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Biosynthesis and Characterization of Algae oil Obtained from *Chlorella vulgaris*

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Authors' contributions

This work was carried out in collaboration among all authors. Author IS designed the study, searched for the literature, performed the laboratory and statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors GOA, OA & FOC managed the initial aspects of sample preparation and protocol write up. Authors SV and JR supervised and guided the conduct of the Laboratory analysis. All authors read and approved the final manuscript.

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ABSTRACT

Chlorella vulgaris was isolated from the African Regional Aquacultural Centre Aluu, Rivers State. The culture was grown and was analysed for oil contents using three different extraction methods and was also analysed for pigments and the algae oil was characterized. Pigment analysis of the biomass had 7-12000 µg/g beta carotene, Astaxantin, Cantaxantin, Chlorophyll-a and Chlorophyll-b was 550,000 µg/g, 362,000 µg/g, 250-9630 µg/g and 72-5770 µg/g. Free fatty acid composition of the algae oil was tetradecanoic acid with a low peak height of 5.07%. The maximal peak height and component was n-hexadecanoic acid with the 17.83 minutes retention time and peak area of 18.24%, followed by the 9-octadecanoic acid (Z) Hexyl ester which had a peak area of 5.77% and

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a retention time of 24.80 minutes. The specific growth rate of 0.041 mg/Lday^{-1} for mixotrophic condition while heterotrophic had SGR of 0.054 mg/Lday^{-1}. The study further identified that algal biomass from *C. vulgaris* has the potential of serving as both nutraceuticals and bioenergy feedstock. There is need for further studies around the algae oil oriented optimization as a veritable tool for biotechnological advancements.

Keywords: Biofuels; soxhlet; sustainable; chlorella vulgaris; free fatty acids; bioenergy.

1. INTRODUCTION

The Global energy consumption is has evolved over the years and this has remained complex for quite some time now. These changes have mainly attributed to population, economic activity, commercial and technological advancements increases, so also the energy-use and its fluctuations. Hence, the global energy-use varies across the world depending the available resource and technological advancements [1] Although fossil fuels are still being produced, under various geochemical processes, they are consumed faster than they are formed. The sources of these fuels are therefore finite and exhaustible [2]. In addition, fossil fuels are found to be major contributors to greenhouse gas (GHGs) emissions to the biosphere, and in 2006 energy associated CO_2 emissions were estimated at 29 Gtonnes [3]. The treaty signed in 1997 in Kyoto, known as Kyoto protocol, advocated for a 5.2% reduction in worldwide greenhouse emissions from the 1990 levels [4].

Microalgae are photosynthetic microorganisms that utilize sunlight, CO_2, minerals and wastewater. They do not require large area of arable land for their cultivation, compared to terrestrial plants [5]. They have been used in the production of animal feed, cosmetics, polymers and cosmetics [5] but the current interest in the use of Microalgae is for the production of biofuels, was motivated because they can accumulate as much as 70% of the dry weight large lipid fractions and other compounds. However, Microalgae was found to remediate effluents [6] hence suitable for growth in wastewater feedstock. Microalgae require carbon dioxide, light, pH, temperature and nutrients. Carbon dioxide supplies carbon for the production of Biomass, the sources of CO_2 for Biomass production can come from industrial exhaust (15% CO_2 above). Light supplies energy, though if it is much can affect growth due to photoinhibition. pH provide suitable medium for growth, a pH of 6-8 though there exist acidophilic algae that can grow in pH as low as 2-3. Temperature (mainly 20-30 degrees) for ideal growth, though biomass production increases with increase in temperature. However different species have different adaptability with respect to pH and temperature [7]. Generally, extraction and production of biofuels from microalgal biomass is more expensive and technologically more challenging, than growing crops. Its production requires light, inorganic nutrients, water, CO_2 and temperature regime that has to be controlled and monitored closely [8-9].

2. MATERIALS AND METHODS

2.1 Water Sample Collection

Water sample was collected from New Calabar River, Port Harcourt, Rivers State. Water Sample was collected in a sterile container and was transferred to laboratory for the study. The microalgal culture (*Chlorella vulgaris*) was obtained from a Fish pond in African Regional Aquacultural Centre Aluu, Port Harcourt, Rivers state.

2.2 Growth conditions and Monitoring

Chlorella spp. was isolated from African Regional Aquacultural Centre, Aluu, Rivers State Nigeria. The pure strain was isolated using a solidified Brilliant Green media, supplemented with 100µg/ml Nystatin and 62.5µg/ml Chloramphenicol and 0.02mg/L Cyanocobalamin was added as a source of trace elements. Additional *Chlorella* sp. biomass was obtained from the Department of Microbiology, University of Port Harcourt. The algal culture was incubated at room temperature for 15 days with continuous aeration and 12:12 hour photoperiod under artificial illumination of 2000 lux. The microalgal culture was separated by centrifuging at 4000 xg. The biomass pellet was dried at 80^0C for about 1 hour until stable weight has obtained. The biomass productivity was calculated using the standard formula. The biomass accumulated was measured using UV visible spectrophotometer at wavelength of 680nm while the cell dry weight

was determined from the cell pellets after centrifugation and dewatering. The pellets were dried in a muffle furnace at over 600 ^{o}C. The dried pellets were placed in a desiccator over night after which the biomass was weighed in triplicates.

The Biomass productivity was calculated using the following formula [10]

$$Biomass\ Productivity\ \left(\frac{g}{L}.d\right)\frac{Final\ dry\ weight}{Initial\ weight \times Total\ number\ of\ culture\ days \times Vol} \times 100 \quad (1.0)$$

The Lipid productivity was calculated using the following formula

$$Lipid\ productivity\ \left(\frac{g}{L}.d\right) = \frac{Lipid\ content\ of\ cells(g/g)\ x\ Dry\ cell\ weight\ (g/L)}{Cultivated\ period(d)} \quad (2.0)$$

2.3 Extraction of Algal Lipids using Bligh and Dyer Method

The algal lipids were extracted by using Bligh and Dyer method. 1 gram of dried algal biomass was treated with 3mL of methanol and chloroform (1:2) ratio and then incubated at room temperature for about 24 hours. The whole blend has centrifuged at 4000 rpm for about 5 minutes and then the supernatant solution was collected. To the reactant solution 2mL of chloroform was added in to the pellet and then centrifuged to collect the supernatant solution followed by the addition of 1% KCl (2ml) to the supernatant solution, two separate layers were formed. The bottom layer contains lipids was completely removed and weighted. The percentage of lipid was calculated using the following formula;

$$Lipid\ content\ (\%) = \frac{Weight\ of\ the\ lipid}{Dry\ weight\ of\ the\ culture} \text{x}\ 100 \quad (3.0)$$

2.4 Bligh and Dyer Methods

One gram (1g) of dried algal biomass was treated with 3mL of methanol and chloroform (1:2) ratio and then incubated at room temperature for about 24 hours. The whole blend has centrifuged at 4000 rpm for about 5 minutes and then the supernatant solution was collected. To the reactant solution added 2mL of chloroform in to the pellet and then centrifuged to collect the supernatant solution followed by the addition of 1% KCl(2ml) to the supernatant solution, two separate layers were formed. The bottom layer contains lipids was completely removed and weighted. The percentage of lipid was calculated using the following formula [11]:

$$Lipid\ content\ (\%) = \frac{Weight\ of\ the\ lipid}{Dry\ weight\ of\ the\ culture} \text{x}\ 100 \quad (4.0)$$

2.5 Soxhlet's Extraction Process

The algal biomass was packed and then transferred in to the Soxhlet's extraction tube. About 350mL of n-hexane was poured into a round-bottomed flask and then the organic solvent was heated using a heating mantle for about 5 hours at a temperature of 80^{0}C. After completing the reaction, the algal oil was separated from the organic solvent by using distillation process. The percentage of lipid content was calculated using standard formula given [12]

$$Lipid\ content\ (\%) = \frac{Weight\ of\ the\ lipid}{Dry\ weight\ of\ the\ culture} \text{x}\ 100 \quad (5.0)$$

2.6 Fatty Acid Profile Analysis

The qualitative analysis of fatty acids was analysed using GC-MS analytical technique. An Agilent 6890 gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15m Alltech EC-5 column (250µ I.D., 0.25µ film thickness). A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35C, hold for 2 minutes, then ramp at 20C per minute to 300C and hold for 5 minutes. The helium carrier gas was set to 2ml/minute flow rate. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan. Identification of the components of the purified compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instrument software.

3. RESULTS AND DISCUSSION

3.1 Lipid Composition of Algae oil from *C. Vulgaris*

The result presented in Fig 1. shows the chromatogram of the lipid components and their elution time. The total ion concentration as a

measure of the m/z ratio are presented in the projections below, Table 2 describes the Free fatty acid composition of the algae oil the first to elute compound was tetradecanoic acid with a low peak height of 5.07%. The maximal peak height and component was n-hexadecanoic acid with the 17.83 minutes retention time and peak area of 18.24%, followed by the 9-octadecanoic acid (Z) Hexyl ester which had a peak area of 5.77% and a retention time of 24.80 minutes. The results in Fig. 1. shows the Gas chromatography Mass Spectrometry of the algae oil and their structural components. The Fig. 2-10 shows the structural component of algae oil acids.

3.2 DISCUSSION

Pigments are very important components of a phyto-structural backbone of any plant. It also adds a number of beneficial attributes to the morphological composition of microalgae, due to the composition of the phytochemical components and the antinutrients and uses due to the presence of steroids. During this study, the biomass had 7-12000 µg/g β-carotene, Astaxantin, Cantaxantin, Chlorophyll-a and Chlorophyll-b was 550,000 µg/g, 362,000 µg/g, 250-9630 µg/g and 72-5770 µg/g. The vitamins B7, B12, B9, B3 and C was 191.6 mg/100g, 125.9 mg/100g, 23.8 mg/100g and 26.9 mg/100g. This composition was in tandem with the previous report of Saffar *et al*. (2016) that observed that β-carotene 1013.1µg/g which was considerably the highest pigment concentration. This investigation further in tandem with the report [13] who also observed that *Chlorella vulgaris* from this study has a β-carotene was 18.42 µg/g and Cryptoxanthin was 1.99 µg/g. Their study although observed a lower a lower concentration of steriods and phytochemicals revealed that the presence of lutein richness for which these pigment can be used to treat eye-defects and other related illnesses. On the other hand they could be used as food supplements as souces of vitamins and other trace minerals.

Table 1. Percentage lipid yield using three different extraction processes

Microalgae	Lipid yield (%)		
	Modified Bligh & Dyer	Bligh & Dyer	Soxhlet Extraction
Chlorella vulgaris	77.2 + 0.002	75.6 + 0.003	73 + 0.001

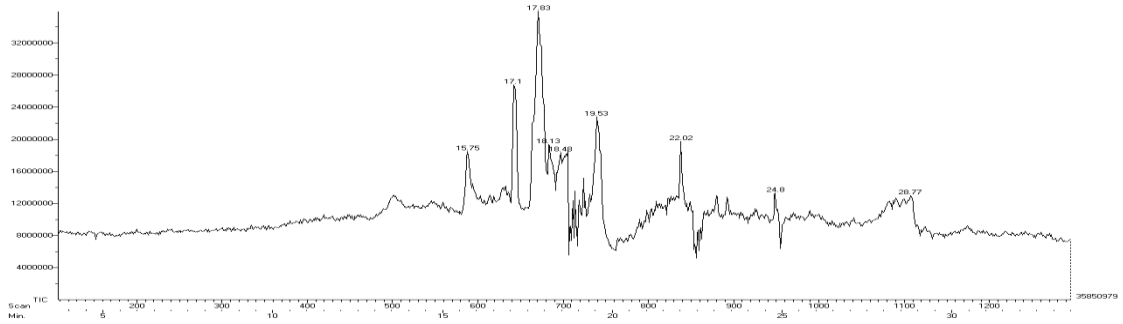

Fig 1. Gas Chromatogram of Lipid extracted from *C. vulgaris*

Table 2. Free fatty acids composition of *Chlorella Vulgaris*

Peak No.	RT (Min.)	Compound Name	Peak area (%)	Peak Area
1	15.75	Tetradecanoic acid	5.07	1817644.64
2	17.10	Pentadecanoic acid	11.24	4029650.04
3	17.83	n-hexadecanoic acid	18.24	6539218.57
4	18.13	hexadecanoic acid	6.85	2455792.06
5	18.48	n-hexadecane	8.14	2918269.69
6	19.53	Oxacyclotetradecane,2-11-dione	14.25	5108764.51
7	22.02	9-octadecanoic acid	13.58	4868562.95
8	24.80	9-octadecanoic acid (Z) Hexyl ester	5.77	2068601.49
9	28.77	9,10-secochola,5,7,10(19) triene	16.86	6044475.06
		Total	100%	35850979.00

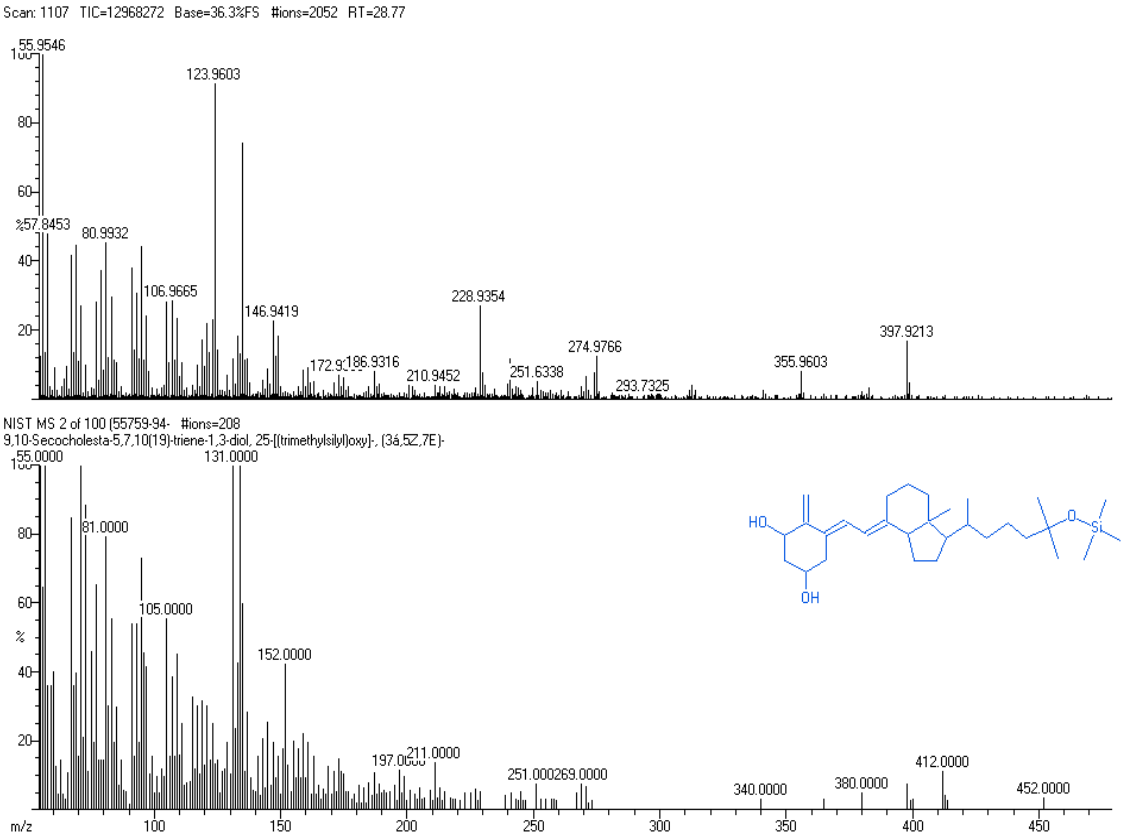

Fig. 2. MS/Scale and structural description for 9,10-secochola,5,7,10(19) triene

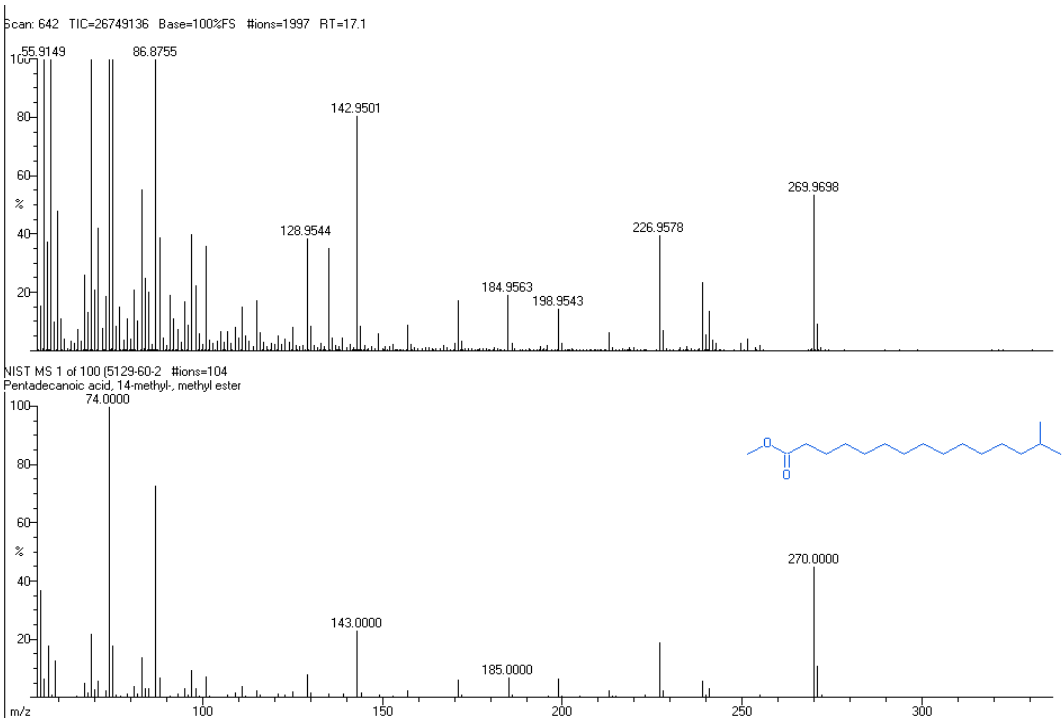

Fig. 3. MS/Scale and structural description for pentadecanoic acid

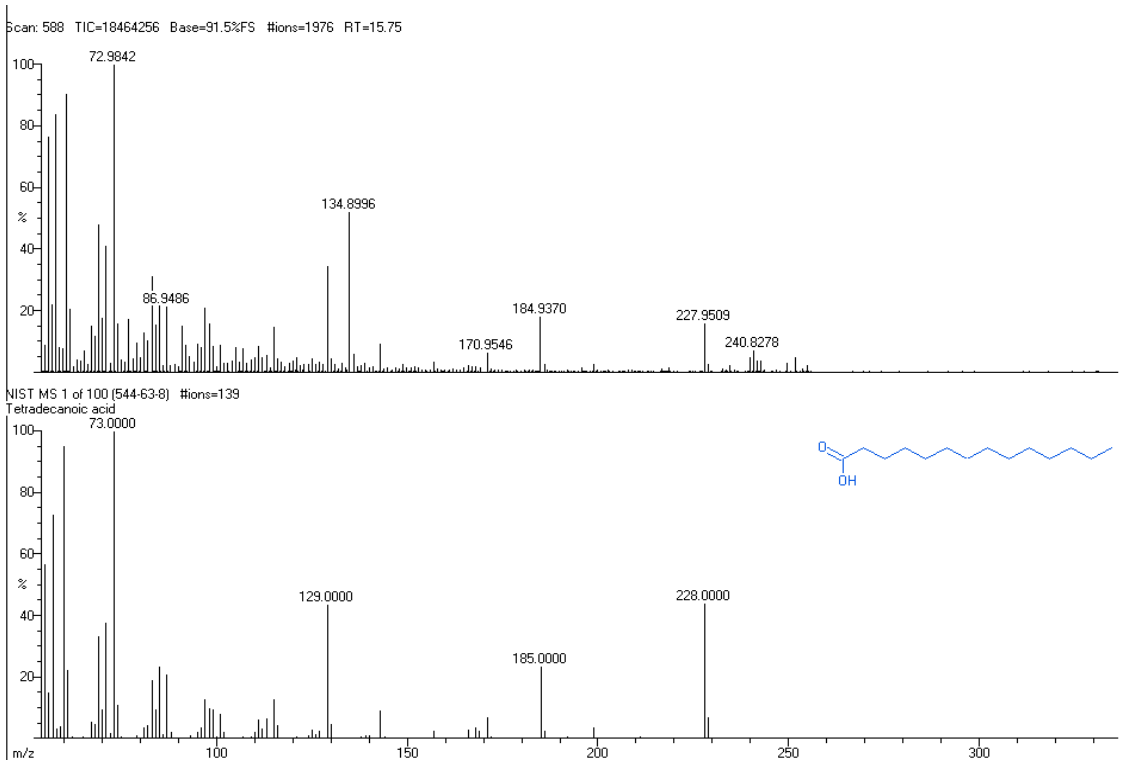

Fig. 4. MS/Scale and structural description for tetradecanoic acid

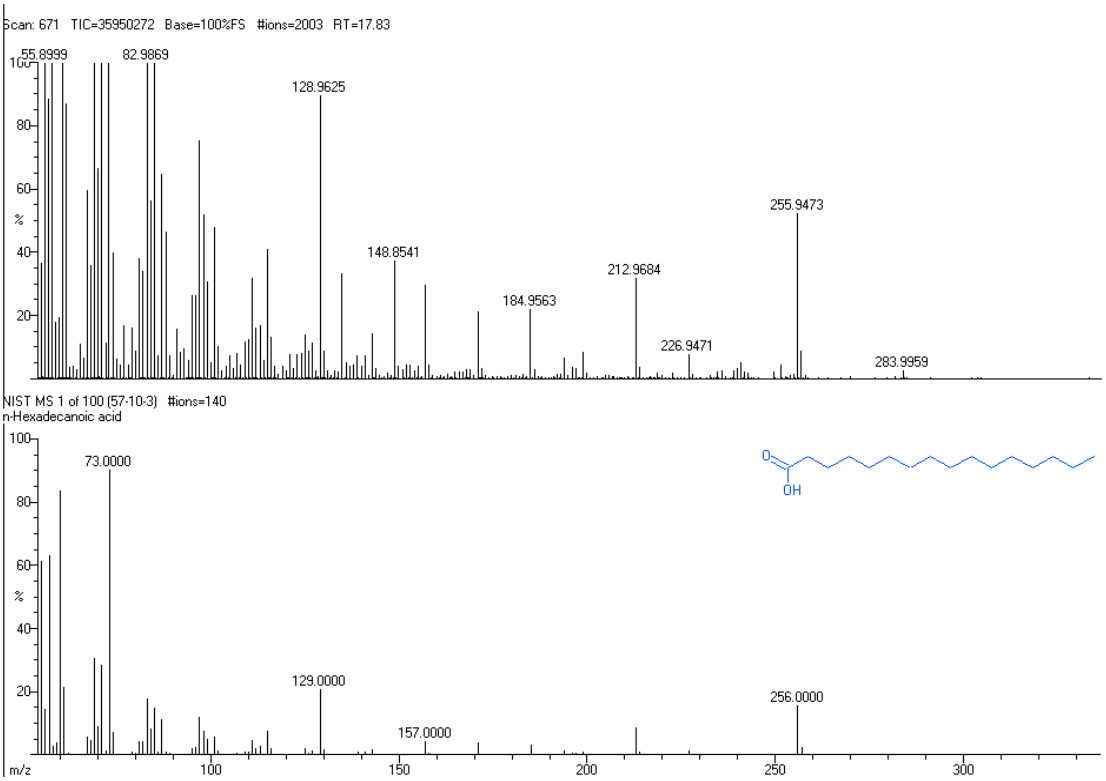

Fig. 5. MS/Scale and structural description for tetradecanoic acid

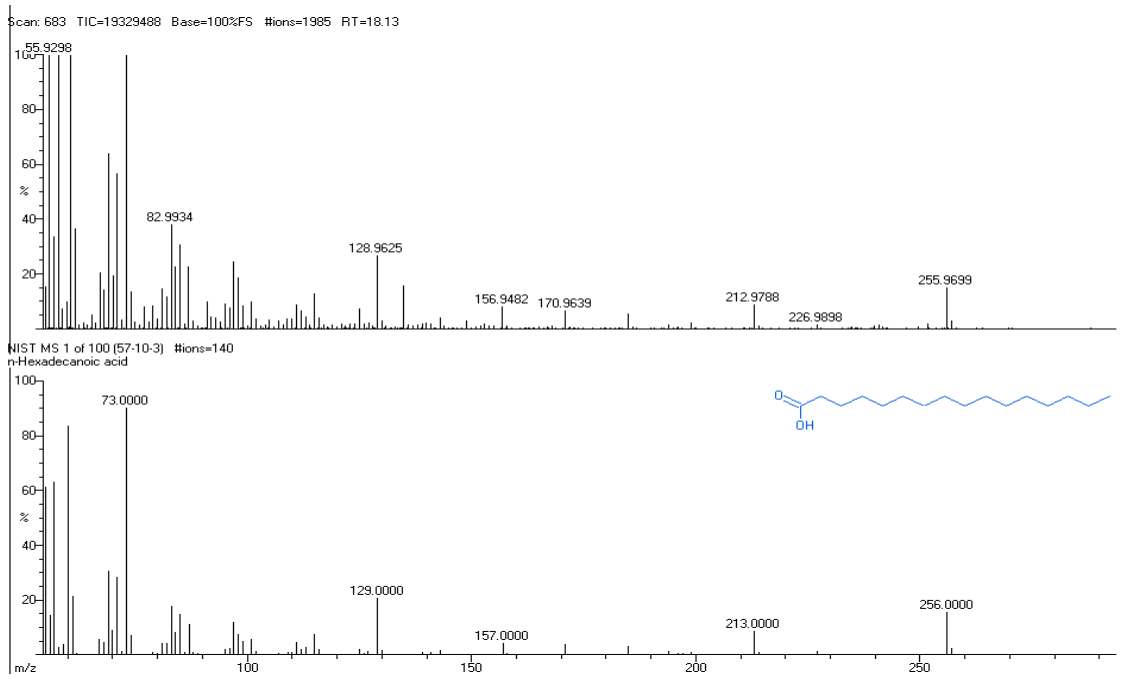

Fig. 6. MS/Scale and structural description for n-hexadecanoic acid

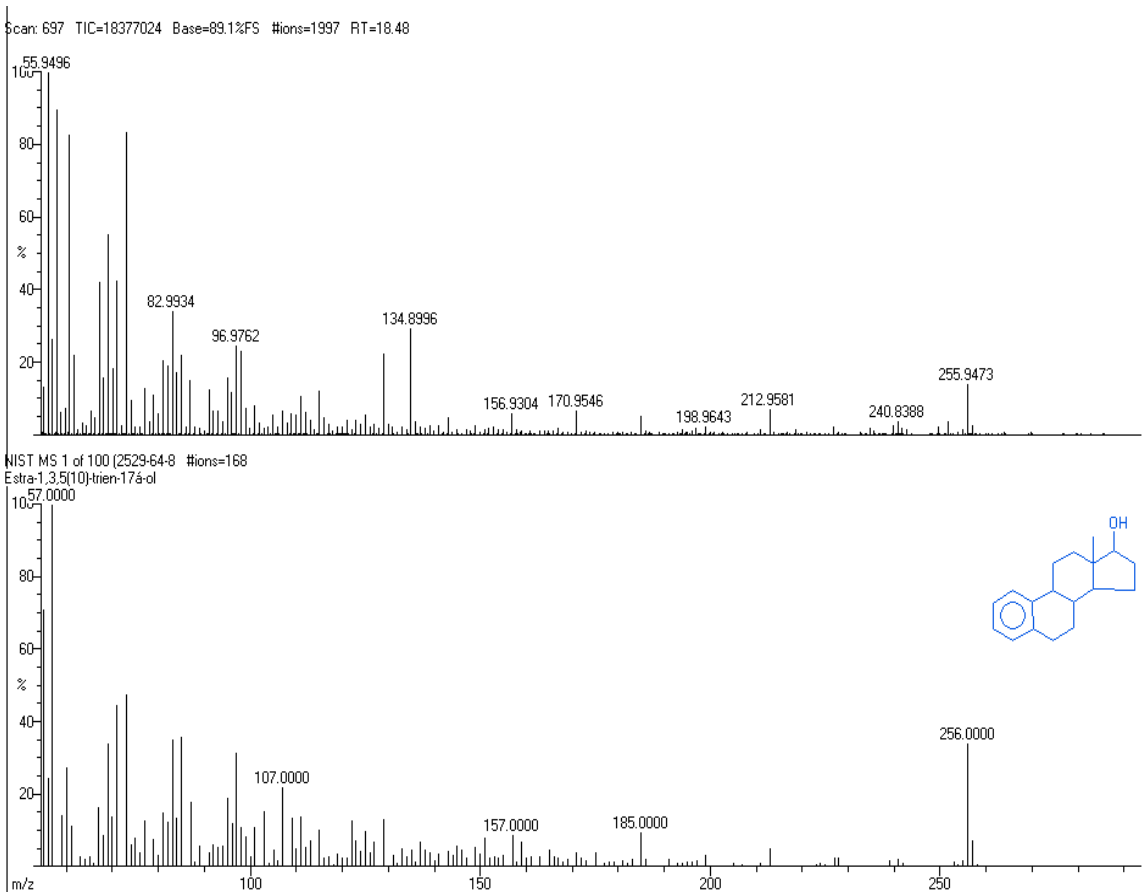

Fig. 7. MS/Scale and structural description for tetradecanoic acid

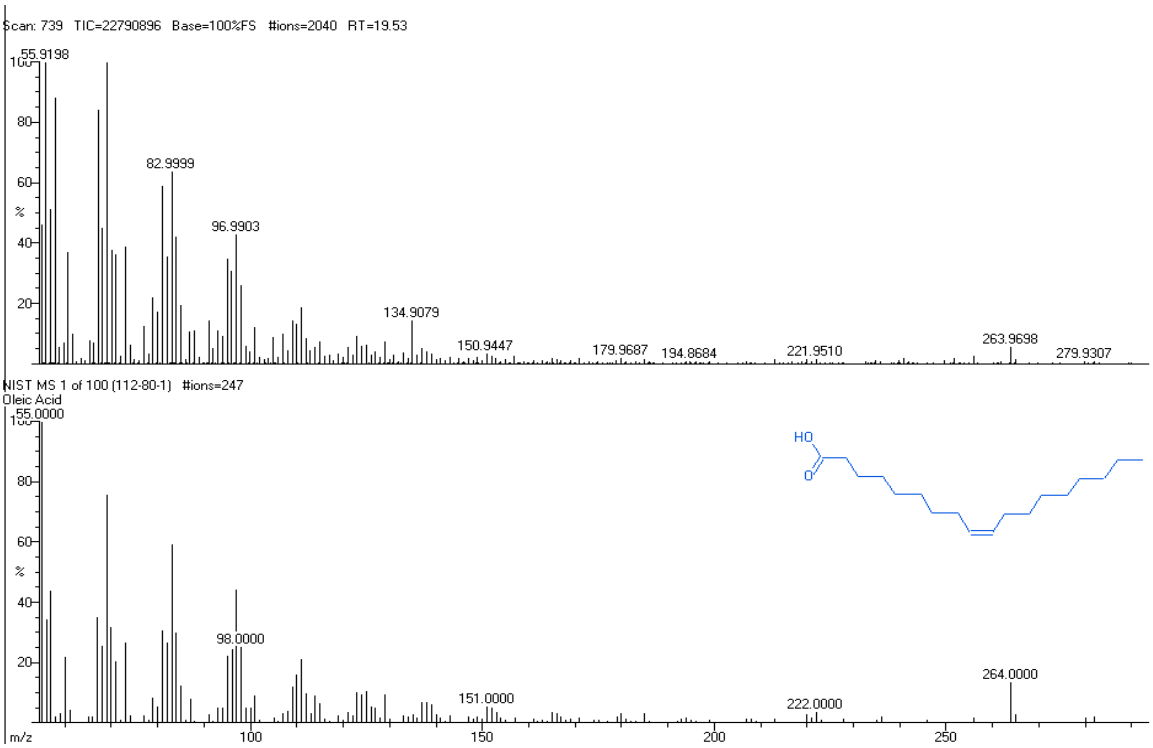

Fig. 8. MS/Scale and structural description for n-hexadecane

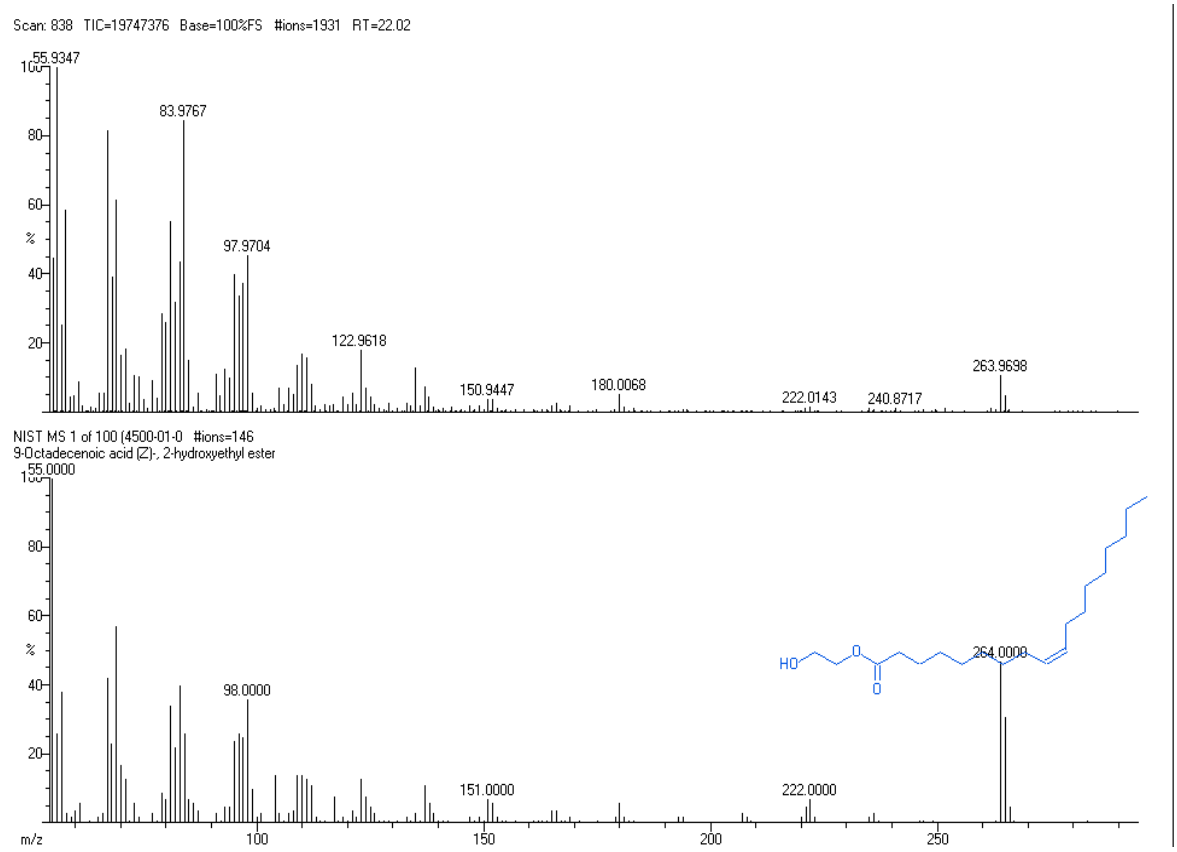

Fig. 9. MS/Scale and structural description for n-hexadecane

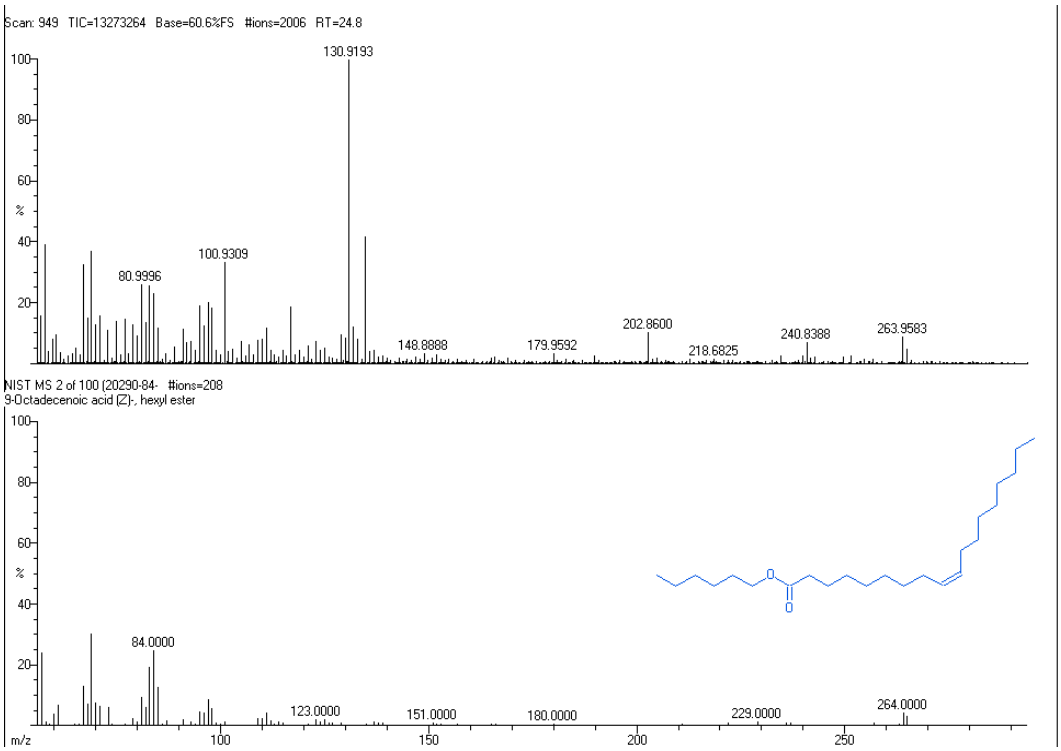

Fig. 10. MS/Scale and structural description for oxacyclotetradecane,2-11-dione

Lipid yield and extraction process contributes to the commercialization of biofuels from microalgae. In this present study, evaluated the efficacy of using solvent extraction protocols, on biofuel using Bligh and Dyer approach, Modified Bligh and Dyer and Soxlet extraction process, this study further revealed, Modified Bligh and Dyer had a yield of 77.2%wt, while the conventional, Bligh and Dyer of 75.6%wt, and soxlet was 73%wt. The work of Verma & Balomajumder [14] suggested in their study that lipid yield of 49.52% while Ultrasonication had a better yield in term of breaking the cell walls of the microalgae for industrial or commercial lipid yield as they reported a lipid yield of 56.2%, although was reported a lower yield than was observed for the present study. In a related study by Selvarajan et al. [15] previously reported a lower yield of lipid from *Chlorella vulgaris* of about 42.1%wt. The process of soxlet extraction has identified to have a higher amount of lipid using an optimal temperature and solvent especially for recollection of lipid dissolved in the solvent. The work of Wang [16] suggest a combination of extraction processes such as Lazer-desorption technique has the potential was reported to release better yield from the algae. Algal recovery and dewatering, as a crucial downstream process have also impeded the process of refining of oil from microalgae. Yield prior to optimization of algae from *Chlorella* sp. could be increased from 18.29% to 80% lipid [17].

Characterization of lipid associated with *Chlorella* oil induced by the growth condition and cultivation medium. Lipids are of central critical importance to the biorefinery and pharmaceutical processes. The quality of lipid is an indication of the cultivation process, nutrient uptake and assimilation. The lipid component indicated the presence of tetradecanoic acid (Myristic acid) commonly seen in nutmeg oil, Pentadecanoic acid, n-hexadecanoic acid, hexadecanoic acid (Palmitic acid) a saturated lipid, oxacyclotetradecane, 9-octadecanoic acid (z) also called methyl-oleate it's a waxy substance also found in the tallow of animal milk. Over 70% of the lipid is composed of saturated fatty acids. Pentadecanoic acid have been reported in Daphnia magna, a marine microalgae known for its nutraceutical value, it is found in milk rich foods. 9-octadedecanoic acid (Oleic acid) found in vegetable oils, and fats. It is an odourless mono-saturate with ω-9 fatty acids, n-hexadecane is also cetane (C:16), a volatile oil component and forms the basis of characterization of oils and categorizes the combustion ability of oil components.The findings of the present study corroborates the earlier

report of Bi and He [18] whose study revealed Palmitic acid (C16:0), Stearic acid (C18:0) and Linoleic acid (C18:3). Their investigation revealed a 60% saturated fatty acid which makes them good candidates of biofuel production. In another study, Adamakis et al. (2018) [12] reported that the presence of both saturates and mono-unsaturates especially palmitic acid (C16:0) and Stearic acid (C18:0) possess the potential for bioconversion into biofuels production. Furthermore, Chankhong [19] also reported 67.59% unsaturated fatty acid further citing that Palmitic acid, stearic ,oleic and Linoleic acid were the fatty acids needed for biodiesel production just as observed in the present study. The study was further agreed to by the account of Batista et al. [20] reported that microalgae had 92.94% unsaturated and 7.006% v/v saturates which differed from the account of the present study, while the fraction present was C16-C20 making up the free fatty acid composition. The report of Al-lwayzy et al. [21] again concur with the report of [20] that lipid production vary significantly with the species of algae type and species. The presence of polyunsaturated long chain fatty acids like the Docosatetraenoates (C22:4) and Octadecanoate (C18:4). Although citing that algae oil from *Euglena* sp. were competitive alternative to *Chlorella* sp oils. The study further concretizes or butresses the role of microalgal-base industry for a robust biofuels production base. Although this cannot be over-emphasized, the application of adequate and well optimized culture media and process has the potential to revamp the biofuel industry. The downstream process suggest every process in the value-chain has the potential to serve as a veritable resource for any industry [22]. The evolution of better lipid extraction protocol has helped in the development and commercialization of biodiesel by transesterification and fuel by Pyrolysis is a valueable roadmap for the commercial production of greener and sustainable energy production. The work of Alves da Silva & Fonseca [23] and Chisti [24] supports the application of indigenous microalgae in the development of natural alternative to the conventional high value compounds that is of importance to man and his development.

4. CONCLUSION

The results of this study showed that significant amount of quality oil from Chlorella can be achieved or realised via Bligh and dyer; and Soxhlet extraction methods which is also rich in Fatty *acid* methyl esters that are increasingly gaining global attention for being utilized in the Biodiesel, emulsifying agents, detergents and in leather works. And the quality and quantity of the oil can be attributed to the cultivation methods employed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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