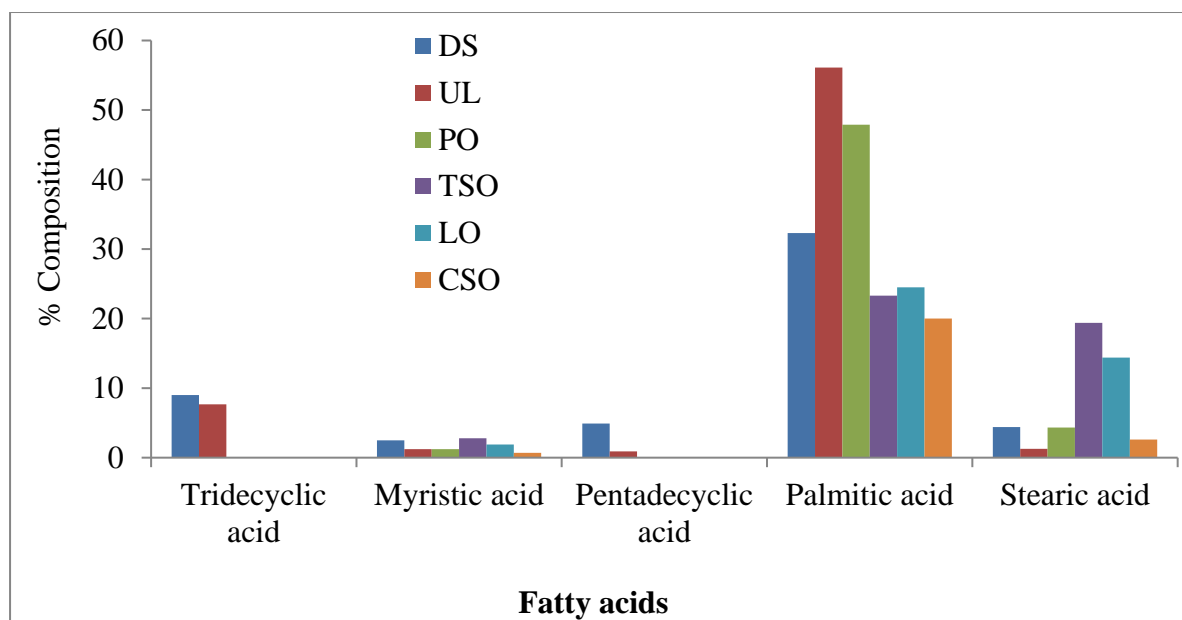
a low amount of fatty acid content in microalgae biomass has been considered the major challenge for use in biodiesel production [19, 20]. Therefore, these challenges can be reduced to the barest minimum if microalgae cultivation can be done using a large quantity of POME to obtain uniform high lipid content for enhanced biodiesel production.

A report by Cai *et al.*, 2013 [21] showed that the pool of fatty acids micro molecules is produced within the chloroplast of algae due to excess carbon synthesis during photosynthesis. Solvent interaction facilitates the transportation of available fatty acids endoplasmic reticulum (ER) from chloroplast for the synthesis of triacylglycerol (TAG). This resulted in the formation of oil bodies in the cytosol. Acetyl-CoA carboxylase (ACCase) is the key enzyme catalyzing the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate. Once the lipid is formed, its extraction process varies based on the methods and solvent involved in the extraction.

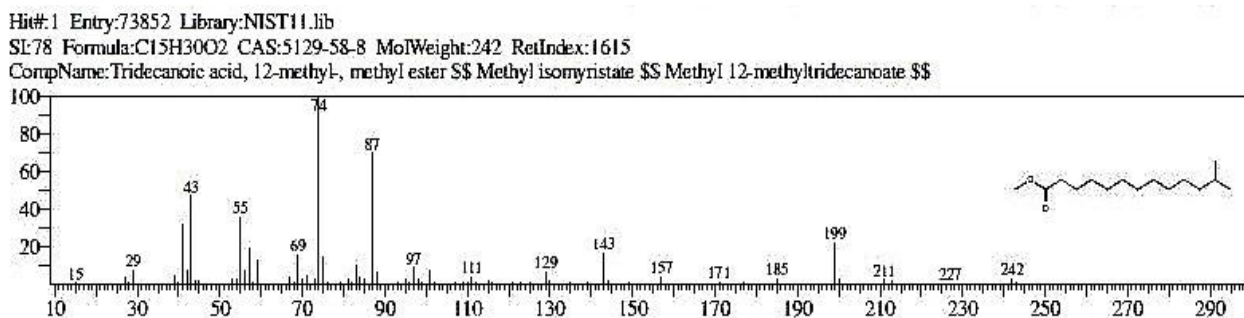
In the current study, lipid was extracted using hexane due to its cheap and non-hazardous properties. When the algal wall breaks, the solvent penetrates the cell and reacts with the lipid,

thereby forming the lipid complex of the solvent. Such complexes spread into the cell cytoplasm where they can be removed via purification technique [21].

The production of FAME obtained from this study was confirmed by the results of the GC-MS analysis. The chromatogram containing the peaks of individual FAME is shown in Figure 4-8. The identified FAME from this study is reported to have characteristics of high carbon number and a single bond. These features define them as good-quality biodiesel [19]. Therefore, the use of POME-grown algae for biodiesel production remains an attractive field of research. Hence, biodiesel production from wastewater-grown algae is an alternative technology that can go a long in providing an alternative source of bioenergy with reduced production costs. The importance of this technology is its attempts to improve the economy of biodiesel production.



**Fig. 3.** Comparison of fatty acid composition of POME grown algae with the different feedstock. DS- this study, UL- *Ulva Linza* biomass [22], PO- palm oil [23], TSO- tallow seed oil [20], LO- lard oil [24], CSO-cotton seed oil [20].



**Fig. 4.** GC-MS result for identification of tricyclic acid methyl ester (C13:0)

Hit#:1 Entry:23315 Library:NIST11s.lib

SE:89 Formula:C16H32O2 CAS:5129-66-8 MolWeight:256 RefIndex:1715

CompName:Tetradecanoic acid, 12-methyl-, methyl ester \$\$ Methyl 12-methyltetradecanoate \$\$ Methyl tetradecanoate, 12-methyl \$\$

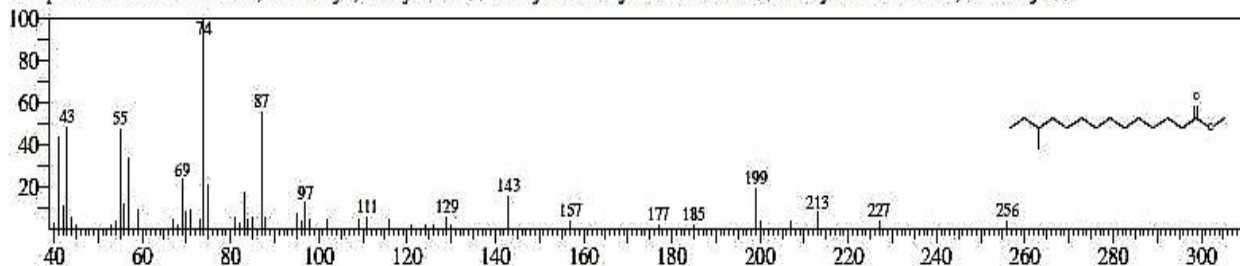


Fig. 5. GC-MS result for identification myristic acid methyl ester (C14:0)

Hit#:1 Entry:84388 Library:NIST11s.lib

SE:88 Formula:C16H32O2 CAS:7132-64-1 MolWeight:256 RefIndex:1779

CompName: Pentadecanoic acid, methyl ester \$\$ Methyl n-pentadecanoate \$\$ Methyl pentadecanoate \$\$ n-Pentadecanoic acid methyl ester \$\$

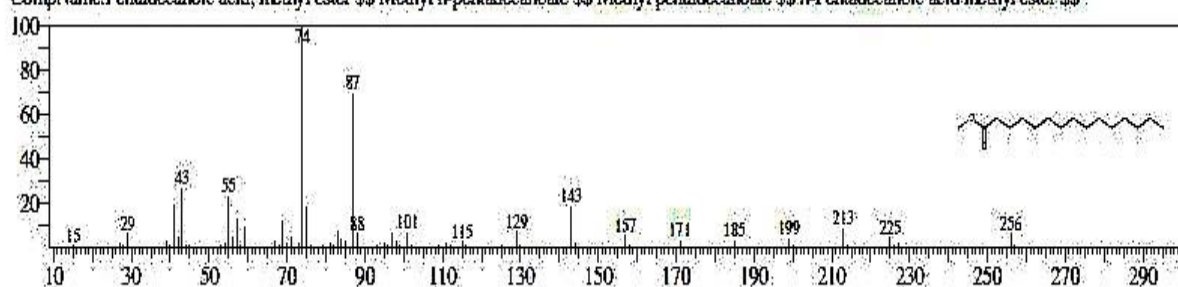


Fig. 6. GC-MS results for identification of pentadecylic methyl ester (C15:0)

Hit#:1 Entry:24296 Library:NIST11s.lib

SE:94 Formula:C17H34O2 CAS:112-39-0 MolWeight:270 RefIndex:1878

CompName: Hexadecanoic acid, methyl ester \$\$ Palmitic acid, methyl ester \$\$ n-Hexadecanoic acid methyl ester \$\$ Methlene 2216 \$\$ Methyl hexadecanoic acid methyl ester

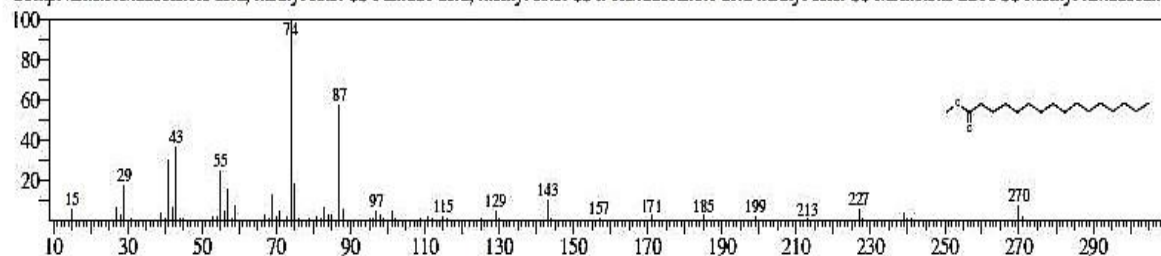


Fig 7. GC-MS result for identification of palmitic acid methyl ester (C16:0)

Hit#:1 Entry:26033 Library:NIST11s.lib

SE:93 Formula:C19H38O2 CAS:112-61-8 MolWeight:298 RefIndex:2077

CompName: Methyl stearate \$\$ Octadecanoic acid, methyl ester \$\$ Stearic acid, methyl ester \$\$ n-Octadecanoic acid, methyl ester \$\$ Kemester 9718 \$\$ Me

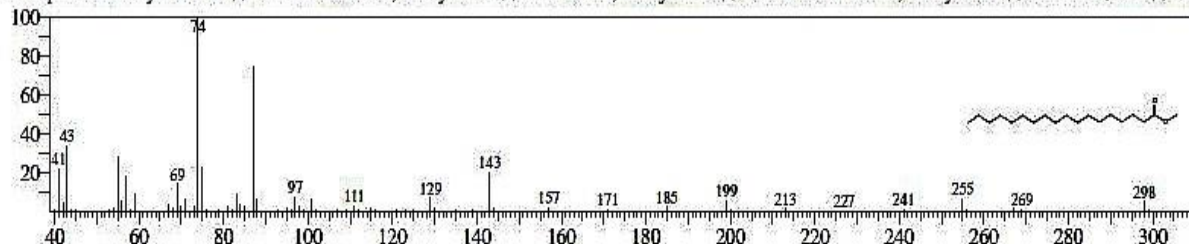


Fig 8. GC-MS result for identification of stearic acid methyl ester (C18:0)

### 3.2 Characterization of FAME obtained from POME Grown Algae

The fuel characteristics of biodiesel obtained from wastewater-grown microalgae were compared with ASTM biodiesel standards. The properties of the biodiesel determined include water and sediment, kinematic viscosity and heating value.

**Table 2.** Characteristic of FAME

Properties	Unit	°ASTM D6751-02	Present study
Water and sediment	% m/m	0.05 max	0.005
Kinematic viscosity	mm <sup>2</sup> /S	1.9-6.0	1.8
Cetane number (CN)	-	min 51 max 65	65

### 3.3 Water and Sediment

The water and sediment content of 0.005% (v/v) obtained from this study fall within the specification of the ASTM standard. In biodiesel, a water specification below 500 ppm (0.05%) is considered pure. The water and sediment reported from this research were lower than 0.043 % (v/v) of FAME from the oil of *Jatropha caucous* seed [25]. The low amount of water content obtained from this study was due to the proper storage of the biodiesel, sourced from the fact that biofuel is more hygroscopic than diesel oil. High water and sediment in fuel cause problems such as microbial growth and corrosion of the fuel engine parts.

### 3.4 Kinematic Viscosity

The kinematic viscosity value obtained is 1.8 mm<sup>2</sup>/S, which is relatively within the range of ASTM standard from the lower limit but slightly lower than 2.4 mm<sup>2</sup>/S obtained in coconut oil methyl ester [2]. Kinematic viscosity determines the thickness of a fluid. Furthermore, fuel with too low viscosity provides a very fine spray, the drops having a very low mass and speed. This resulted in partial penetration and the formation of black smoke specific to combustion in the absence of oxygen (near the injector) [2]. This viscosity reading was determined at a shear stress of 55.6 D/cm<sup>2</sup>. Shear stress is an engineering property of moving fluid. It is important in understanding the structure of a flow field, understanding the phenomena of corrosion, saltation and the build-up of pipe deposits. Thus, shear stress is the force required by a particular liquid to reach the elastic limit.

### 3.5 Heating value

The FAME obtained from this study has a heating value of 39 MJ/kg. This value is within the standard ASTM specification of biodiesel (Table 2). The heating value obtained from this research is comparable with 41 MJ/kg of microalgae oil reported in the literature [20]. The heating value of biodiesel indicates the amount of heat produced by the complete combustion of a unit quantity of fuel. This property was reported dependent on the number of carbon and molecular weight of biodiesel [25].

## 4. CONCLUSION

Based on the results of biodiesel analysis, the components of FAME confirmed in microalgae biomass are mostly saturated fatty acid, and they include; tridecylic acid (C13:0), myristic acid (C14:0), pentadecylic acid (C15:0), palmitic acid (C16:0) and stearic acid (C18:0). These indicated that the biomass obtained from *C. sorokiniana* after POME treatment has the potential to be used for bioenergy production. The findings of this study could contribute to proving an alternative method of FAME production that is less costly.

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