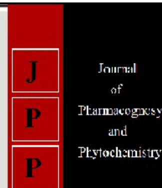
Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com


E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(6): 01-06
Received: 01-09-2016
Accepted: 02-10-2016

Adesalu TA
Department of Botany, Faculty of Science, University of Lagos, Akoka, Nigeria

Temenu TO
Department of Botany, Faculty of Science, University of Lagos, Akoka, Nigeria

Julius ML
Department of Biological Sciences, St. Cloud State University, St. Cloud, MN 56301 U.S.A

Correspondence
Adesalu TA
Department of Botany, Faculty of Science, University of Lagos, Akoka, Nigeria

Molecular characterization, lipid analysis and GC-MS determination of bioactive compounds identified in a West African strain of the green alga *Oedogonium* (Chlorophyta)

Adesalu TA, Temenu TO and Julius ML

Abstract
In this study, the species of *Oedogonium* was subjected to two weeks batch culturing using Bold's Basal Medium (BBM). The DNA of the pure culture obtained was extracted using DNeasy® Plant Mini Kit following the protocols, conserved DNA regions of chloroplast genome corresponding to SSU/18S rDNA and Rbcl were amplified and Polymerase Chain Reaction (PCR) product was sequenced. Based on morphology and DNA barcoding, isolates were identified as *Oedogonium tenerum* for SSU/18S rDNA and *Oedogonium cardiacum* Wittrock ex Hirn (Chlorophyta) for Rbcl. Lipids analysis and the active compounds of *Oedogonium* strain were evaluated using Agilent 7890A Gas Chromatography (GC) system and Agilent 5975C inert Mass Spectrometer Detector. Analysis of the sample lipids showed 12.98% lipid content while GC-MS analysis of hexane extract of *Oedogonium* strain (KU680468 (SSU/18S rDNA); KU680573 Rbcl) revealed the existence of Eicosane, 1-Nonadecene, Tricosane, Heneicosane and Nona hexacontanoic acid adding up to 70.27%. The minor constituents include Butylated hydroxytoluene, Nonadecane, 1-Docosene adding up to 29.3%. The importance of some of these components in medicine includes anti-viral and anti-oxidant properties. Other importance of these hydrocarbons are found in food preservatives and majorly in the petrochemical industry for the production of candles, biopesticides (insect pheromones), paraffin wax and other by-products of petroleum. The result implied a closely related but unique strain which is probably endemic to West Africa and Nigeria in particular as other strains are from North America.

Keywords: *Oedogonium,* GC-MS, Lipids, DNA extraction, PCR, Active compounds

1. Introduction

Algae are heterogeneous complex organisms which comprise the dominant photoautotrophs in many aquatic environments, [1,2] simple microscopic or macroscopic, unicellular to multicellular plants and are competent converters of sun energy to useful biochemical products like oil [3] The green algae are the most diverse group of algae with more than 7000 species growing in a variety of habitats [4] The green algae include unicellular and colonial flagellates as well as various coccoid and filamentous forms, and macroscopic multicellular seaweeds. The freshwater green micro alga, *Oedogonium,* according to [5] is a key target species for the bioremediation of waste waters and for its utilization in energy production via gasification with coal. Numerous authors [6, 11] have documented lipid production in *Oedogonium* spp. identifying it as an organism with potential for biofuel production and valuable biomass source for bioenergy applications [12]. Based on essential oils in *Oedogonium,* it has a higher devolatilization rate and higher char reactivity which makes it easier to be gasified with lignite and used as source of energy fuels [5]. Others have identified bio compounds in *Oedogonium* spp. [9, 13] some particularly useful as antimicrobial products [14, 17]. One issue is that strains of *Oedogonium* vary in their biochemical composition [18] and differ in production of these compounds in varying environments [19]. Over 1400 varieties and forms of *Oedogonium* have been described [20]. This creates difficulty in understanding the potential and variation in biochemical products for commercial exploitation. Here we present information for an undescribed species of *Oedogonium* from Nigeria. The species is characterized molecularly and biochemical composition is detailed. The hope is this will act as an example for detailing potentially useful algal strains for commercial application.

2. Materials and Methods

2.1 Description of study area

The water sample was obtained from a running canal at the back of University of Lagos with coordinates of N06.50994, E03.38885.

The canal is characterized by aquatic vegetation dominated by macrophytes, *Ageratum conyzoides* and *Sida acuta.* The algae sample was submerged in the water as a network of filamentous green algae.

2.2 Collection of sample
Environmental samples for biological analysis were collected by direct method without preservation. The sample was then taken to the laboratory for culturing. Water sample for the analysis of physical parameters was also collected in a clean plastic bottle. Samples were taken to the laboratory for further studies.

2.3 Laboratory processes
In the laboratory, the work area was cleaned with Absorbent Cotton Wool B.P and 70% ethanol to get rid of any other micro-organisms that could contaminate the water samples containing the live algal forms. Substantial weights of the sample were cultured in 3 conical flasks with 5ml of BBM nutrient and 100ml of distilled water added to each. These conical flasks were kept close to the window to ease light penetration for photosynthesis. The conical flasks were shaken twice daily to ensure uniform mixture of nutrients and to prevent clumping. After a week of culturing, increase in biomass was observed in the conical flasks indicating growth of the algae in the sample. The samples were prepared on slides and viewed under the microscope. Species of *Oedogonium* and *Cladophora* were observed since its environmental sample.

2.4 Preparation of Bold's Basal Medium (BBM) Nutrient
The Bold Basal Medium was derived from a modified version of Bristol's solution [21]. The medium lacks vitamins and some trace metal concentrations are high. This is a useful medium for many algae especially green algae. The reagents were weighed using a sensitive weighing balance. After weighing, the reagents were all dissolved in 1000ml of distilled water and sterilized using the autoclave. This was then used as the culture media.

2.5 Analysis of Physical Parameters
The pH and conductivity, air and water temperatures and salinity were analyzed in-situ using HANNA Combo pH and EC Multi Meter Hi 98129, Mercury-in-glass thermometer, Refractrometer and Secchi disc respectively.

2.6 DNA extraction, PCR amplification and Sequence
Algal cultures preliminarily identified as *Oedogonium* sp. were centrifuged for 15 min. in 15 ml centrifuge tubes. The pellet was then retrieved and immersed in liquid nitrogen for 2 min. Frozen pellets were then ground and biomass was transferred to a 1.5ml centrifuge tube. Total nucleic acids were extracted with a DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). Extracted DNA was amplified via PCR targeting portions of SSU rDNA (~1550 nucleotide) and rbcL (~1250 nucleotide). The volume of each PCR was 50µL: 1.0 µL purified DNA; 5 µL 10× buffer; 2.5mM $MgCl_2$ (5µL of 25mM stock); 0.2 mM each dATP, dCTP, dGTP, dTTP (1 µL each of 10 mM stocks); 0.1 µM each primer (0.25 µL each of 20 µM stocks); 0.2 units Taq polymerase; and ddH_2O to a final volume of 50 µL. PCR conditions for SSU followed [22]. The crude PCR product was purified using ExoSAP-IT (USB Corporation, Cleveland, Ohio) following manufacturer's specifications. The pure PCR product was run in a FlashGel System (Lonza, Rockland, ME) to confirm nucleotide lengths of amplified products. Forward and reverse strands were cycle sequenced with BigDye (Applied Biosystems, Foster City, CA, USA) using a combination of nested primers (Table 1). Sequences were resolved with an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence chromatograms were edited and assembled into contigs with Sequencher ver. 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Assembled sequences for each target gene were compared to other taxa to confirm identification using NIH's BLAST system (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1: Combination of nested primers used for the sequencing of *Oedogonium*

Name	Marker	Sequence (5' to 3')
SSU11	SSU	AAC CTG GTT GAT CCT GCC AGT
SSU11+	SSU	TGA TCC TGC CAG TAG TCA TAC GCT
SSU301+	SSU	ATC ATT CAA GTT TCT GCC C
SSU515+	SSU	TGG AAT GAG AAC AAT TTA A
SSU1004+	SSU	CGA AGA TGA TTA GAT ACC ATC G
SSU1451+	SSU	TGT GAT GCC CTT AGA TGT CCT GG
ITS1DR2	SSU	CCT TGT TAC GAC TTC ACC TTC C
SSU1147–	SSU	AGT TTC AGC CTT GCG ACC ATA C
SSU568–	SSU	CAG ACT TGC CCT CCA ATT GA
rbcL66+1	rbcL	TTA AGG AGA AAT AAA TGT CTC AAT CTG
RbcL527+	rbcL	AAA ACA TTC CAA GGT CCT GCT
RbcL1255–2	rbcL	TTG GTG CAT TTG ACC ACA GT
RbcL587–	rbcL	GTC TAA ACC ACC TTT TAA MCC TTC
nd6+1	rbcL	GTA AAT GGA TGC GTA TG

2.7 Analysis of Total Extracted Lipids
For Total extracted lipids quantification, the protocols of [23, 24] was followed. Two hundred milliliters of culture liquid was sub-sampled and filtered onto a 47 mm glass fiber filter, pore size ~0.8 nm, and placed into a labeled plastic Petri dish and stored in a −80 ^{0}C freezer. Filters were removed from the freezer and placed in a plastic desiccator. The desiccator was then placed in a drying oven at 60 ^{0}C for 24 h. Upon removal from the drying oven the desiccator was connected to a vacuum pump for 1 h to remove water vapour. The filters were immediately removed from the desiccator and weighed with a Mettler AG245 analytical scale. Filters were then placed in individual Soxhlet extraction apparatus with 80 ml of a 2:1 methanol and chloroform solution. Extraction was allowed to proceed for 8–20h. At the end of the extraction period, the extract was transferred to a Kuderna-Danish

Concentrator. The extract was condensed to 1ml and the extraction apparatus was rinsed with an additional 1ml of 2:1 methanol and chloroform. This 2ml of extract was transferred to a dry weigh ampoule and the solution was placed under a nitrogen stream until dry. The ampoule was then weighed on a Mettler AG245 analytical scale. Based upon these values mg/L of lipid was calculated for culture flask.

2.8 Gas Chromatography Mass Spectrometry (GC-MS) Analysis

The GC-MS analysis was done at the University of Lagos central research laboratory. The GC-MS uses the principle of separation technique, sample was analyzed in Agilent 7890A Gas Chromatography (GC) system equipped with a HP-5 column (30ml x 250µm ID, 0.25µm film thickness, Agilent 122-0132) and Agilent 5975C inert Mass Spectrometer Detector with triple-axis detection using Helium as carrier gas. The flow rate of 1.50ml/min; injector and column oven temperature 280 ^{0}C and 80 ^{0}C; injector mode split ratio of 20:1. GC-MS consists of an injection port at one end of a metal column packed with the analyte solution and a detector at the other end of the column. It is made up of the mobile phase which is the Helium gas and a stationary phase which serves as the column. The sample is injected into the injector with a liner under, the syringe picked the sample and the mobile phase and the Helium gas propelled the sample from the liner down the column where separation into different components occurs. The injection port was maintained at a temperature 80 ºC for 4 mins, it increased 10 ºC per min to 24 ºC for 20 min. The run time was 40 minutes. The column used was a metal tube (HP5MS) often packed with a sand-like material to promote maximum separation. As the sample moved through the column, the different molecular characteristics determined how each substance in the sample interacted with the column surface. The column allowed the various substances to partition themselves and the various components in the sample separated before eluting from the column. The length of the column was 30m, internal diameter of 0.320mm and thickness of 0.25µm. The amount of time at which each particular component elute from the compound was retained in the GC column known as the retention time which helped in differentiating between the components such that if two samples do not have equal retention times, those samples are not the same substance.

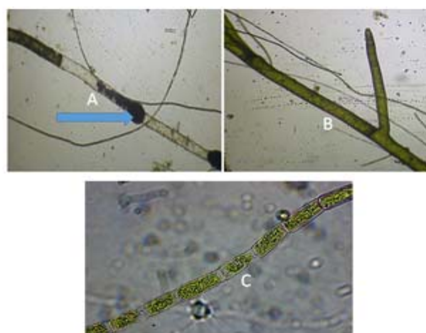
2.9 Identification of components

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns and Fiehn Mass Spectra Libraries. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The Name, Molecular weight and Structure of the components of the test materials were ascertained.

3. Results

3.1 Biological analysis

Olympus XSZ-N107 light microscope with attached Moticam 580 was used for the observation of the sample. Observations revealed the presence of *Cladophora* and *Oedogonium* with *Oedogonium* in abundance (Plates: A-C). *Oedogonium* was isolated as described earlier and fortified with 5ml BBM to 95ml sample, placed close to window pane for light penetration.



Plates A-C: *Oedogonium* sp. (A and C); Note: arrow showing sex cell in A; *Cladophora* sp. (B)

3.2 Physical parameters

The results of the physical parameters of the water sample showed the freshwater status of the sampling location with salinity value of 0‰, the water was slightly acidic with pH 6.28. The conductivity recorded 524.0µS/cm while air and water temperatures were 28 ^{0}C and 26 ^{0}C respectively.

3.3 DNA extraction, PCR amplification and Sequence

The pure PCR product run in a Flash Gel System (Lonza, Rockland, ME) confirmed nt lengths of amplified products (Figure 1). A partial SSU rRNA fragment 1541 nt in length and a partial RBCL fragment 1243 nt in length were recovered. BLAST results for the partial SSU rRNA fragment identified a 98% similarity to an existing *Oedogonium tenerum* sequence (DQ078296.1) while BLAST results for the partial RBCL fragment identified a 99% similarity to an existing *Oedogonium cardiacum* sequence (EF113458.1).

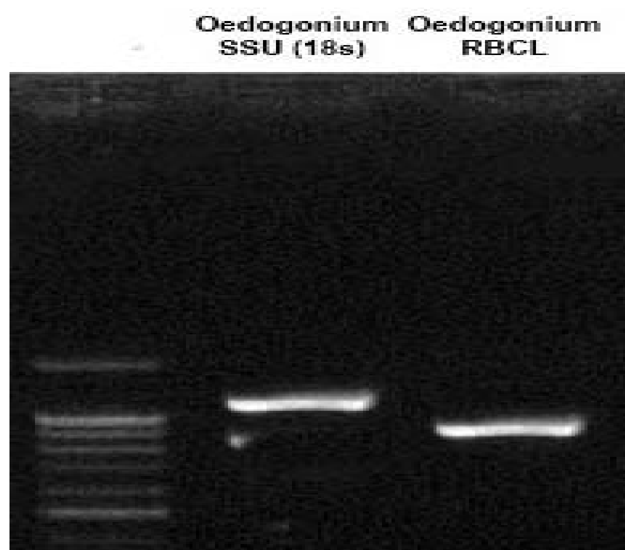


Fig 1: Agarose gel purification of the *Oedogonium* strain

3.4 Total Extracted Lipids

From the analysis, the lipid extract was 12.98% as presented in Table 2.

Table 2: Lipid extracts biomass of the *Oedogonium* sp.

	Vial	Vial+Lipid	Lipid	Filter	Filter+Biomass	Biomass	Lipid%
Oedogonium strain	13052.52	13058.26	5.74	116.47	160.69	44.22	12.98055179

3.5 Gas Chromatography Mass Spectrum

Eight compounds were identified in this strain of *Oedogonium* sp. (KU680468, KU680573) by GC-MS, analysis revealed a high composition of esters (hydrocarbons) although some appear twice. The active principles with their retention time (RT), molecular formula, molecular weight, nature of the compound, composition percentage and quality in the hexane extract are presented in Table 3 and Figure 2.

Table 3: Bioactive compounds present in the hexane extract of *Oedogonium* sp.

No	Name of compound	Molecular Formula	Molecular weight (g/mol)	Nature of the compound	RT (mins)	Percentage composition	Quality
1	Butylated hydroxytoluene	$C_{15}H_{24}O$	220.35	Phenol	11.81	6.6	97
2	Nonahexacontanoic acid	$C_{69}H_{138}O_2$	999.8	Alkanoic acid	14.66	9.18	55
3	Nonahexacontanoic acid	$C_{69}H_{138}O_2$	999.8	Alkanoic acid	15.76	4.08	70
4	Nonadecane	$C_{19}H_{40}$	268.52	Hydrocarbon	17.53	6.75	64
5	Nonadecane	$C_{19}H_{40}$	268.52	Hydrocarbon	18.16	5.56	92
6	Eicosane	$C_{20}H_{42}$	282.55	Aliphatic hydrocarbon	18.25	13.4	68
7	Heneicosane	$C_{21}H_{44}$	296.57	Aliphatic hydrocarbon	18.41	9.78	92
8	Eicosane	$C_{20}H_{42}$	282.55	Aliphatic hydrocarbon	18.5	8.63	90
9	1-Nonadecene	$C_{19}H_{38}$	266.51	Hydrocarbon	18.55	10.67	89
10	1-Docosene	$C_{22}H_{44}$	308.58	Hydrocarbon	18.78	6.74	86
11	Tricosane	$C_{23}H_{48}$	324.63	Paraffin hydrocarbon	19.44	15.75	95
12	Tricosane	$C_{23}H_{48}$	324.63	Paraffin hydrocarbon	19.68	2.86	95

The prevailing compounds were Tricosane (15.72%), Eicosane (13.40%), 1-Nonadecene (10.67%), Heneicosane (9.78%), Nonahexacontanoic acid (9.18%), Nonadecane (6.75%), 1-Docosene (6.74%) and Butylated hydroxytoluene (6.60%). Figures 3-5 show the mass spectrum and structures of petrochemical important phytochemical constituents of *Oedogonium* sp. (KU680468, KU680573). Butylated hydroxytoluene and Nonahexacontanoic acid are alkanoic acid used in cosmetics. Nonadecane, Eicosane, Heneicosane and Tricosane belong to the higher alkane group that is useful in the production of biopesticides, candles, aviation fuels, lubricating oil and serve as anti- corrosive agents while 1-Docosene and 1-Nonadecene make up the alkene group useful as turbine and gear oils.

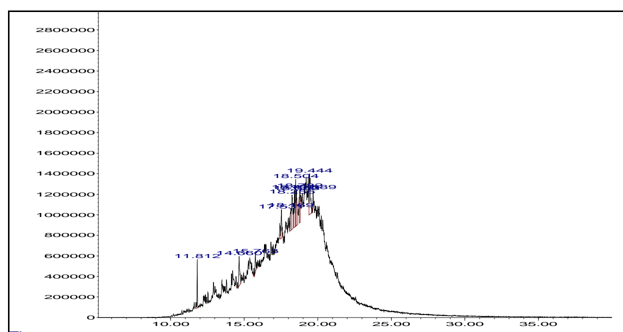


Fig 2: GC-MS chromatogram of the hexane extract of *Oedogonium cardiacum*

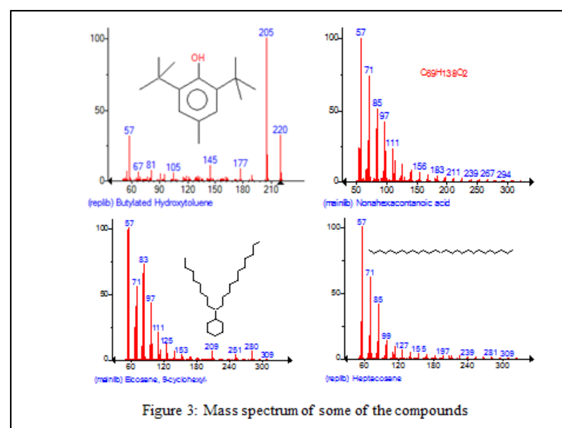


Figure 3: Mass spectrum of some of the compounds

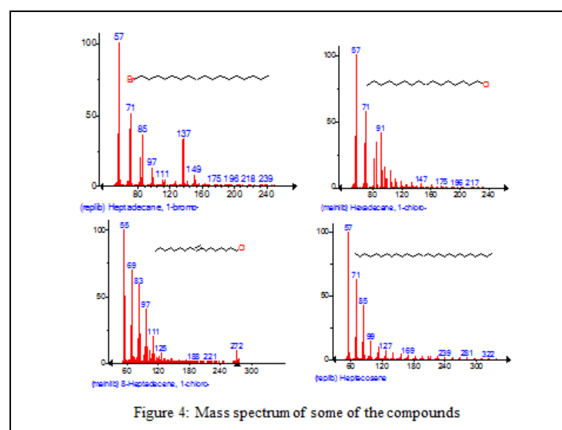


Figure 4: Mass spectrum of some of the compounds

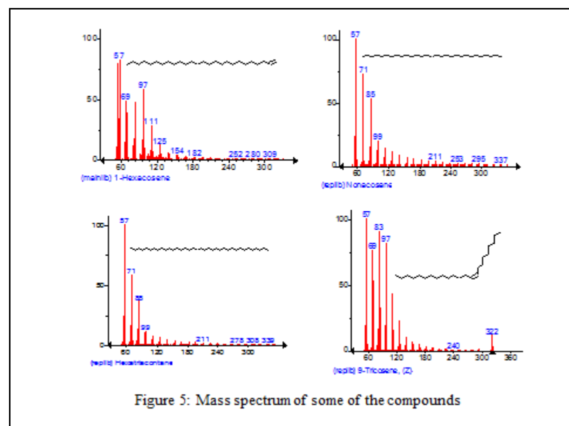


Fig 5: Mass spectrum of same of the compounds

4. Discussion

From the GC-MS analysis carried out on the fresh water green alga, *Oedogonium* using hexane extract (hot method), eight volatile components were produced. Some of these compounds are hydrocarbons which are good raw materials in the petrochemical industry for candles, lubricants, jet fuels and biopesticides while the other two are acids which could be used as additives in soaps and other cosmetics production. Butylated hydroxytoluene can be used as food preservatives, antioxidant and antiviral properties [25] Eicosane, one of the compounds in paraffin wax, is used in making candles while Tricosane uses have been reported by [26] for biopesticides, aviation fuel and lubricating oil. The GC-MS result has shown that these hydrocarbons were the major content of the algal oil. Hexane which is a non-polar solvent was used to extract these bioactive compounds from the *Oedogonium* species because of its high stability, low greasy residual effects, boiling point and low corrosiveness. From the work on extraction of oil using different solvents on *Ulva compressa* [27], the highest oil extraction yield of 7.33% was achieved from the *Ulva compressa* biomass using the n-hexane solvent. This solvent system was adopted for further extraction study with other algal biomass. From the literature, it was observed that the same solvent (hexane) was utilized for the extraction of triglycerides using the bond elut procedure [27]. Oil extraction from this same alga species was carried out using n-hexane-2 isopropanol [28] and n-hexane-ethyl alcohol [29] as solvent mixtures yielded 5.62% and 5.31% respectively. Therefore, this shows hexane as the most widely used solvent for the extraction of oil from micro and macro algae [30]. It is less toxic, has minimal affinity towards neutral lipid contaminants and higher selectivity towards neutral lipid fraction [28]. Many research works have already been established on the extraction of value added products from macro algal species with the use of hexane as extracting solvent [31]. According to [32] hexane was used as a solvent for oil recovery from fresh water algae for the biodiesel production. Less work has been done on *Oedogonium* species.

5. Conclusion

The presence of the large number and increase in minimum quality above 90 of aliphatic hydrocarbons compared to the number of other components in the extract suggests that the sample is a potential source of biofuels. This study is in conformity with [33], who reported that hexane extracts of *Oedogonium* and *Spirogyra* species yielded more volatile compounds (methyl ester) in *Oedogonium* than *Spirogyra*. The study of volatile compounds of various microalgae is mandatory for reduction of greenhouse gases, because microalgae based biofuel contain less sulphur or nitrogen compared to fossil fuel. There is a dearth of literature on extraction of volatile oil from *Oedogonium* in Nigeria in particular. Therefore, this study has established the endemic nature of *Oedogonium* strain using molecular techniques and also been able to showcase the potentials of using this alga (*Oedogonium*) as an alternative source of fuels, its medicinal values and petrochemical importance.

6. References

1. Round FE. The ecology of algae. Cambridge University Press, 1981, 653.
2. Wetzel RG. Limnology (2nd edn.) Philadelphia, Saunders, 1983, 853.
3. Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug JH, Posten C *et al*. Second generation biofuels: High-Efficiency microalgae for biodiesel production. Bioenerg Res. 2008; 1:20-43.
4. Mongillo J, Zierdtarshaw L. Encyclopedia of Environmental Science. Greenwood, United States. 2000; 504.
5. Zhu Y, Kwong CW, van Eyk PJ, de Nys R, Wang D, Ashman PJ. Pyrolysis Characteristics and Char Reactivity of *Oedogonium* sp. and Loy Yang Coal. Energy Fuels, 2015; 29(8):5047-5055.
6. Hossain ABMS, Salleh A. Biodiesel Fuel Production from Algae as Renewable Energy. American Journal of Biochemistry and Biotechnology, 2008; 4(3):250-254.
7. Haq I, Muhammad A, Hameed U. Comparative assessment of Cladophora, Spirogyra and Oedogonium biomass for the production of fatty acid methyl esters. Prikl. Biokhim Mikrobiol. 2014; 50(1):80-84.
8. Singh RK, Tiwari SP, Rai AK, Mohapatra TM. Cyanobacteria an emerging source for drug discovery. J. Antibiotics. 2011; 64: 401-412.
9. Tonetto AF, Oliviera KSM, Domingues TM. Analysis of Biochemical Composition of Three Stream Macroalgae Species. International Journal of Applied Science and Technology. 2014; 4(5):111-115.
10. Munir N, Imtiaz A, Sharif N, Naz S. Optimization of growth conditions of different algal strains and determination of their lipid contents. The Journal of Animal & Plant Sciences. 2015; 25(2):546-553.
11. Neveux N, Magnusson M, Mata L, Whelan A, de Nys R, Paul NA. The treatment of municipal wastewater by the macroalga *Oedogonium* sp. and its potential for the production of biocrude. Algal Research, 2016; 13:284-292.
12. Lawton RJ, de Nys R, Paul NA. Selecting reliable and robust freshwater macroalgae for biomass application. PLoS One. 2013; 8(5):e64168.
13. Neveux N, Yuen AKL, Jazrawi C, Magnusson M, Haynes BS, Masters AF *et al*. Biocrude yield and productivity from the hydrothermal liquefaction of marine and freshwater green macroalgae, Bioresource Technology, 2014, 334-341.
14. Pérez-Gutiérrez RM. Isolation and identification of antibacterial compounds from *Oedogonium capillare* leaves. Latin American and Caribbean Bulletin of Medicinal and Aromatic Plants, 2006; 5(1):15-19.
15. Singh DP, Trivedi RK. Environmental, Economic and Financial Aspects of Production of Biodiesel from Algal

Biomass, the International Journal of Science & Technoledge. 2014; 2(1):32-38.
16. Kamble SM, Rokde AU, Ashok M, Chavan AM. Antifungal activity of algal extracts against plant pathogenic fungi. I. Multidisciplinary Research Journal. 2012; 2(3):23-24.
17. Snehalatha D, Digamber RB. Antifungal activity of freshwater green-algae (Chlorophyceae), European Journal of Pharmaceutical and Medical Research. 2015; 2(5):1394-1402.
18. Lawton RJ, de Nys R, Skinner S, Paul NA. Isolation and identification of *Oedogonium* species and strains for biomass applications. PLoS One. 2014; 6-9(3):e90223.
19. Lawton RJ, de Nys R, Magnusson ME, Paul NA. The effect of salinity on the biomass productivity, protein and lipid composition of a freshwater macroalga. Algal Research, 2015; 12:213-220.
20. Guiry MD, in Guiry MD, Guiry GM. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway, 2016. http://www.algaebase.org;; searched on 05 September 2016.
21. Bold HC. The morphology of *Chlamydomonas chlamydogama* sp. nov. Bull Torrey Bot Club. 1949; 76(2):101-108.
22. Alverson AJ, Jansen RK, Theriot EC. Bridging the rubicon: phylogenetic analysis reveals repeated colonizations of marine and fresh waters by thalassiosiroid diatoms. Mol Phylogenet Evol. 2007; 45:193-210.
23. Sicko-Goad L, Evans MS, Lazinsky D, Hall J, Simmons MS. Effects of chlorinated benzenes on diatom fatty acid composition and quantitative morphology. III. 1,2,3-Trichlorobenzene. Arch. Environ. Contam. Toxicol. 1989a; 18:647-655.
24. Sicko-Goad L, Evans MS, Lazinsky D, Hall J, Simmons MS. Effects of chlorinated benzenes on diatom fatty acid composition and quantitative morphology. IV. Pentachlorobenzene and comparison with trichlorobenze isomers. Arch. Environ. Contam. Toxicol. 1989b; 18:656-668.
25. Ash M, Ash I. Handbook of Preservatives. Synapse Information Resources, U.S.A. 2004, 873.
26. Olaniran OA, Sudhakar AVS, Drijfhout FP, Dublon IAN, Hall DR, Hamilton JGC *et al*. A male-predominant cuticular hydrocarbon, 7-methyltricosane, is used as a contact pheromone in the western flower thrips *Frankliniella occidentalis*. Journal of Chemical Ecology. 2013; 39:559-568.
27. Kaluzny MA, Duncan LA, Merritt MV, Epps DE. Rapid separation of lipid classes in high yield and purity using bonded phase columns. J. Lipid Res. 1985; 26:135-140
28. Lee S, Yoon B, Oh H. Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. Biotechnology Techniques, 1998; 12(7):553-556.
29. Meziane S, Kadi H, Lamrous O. Kinetic study of oil extraction from olive foot cake. *Grasasy Aceites* 2006; 57:175-179.
30. Miao X, Wu Q. Biodiesel production from heterotrophic microalgal oil. Bioresource Technology, 2006; 97(6):841-846.
31. Aresta M, Dibenedetto A, Carone M, Colonna T, Fragale C. Production of biodiesel from macroalgae by supercritical CO2 extraction and thermochemical liquefaction. Environ. Chem. Lett. 2005; 3:136-139.
32. Vijayaraghavan K, Hemanathan K. Biodiesel Production from Freshwater Algae, Energy and Fuels-a journal of Science, 2009; 23:5448-5453
33. Hossain SA. Biodiesel Fuel Production from algae as Renewable Energy. American Journal of Biochemistry and Biotechnology, 2008; 4(3):250-254.