Int.J.Curr.Microbiol.App.Sci (2017) *6*(5): 1801-1810

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 5 (2017) pp. 1801-1810**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.605.196

Extraction and Profiling of Lipids of Isolated Strain *Chlorella* fw-12 by GC-MS

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Keywords

Algae culture, Microalgal lipids, Transesterification, GC-MS, biodiesel.

Article Info

Accepted:
17 April 2017
Available Online:
10 May 2017

A B S T R A C T

In search of potential source of biodiesel, four microalgal strains were finally isolated. In the first step of investigation, 25 samples from aquatic bodies of various districts of north India were collected and processed, resulting in a total of 4 microalgal strains viz. *Nostoc* sp., *Chlorella* sp., *Chlorococcum* sp. and *Oscillatoria* sp. were isolated and grown in pure culture in culture room. On the basis of observations, maximum lipid content was recorded as 19.88% (on dry biomass basis) in *Chlorella* fw-12 (at 28th day) under standard growth conditions in liquid BG-11 medium. Extracted lipid was purified by trans-esterification and characterized by GC-MS technique in respect to lipid quality (profiling of lipid constituents). A total of 10 chemical compounds were detected as saturated hydrocarbons namely n-Decane, n-Undecane, n-Dodecane, n-Tridecane, n-Tetradecane. Other than hydrocarbons, a high percentage of fatty acids (61.44%) was also recorded, major fatty acid recorded were C19:1, C19:2 and C19:3, which were methyl hexadecanoate (palmitate) (30.20%), methyl 9, 12, 15-octadecatrienoate (linolenate) (11.03%), methyl octadecaenoate (elaidate) (8.74%), methyl 9, 12-octadecadienoate (linolenate) (7.51%) and methyl 5, 9, 12-octadecatrienoate (gamma-linolenoate) (2.07%), which makes better quality of algal biodiesel. Thus, selected *Chlorella* fw-12 unicellular green algae would be a potent source of qualitative biodiesel.

Introduction

The sustainability of algal biofuel production systems is achievable. There is regular research on biodiesel fuel into finding more and more appropriate source to enhance oil yield in algal cells. Fossil fuel energy resources are diminishing rapidly and most importantly the liquid fossil fuel will be diminished by the year about 2050. Additionally, fossil fuels are directly related to air pollution, land and water degradation. In order to replace all fossil fuel usage entirely, a large amount of biomass is required to manufacture sufficient bio-oil. Research on biofuel presently focuses on high amount of oil yield. In these circumstances, biofuel from renewable sources like microalgae can be a substitute to reduce our dependency on fossil fuels and assist to maintain the healthy global environment and economic sustainability (Chisti, 2007). Over 50,000 microalgal species are present globally in aquatic and terrestrial environment,

implying their widespread availability. Different microalgal species may vary in their lipid content from about 5% to 75% by weight of dry biomass (Um and Kim, 2009; Sydney *et al*., 2010). Microalgae has ability to utilize non-arable land and waste water resources with few competing uses, which makes algal biofuel production systems superior to biofuels based on terrestrial biomass. It has developed great interest in government sector, non-government organization, and private sector and among researchers. Recent initiatives clearly indicate this interest at all levels of government and in private sector in the development of algal biofuels technologies and enterprises. Production of biofuel from feed stocks generally consumed by humans or animals can produce a problem, which can be the root cause of worldwide dissatisfaction.

Fatty acids are the major component of lipids, and the physical, chemical, and physiological properties of a lipid class depend primarily on its fatty acid composition. Lipids and fatty acids are the primary metabolites of microalgae to produce biodiesel. *Chlorella vulgaris* (Gouveia *et al*., 2009), *Scenedesmus obliquus* (Da-Silva *et al*., 2009; Gouveia *et al*., 2009; Sahu *et al*., 2013), have been extensively studied. In order to define a lipid class, it is must to define its fatty acids also. Fatty acids are compounds which are synthesized naturally through condensation of malonyl coenzyme A units by a fatty acid synthase complex. They usually contain even numbers of carbon atoms in straight chains (generally C_{14} to C_{24}), either saturated or unsaturated. Synthesis of neutral lipids in the form of (triacylglycerides) TAG can be induced in many species under stress conditions. These lipids are suitable initiators for biodiesel production (Miao and Wu, 2006; Hu *et al*., 2008). Trans-esterification of these algal lipids convert crude lipids into biodiesel on reaction with alcohol (generally methanol) and sodium hydroxide act as catalyst and glycerol is produced as byproduct.

Materials and Methods

Sampling and isolation of microalgae

Samples were collected in five stages as these all stages include the probability of finding different species of microalgae due to season change (summer to rainy and rainy to winter) favours the change in environmental conditions (Tables 1 and 2). All the water samples were collected in blue capped sample containers (around 100 ml water). All the samples were stored at 4^0C in refrigerator.

Enrichment of samples

Enrichment culture techniques were used to obtain unialgal colonies of microalgae using Bold Basal medium (Bold, 1949; Bischoff and Bold, 1963) in culture room at 3.5 klux light intensity with 16:8 light and dark period and 28 ± 2^0C temperature. The pH of the medium was maintained at 8.0for optimal growth of cultures (Table 3).

Sterilized Bold Basal medium (BBM) was poured in 250 ml conical flasks and autoclaved. Each 1 ml of collected water samples were inoculated in each flask and kept in culture room for a period of 10-15 days, which is maintained with optimal light intensity and temperature.

After incubation period green coloured growth of algal cells was observed and this was picked with the help of Pasteur pipettes in order to obtain unialgal cells. Unialgal cell was re-cultured in another 250 ml conical flask containing 100 ml sterilized Bold Basal medium, incubated again for 10-15 days.

Solid agar based medium was prepared by dissolving 15-18 g of purified agar in 1 liter

of medium and autoclaved before use for maintenance of the cultures. Plates were incubated in culture room for a period of 10-15 days. The isolated colonies were picked up repeatedly and microalgae strains were purified by repeated sub culturing, plating and streaking on the appropriate medium. Isolated microalgae strains from selected habitat were grown and maintained in Bold Basal medium (BBM) under similar conditions. Cultures were regularly streaked on plate having appropriate solid medium to maintain purity of cultures which was examined several times by microscopic observation at regular intervals. Discrete colonies were inoculated in fresh medium and used for the study at exponential phase.

Micromanipulation

Isolation of microalgal cells from different type of aquatic samples was carried out by using solid agar based medium as well as broth Bold Basal medium. Greenish colonies of cells was several times recultured on freshly prepared agar plates and also subcultured in liquid medium. After incubation for 15-20 days, 2-3 types of growth of cells were visible. Microalgal colonies obtained from Petri dishes incubated according to above mentioned methods were transferred carefully to the conical flasks of 250 ml containing 100 ml liquid BBM with the help of inoculation loop. All the flasks were placed onto rack maintained with light intensity and temperature. After incubation for 15-20 days, color of transparent medium turns green. Some of the flasks show web like growth of microalgal cells of different genera. Out of these multi algal colonies, Pasture pipptes were used to find out unialgal growth. In this method, three drops of sterilized BBM was kept on a glass slide. A little quantity of algal cells was sucked with the help of sucker and poured into first drop of sterilized medium. Again suck it gently and transfer into second and third drop in order to find unialgal cells. Finally transfer it gently to a conical flask containing 100 ml sterilized BBM. After the incubation period of 15-20 days unialgal cell growth appeared. Unialgal cells were then transferred to BG-11 medium.

Serial dilution method

Enrichment sample containing a large number of microalgal cells were isolated by serial dilution method. A dilution from 10^{-1}- 10^{-10} was made in sterilized test tubes containing 9 ml BBM. One ml of enrichment sample was transferred into each test tubes labeled with dilution factor from 10^{-1}- 10^{-10} respectively. Dilution factor 10^{-6}- 10^{-10} was used for spreading onto the freshly prepared agar plates. Petri dishes were placed onto rack maintained with light intensity and temperature. After incubation period of 15-20 days a number of colonies of green color was observed.

For the purpose of axenic strains of microalgal cultures, the method involving triple antibiotic solution was employed (Kaushik, 1987).

Growth and maintenance

Microalgal strains of different isolates were grown and maintained in chemically defined modified BG-11 medium (Stainer *et al*., 1971) at 30 ± 2^0C under a light intensity of 3.5 klux and light: dark cycle of (16: 8) hours. pH of the medium was maintained in the range of 8.0 - 8.2 for optimal growth of all isolates. Solid agar medium was prepared by using 15-18 g of purified agar per litre of BG-11 medium (1.5-1.8%) and autoclaved well before use for maintenance of the isolated cultures. After an incubation period of 14 days, all isolated cultures were subjected to streaking on agar plates of BG-11 medium for maintenance of pure colonies under standard cultural conditions. These colonies were picked up and inoculated in 250 mL flasks

containing 100 mL BG-11 medium and incubated till exponential phase of 14 days. Biomass was harvested on 14th day, 21st day and 28th day and processed for extraction of lipids. Extraction of lipids was carried out by Bligh and Dyer (1959) method.

Analysis

All isolated microalgalstrains were identified accordingly to keys given by Desikachary (1959) and Geitler (1932), for members of cyanophyceae and keys given by Komarek *et al.,* (1983), Hindak (1988) and Prescott (1961), for the members of chlorophyceae. Morphological studies were carried out on the specimens of exponential growth phase. All the measurements on fresh material were performed on each morphological variable by using a light microscope (Olympus, model: CX40RF200). Micrographs were taken by Olympus (CAMEDIA C-5060 WIDE ZOOM) digital compact camera**.** Isolates were identified at genus level on the basis of color of thallus, trichomes and heterocyst etc. All the isolates were grown in 3 replicates for analysis of biomass and lipid and mean of the three replicates was obtained to have mean estimates of biomass weight gravimetrically (g/l) and lipid %.

The lipid profile was done by using gas chromatography (model Agilent technologies 7890A GC system equipped with an Agilent 5975C inert XL EI/CI MSD triple axis detector). The compounds were profiled on a 30mX250µmX0.5µm HP-5MS column. Carrier gas was Helium at the rate of 0.7 mL per minute. The temperature conditions were 40 ^{0}C for one minute with a hold for a minute followed by 250 ^{0}C for ten minutes at the rate of 15 ^{0}C per minute and hold for ten minutes. The instrument contained split/splitters injector (445137A) with auto injection facility. Mass detector (Mass EI) temperature was 260 ^{0}C.

Results and Discussion

In this work four algal species (2 green algae and 2 cyanobacteria) were finally isolated and purely grown in culture room. Isolate number 11, 02, 06 and 09 were finalized, grown in pure culture and carried for biomass and lipid extraction process. Out of these four isolates *Chlorella* fw-12 was produced maximum biomass (Fig. 1) and lipid % (0.93±0.07g/l and 19.88±0.07% on 28th day). Extracted lipid was purified and profiled by GC-MS.

Lipids purification and characterization

Isolate *Chlorella*fw-12was selected as potent microalgae for biodiesel purpose, crude lipid extracted from this isolate was purified via trans-esterification, and fatty acid methyl esters (FAME) were produced. Purified lipid samples were processed and subjected to lipid characterization by GC-MS technique. The results are presented in table 4 and figures 4 and 5.

Microalgae *Chlorella* fw-12 was investigated for lipid profiling of chemical constituents. A number of chemical compounds comprising hydrocarbons, saturated and unsaturated fatty acids were detected. GC-MS profiling of this strain shows a total of 10 chemical compounds which comprised saturated hydrocarbons namely n-Decane, n-Undecane, n-Dodecane, n-Tridecane, n-Tetradecane. Other than hydrocarbons, a high percentage of fatty acids (61.44%) was also recorded, major fatty acid recorded were C19:1, C19:2 and C19:3, which were methyl hexadecanoate (palmitate) (30.20%), methyl 9, 12, 15-octadecatrienoate (linolenate) (11.03%), methyl octadecaenoate (elaidate) (8.74%), methyl 9, 12-octadecadienoate (linolenate) (7.51%) and methyl 5, 9, 12-octadecatrienoate (gamma-linolenoate) (2.07%) (Tables 5 and 6).

Table.1 District wise samples detail

Shamli	Baghpat	Garhmukhteshwar	Meerut	Nanital/ Ranikhat	Total
02	06	04	20	08	40

Table.2 Samples type (type of sampling site)

S.No.	Type of sample	No. of samples
1.	Fresh water samples	08
2.	Sewage water samples	20
3.	Pond water samples	05
4.	Lake water samples	04
5.	Cropland soil samples	01
5.	Shallow water samples	02

Total no. of samples= **40**

Table.3 Details of mono-algal colonies obtained in liquid/solid BBM medium

S. No.	Isolate No.	strain	division
1.	11.	*Nostoc* sp.	Cyanobacteria
2.	02.	*Chlorella* sp.	Green algae
3.	06.	*Chlorococcum* sp.	Green algae
4.	09.	*Ossilatoria* sp.	Cyanobacteria

Table.4 Estimates of dry biomass (g/l) of isolated microalgae

Isolate No.	Dry biomass (g/l)								
	14th day			21st day			28th day		
	Mean (g/l)±SE	S.D.	C.V. %	Mean (g/l)±SE	S.D.	C.V. %	Mean (g/l)±SE	S.D.	C.V. %
11	0.53±0.09	0.15	0.29	0.67±0.03	0.06	0.09	1.23±0.09	0.15	0.12
02	0.73±0.07	0.12	0.16	0.93±0.03	0.06	0.06	0.93±0.07	0.12	0.12
06	0.67±0.03	0.06	0.09	0.97±0.03	0.06	0.06	1.27±0.03	0.06	0.05
09	0.37±0.03	0.06	0.16	0.83±0.03	0.06	0.07	1.13±0.03	0.06	0.05

Table.5 Lipid % of isolated microalgae

Isolate No.	14th day			21st day			28th day		
	Lipid % SE(M) ±	S.D.	C.V. %	Lipid % SE(M) ±	S.D.	C.V. %	Lipid % SE(M) ±	S.D.	C.V. %
11	2.18±0.11	0.19	0.09	4.05±0.02	0.03	0.01	5.44±0.11	0.20	0.04
02	11.17±0.69	1.19	0.11	14.27±0.33	0.57	0.04	19.88±0.07	0.13	0.01
06	8.23±0.22	0.38	0.05	10.77±0.34	0.59	0.05	13.40±0.31	0.53	0.04
09	4.13±0.04	0.06	0.02	5.93±0.09	0.15	0.03	8.52±0.20	0.35	0.04

Fig.1 Biomass (g/l) of isolated microalgae

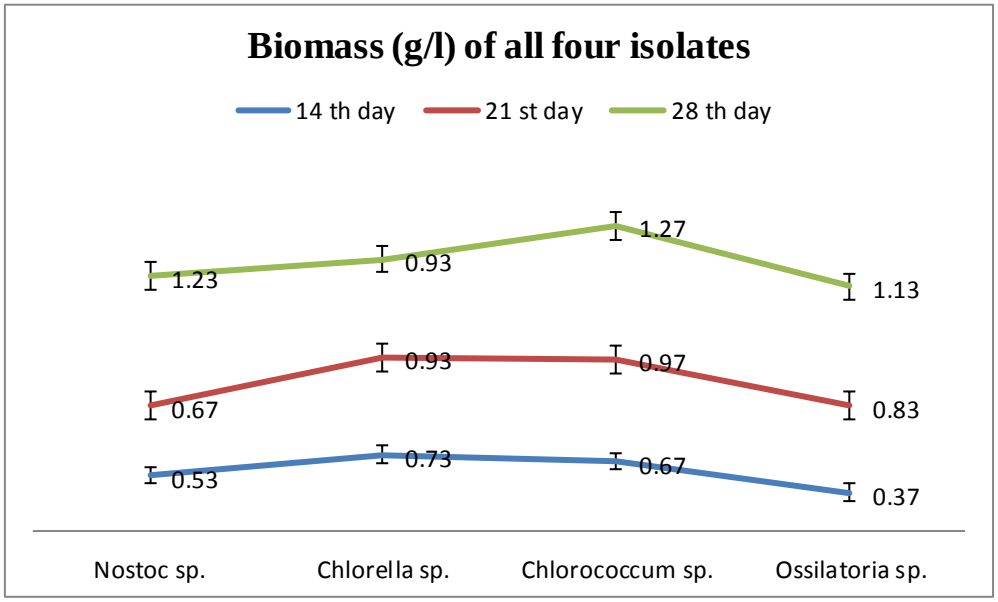
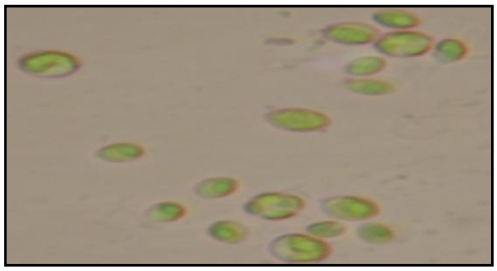


Table.6 Lipid profile of green microalgal strain *Chlorella* fw-12.

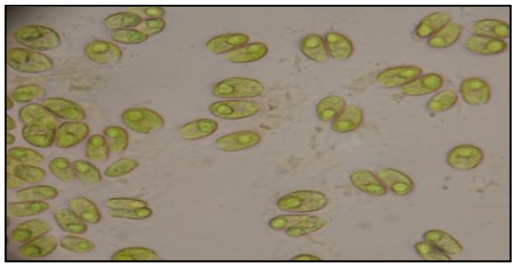
S. No.	Retention time (in minute)	Chemical name	Chemical formula	Shorthand designation	Molecular wt. (g/mol)	Area (in percentage)
1.	3.471	n- Decane	$C_{10}H_{22}$	C10:0	142.28 g/mol	3.71
2.	4.794	n- Undecane	$C_{11}H_{24}$	C11:0	156.30 g/mol	6.16
3.	6.212	n- Dodecane	$C_{12}H_{26}$	C12:0	170.33 g/mol	5.50
4.	7.637	n- Tridecane	$C_{13}H_{28}$	C13:0	184.36 g/mol	5.51
5	9.010	n- Tetradecane	$C_{14}H_{30}$	C14:0	198.38 g/mol	3.40
6.	15.276	Methyl hexadecanoate (Palmitate)	$C_{17}H_{34}O_2$	C17:0	270.45 g/mol	30.20
7.	16.832	Methyl 5, 9, 12-Octadecatrienoate (Gamma- linolenate)	$C_{19}H_{32}O_2$	C19:3	264.40 g/mol	2.07
8.	16.987	Methyl 9, 12-Octadecadienoate (Linolenate)	$C_{19}H_{34}O_2$	C19:2	294.47 g/mol	7.51
9	17.050	Methyl 9, 12, 15-Octadecatrienoate (Linolenate)	$C_{19}H_{32}O_2$	C19:3	292.45 g/mol	11.03
10	17.095	Methyl octadecaenoate (Elaidate)	$C_{19}H_{36}O_2$	C19:1	296.48 g/mol	8.74

Fig.2 Micrographs of isolated microalgae: (a) *Nostoc* sp.; (b) *Chlorella* sp.; (c) *Chlorococcum* sp.; (d) *Ossilatoria* sp.

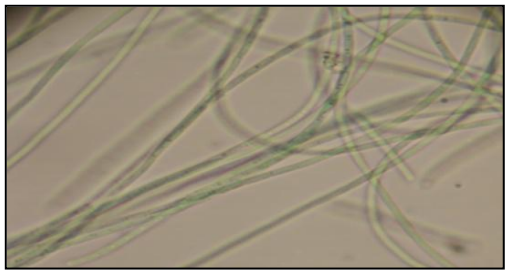
Nostoc sp.



Chlorella sp.



Chlorococcum sp.



Ossilatoria sp.

Fig.3 Lipid (%) of isolated microalgae

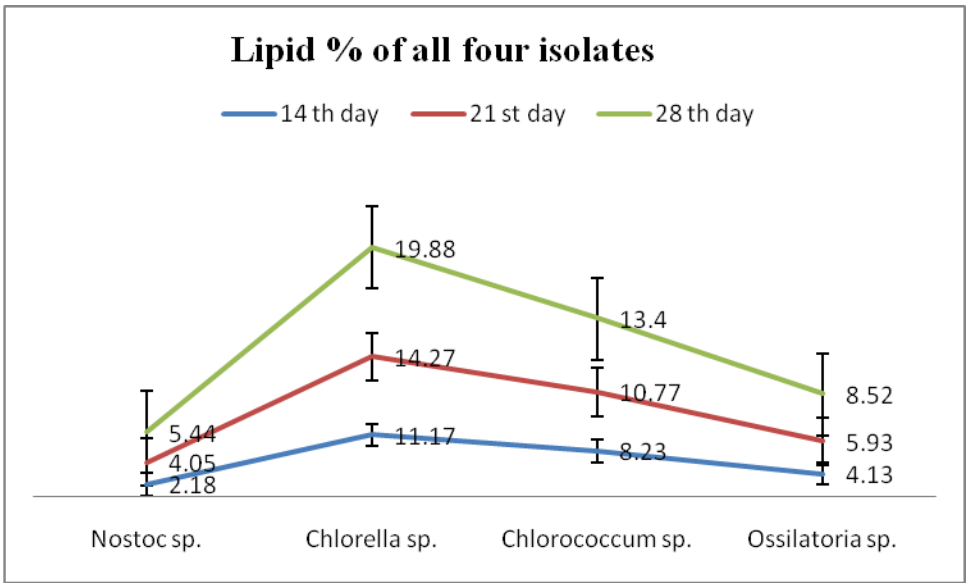
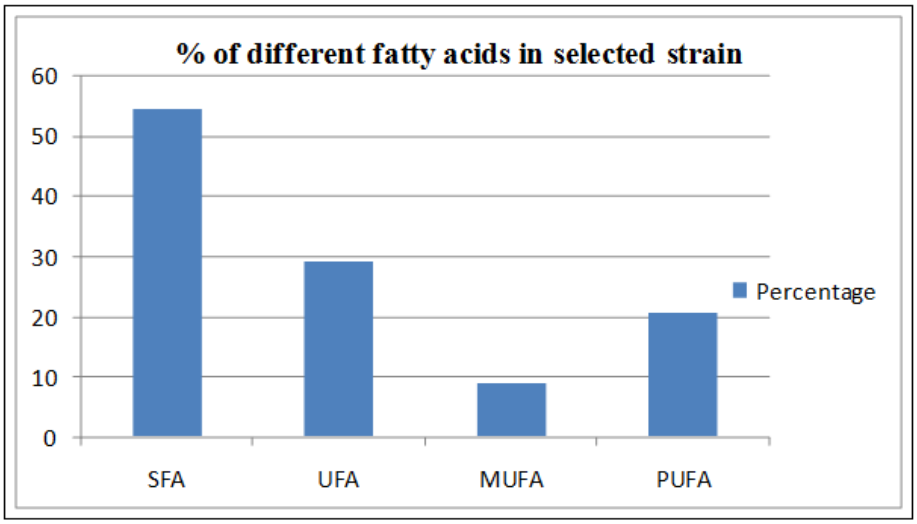
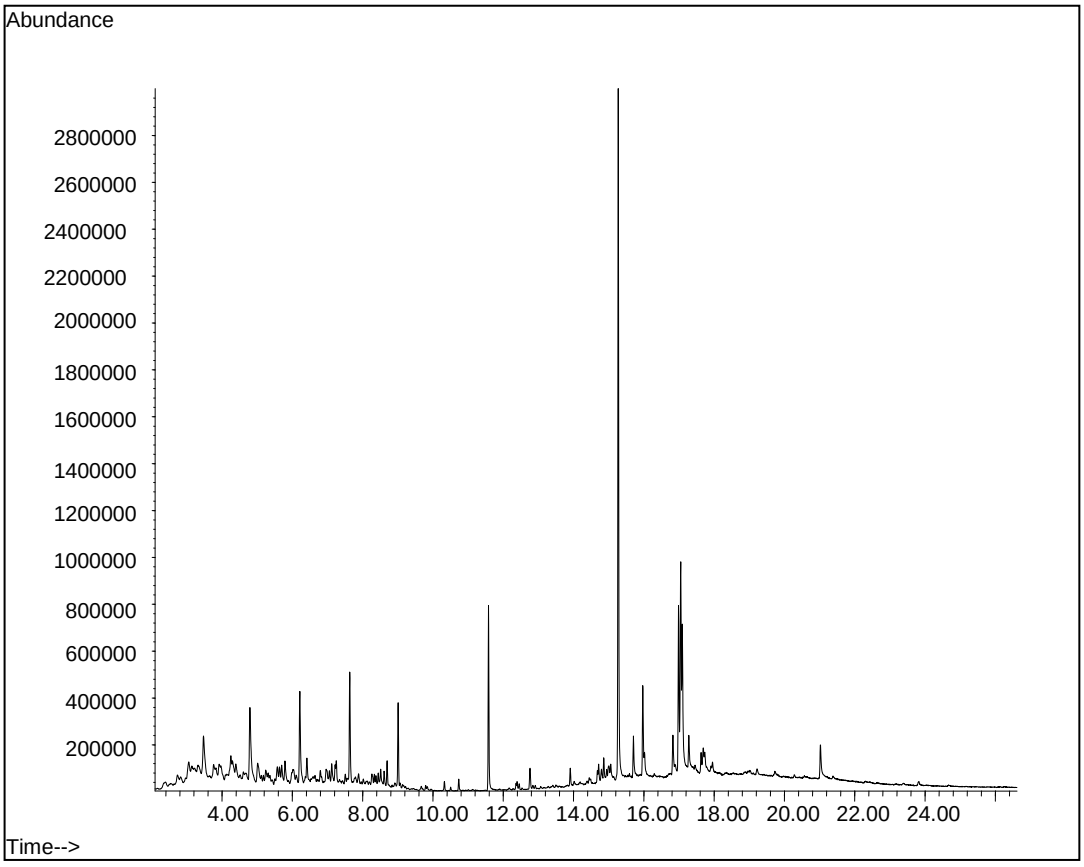


Fig.4 Percentage of different fatty acids in lipids



Abbreviations: SFA- Saturated fatty acids; **USFA-**Unsaturated fatty acids; **MUFA-** Monounsaturated fatty acids; **PUFA-**Polyunsaturated fatty acids

Fig.5 Chromatogram of lipids profile of selected second best green micro algal strain Chlorella fw-12



In support of our isolated potent microalgal isolate *Chlorella* fw-12, a recent study has shown that other green algae *Scenedesmus dimorphus* performed better than other isolates with respect to important growth parameters and lipid content of near about 30% of dry biomass (Gour *et al.,* (2016). Selvarajan *et al.,* (2015) reported 42.1% ± 2.5% (of dry biomass) of lipid in case of *Chlorella vulgaris.* Yang *et al.,* (2012) isolated four microalgal strains from fresh water bodies and lipid was extracted. Lipid percentage on dry biomass basis was observed to be 28% in *Chlorella sp.,* 51% in *Chlamydomona sreinhardtii*, 39% in *Monoraphidium dybowskii* and 43% in *Chlorella sp*.

In conclusion, in this study we discussed on ability of microalgae to use as biofuel in order to replace fossil fuels. Microalgae have its environmental benefits and the fact that, it is a renewable source of energy and can remove greenhouse gases (GHG) also from environment. Now it can be an achievable goal. Selection of oleaginous species out of large number of microalgal species was done. *Chlorella* species are high lipid accumulating green algae among all four isolates. Large amount of fatty acids found in lipid profile of our selected isolate *Chlorella* fw-12, which makes this, as more potential feedstock for biofuel purpose. Major fatty acid recorded were C19:1, C19:2 and C19:3, which were methyl hexadecanoate (palmitate) (30.20%), methyl 9, 12, 15-octadecatrienoate (linolenate) (11.03%), methyl octadecaenoate (elaidate) (8.74%), methyl 9, 12-octadecadienoate (linolenate) (7.51%) and methyl 5, 9, 12-octadecatrienoate (gamma-linolenoate) (2.07%).

Acknowledgement

Authors are very thankful to the University Grants Commission (UGC) New Delhi for financial support and Head, Department of Microbiology, C. C. S. University Meerut for providing all necessary facilities to carry out this research work.

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How to cite this article:

Parveen Kumar, Devendra Kumar, Priyanka Nehra, P.K. Sharma. 2017. Extraction and Profiling of Lipids of Isolated Strain *Chlorella* fw-12 by GC-MS. *Int.J.Curr.Microbiol.App.Sci.* 6(5): 1801-1810. doi: https://doi.org/10.20546/ijcmas.2017.605.196