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Isolation, lipid extraction and profile of *Scenedesmus* fw–28 strain by GC-MS

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Abstract

Isolation of four microalgae was carried out for the purpose of search of potential source of biodiesel. In the first step of investigation, 49 samples from aquatic sites of various districts of north India were collected and processed, resulting in a total of 4 microalgal strains viz. *Phormidium sp., Scenedesmus sp., Chlamydomonas sp.* and *Lyngbya sp.* were finally isolated and grown in pure culture in culture room. On the basis of observations, maximum lipid content was recorded as 43.97% in *Scenedesmus* fw-28 (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), especially the palmitate (palmitic acid) and linolenate (linolenic acid), because these two fatty acids are important for good quality of biodiesel as palmitic acid is a saturated fatty acid (SFA) while linolenic acid is a poly unsaturated fatty acid (PUFA). In *Scenedesmus* fw-28, major fatty acids recorded were C16 : 4, C17 : 0, C19 : 2, C19 : 3 and C22 : 1, which makes better quality of algal biodiesel. Thus, selected *Scenedesmus* fw-28 unicellular green algae would be a potent source of qualitative high quantity biodiesel.

Keywords: Algae isolation, Algae culture, microalgae lipids, transesterification, GC-MS, biodiesel

Introduction

In the present time, world population is facing two major crises of energy demand and global warming, which are relevance to more population growth and hike in industrialization, leads to more and more use of fossil fuels (Rupprecht, 2009). Hence now it is necessary to find out alternatives of replacement of use of fossil fuels. Microalgae could be suitable feedstock for the production of alternative source of energy. Microalgae are the photosynthetic microorganisms, which are attracting more attraction from government, researchers, local and international bodies. With some advantages biodiesel is better fuel than fossil fuels. In the transportation, use of biodiesel and bioethanol has increased immensely (Tabatabaei et al., 2011). Main advantage of biofuel is that it is nontoxic and also lowers greenhouse gases (GHG) (Demirbas, 2009). Microalgae have capability to grow under harsher environmental conditions and have least requirement for nutritional supply; can also be grown on unfertilized land so that they have no competition with agricultural land. Compared with other biomass-derived biofuels, algae-based biodiesel is receiving increasing attentions worldwide in the recent years. Major starting point for successful biodiesel production is the identification of suitable algal strains that possess high constituent amounts of total lipids, in general, but particularly neutral lipids. In several decades back, many investigations were made aiming screening of oil producing algae in Europe, north America, Australia, as well as in many other parts of the world (Sheehan et al., 2009; Carioca et al., 2009; Vijyaraghavan and Hemanathan, 2009; Rodolfi et al., 2009; Blackburn et al., 2009). Aquatic Species Program (ASP) is the good example for the purpose funded by the Department of Energy (DOE) of United States from 1978 to 1996 representing the most comprehensive research efforts to date on fuels from microalgae (Sheehan et al., 2009).

A number of species of microalgae, like *Scenedesmus obliquus*, *Botryococcus braunii*, *Nannochloropsis* sp., *Dunaliella primolecta*, *Chlorella* sp., and *Schizochytrium sp.,* contains large quantities of hydrocarbons and lipids. *Botryococcus braunii,* the colonial green microalgae, has the capability to produce a large number of hydrocarbons as compared to its biomass (Cohen et al., 1999; Chisti, 2007). High amount of oil percentage in microalgae species are reported. It reaches up to 80% and ranges from 20 to 50% (Chisti, 2007; Mata et al., 2010). The microalgae *Scenedesmus* and *Chlorella* have up to 50% lipids in their cells and *Botryococcus braunii* produces the highest oil content of about 80% (Powell and Hill, 2009). Even suffering from adverse and fluctuating environmental conditions, microalgae have the capability to accumulate higher contents of biochemical compounds for their survival like lipids, protein, starch and carotenoids (Lim et al., 2012). Microalgae produce a diversity of cellular lipids that can be used by the food and biofuel industry (Huerlimann et al., 2010). Carbon chain length C14 to C18 are most preferable for biodiesel production. Saturated fatty acids such as C14,

C16, and C18 and unsaturated fatty acids such as C16:1, C16:2, C18:1 and C18:2 are the most suitable for biodiesel quality (Schenk et al., 2008). This is due to the reason that polyunsaturated fatty acids with more than two double bonds (3 or 4) have lesser stability in storage (Knothe, 2006; Cohen, 1999). Transesterification process is the chemical conversion method to convert extracted lipids into biodiesel (Cohen, 1999). Transesterification process is very sensitive method because it depends on type and number of free fatty acids (FFAs), water content, molar ratio of alcohol to lipid, catalyst used temperature of reaction etc. (Sohpal and Singh, 2014).

Materials and Methods

Sampling and isolation of microalgae

Samples were collected in five stages viz. October, March, May, July and September, as these all stages include the probability of finding different species of microalgae due to season change (summer to rainy and rainy to winter), which favors the change in environmental conditions. Sites of collected samples were also arranged quite different in order to find diversified species of microalgae. All the water samples were collected in blue capped sample containers (around 100 ml of water) and stored at 4^0 C in refrigerator. Total 49 samples were collected from different locations (2 from Shamli, 6 from Baghpat, 6 from Garhmukteshwar, 27 from Meerut and 10 from Nainital)

Table: 1 Samples type (on the basis of type of aquatic body).

S. No.	Type of sample	No. of samples
1.	Fresh water samples	8
2.	Sewage water samples	26
3.	Pond water samples	7
4.	Lake water samples	4
5.	Running water samples	2
6.	Shallow water samples	2

Total no. of samples= **49**

Enrichment of samples

Bold Basal medium (Bischoff and Bold, 1963) was used for enrichment culture techniques to obtain unialgal colonies of microalgae in culture room at 3.5 klux light intensity with 16:8 light and dark period and 28 ± 2^0C temperature. pH 8.0 was maintained for optimal growth of cultures. Sterilized Bold Basal medium (BBM) was poured in 250 ml conical flasks and autoclaved. One ml of each water sample was inoculated in each flask and kept in culture room for a period of 10-15 days at optimal light intensity (3 klux) and temperature (28 ± 2^o C).

After incubation of 10-15 days, green colored growth of algal cells was observed which 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, and 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. BBM medium was used for the growth and maintenance of isolated microalgae strains. 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.

Serial Dilution Method

Serial dilution method was used for enrichment samples containing a large number of microalgal cells. 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 every 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 specific light intensities and temperatures. After incubation period of 10-15 days, a number of green colored colonies were observed.

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 were recultured on freshly prepared agar plates several times and also subcultured in liquid medium. After incubation for 10-15 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 given light intensities and temperatures. After incubation for 10-15 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 10-15 days, unialgal cell growth appeared. Unialgal cells were then transferred to BG-11 medium. Axenic strains of microalgal cultures were maintained using, the method of triple antibiotic solution (Kaushik, 1987).

Growth and maintenance

Microalgal strains of different isolates were grown and maintained in chemically defined modified BG-11 medium (Stainer *et al*., 1959) at 30 ± 2ºC 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 14^{th} day, 21^{st} day and 28^{th} day and processed for extraction of lipids following Bligh and Dyer (1959) method.

Analytical methods

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

Lipid profile was determined 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 30 m x250 µm x 0.5 µm 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

In this study, four isolates (number 01, 03, 04 and 17) were finally retained and grown as pure culture in culture room, subjected to recording of visual observations and eventually identified as four algal species, 2 as green microalgae and 2 as cyanobacteria (Table 2). For each of the four pure cultures, biomass was measured and lipids were extracted therefrom. The isolate *Scenedesmus* fw-28 produced maximum biomass and lipid % (3.57±0.12 g/l and 43.97% on 28^{th} day). Extracted lipid was purified and profiled by GC-MS.

Table: 2 Details of mono-algal colonies obtained in liquid/solid BBM medium.

S. No.	Isolate No.	Isolate code	Sampling site	Visual observations	Isolate name
1.	01	ACSW 57	Sewage water sample	Dark green jelly like growth appeared in flask/dots on petri plate	*Phormidium* sp.
2.	03	KFWS 28	Fresh water sample	Light green color liquid appeared in flask	*Scenedesmus* sp.
3.	04	VDSW 68	Sewage water sample	Very light color solution appeared in flask/growth appeared on petri plate	*Chlamydomonas* sp.
4.	17	VGSW 03	Sewage water sample	Yellowish green color appeared on petri plate	*Lyngbya* sp.

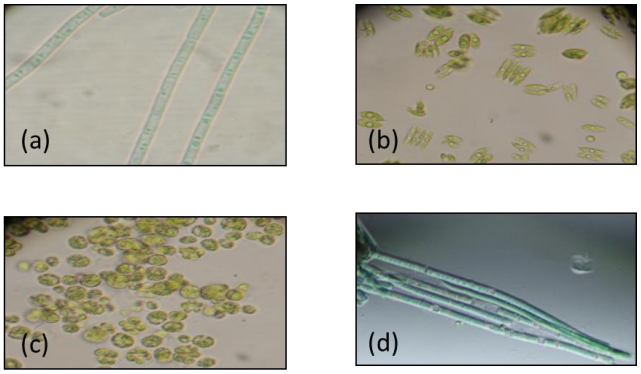

Fig: 1 Micrographs of isolated microalgae: (a) *Phormidium* sp., (b) *Scenedesmus* sp., (c) *Chlamydomonas* sp., (d) *Lyngbya* sp.

Table: 3 Estimates of dry biomass (g/l) of selected microalgae

	Dry biomass (g/l)								
Isolate	**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. %
Phormidium	0.63±0.3	0.06	0.09	1.07±0.03	0.06	0.05	1.47±0.15	0.25	0.17
Scenedesmus	1.43±0.03	0.06	0.04	2.87±0.07	0.12	0.04	3.57±0.12	0.21	0.06
Chlamydomonas	0.63±0.03	0.06	0.09	1.03±0.03	0.06	0.06	1.33±0.03	0.06	0.04
Lyngbya	0.77±0.03	0.06	0.08	1.17±0.03	0.06	0.05	1.67±0.03	0.06	0.03
ANOVA									
Interaction	**Column factor**			**Row factor**			**P value**		
Significant	Highly significant			Highly significant			<0.0001		

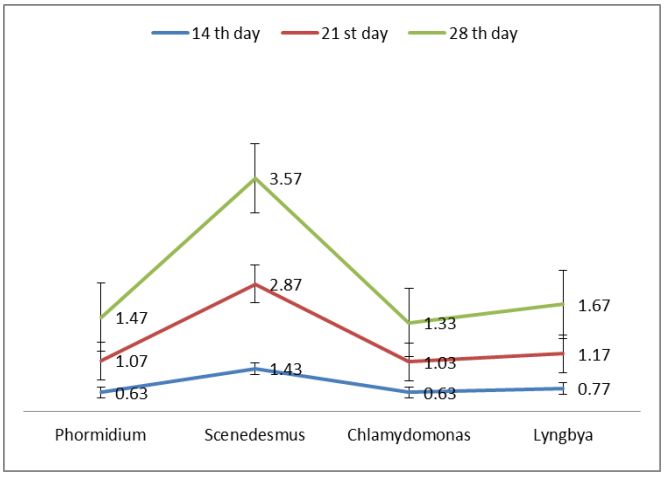

Fig: 2 Biomass (g/L) of isolated microalgae

Table: 4 Lipid (% of DW) of selected microalgae

Isolate	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. %
Phormidium	4.63±0.37	0.64	0.14	7.10±0.15	0.26	0.04	10.62±0.30	0.51	0.05
Scenedesmus	23.70±0.70	1.21	0.05	33.03±0.38	0.67	0.02	43.97±0.38	0.66	0.01
Chlamydomonas	5.12±0.32	0.55	0.11	8.40±0.17	0.30	0.04	12.96±0.13	0.22	0.02
Lyngbya	6.06±0.01	0.02	0.00	8.77±0.07	0.12	0.01	10.08±0.16	0.28	0.03
ANOVA									
Interaction	**Column factor**			**Row factor**			**P value**		
Significant	Very significant			Highly significant			<0.0001		

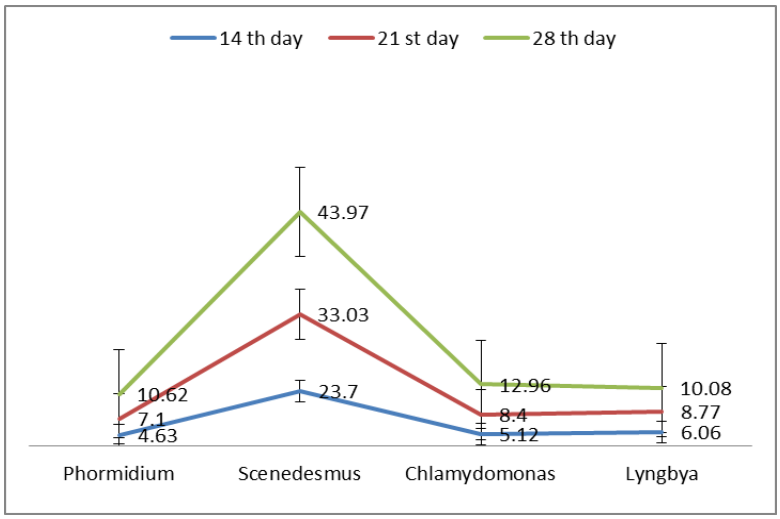

Fig: 3 Lipid (% of DW) of isolated microalgae

Lipids purification and characterization

On the basis of highest lipid content, isolate No. 03 which was identified as *Scenedesmus* fw-28 was 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 of *Scenedesmus* fw-28 were processed and subjected to lipid characterization by GC-MS technique. The results are presented in Table 5 and Figures 2 to 4.

A number of chemical compounds comprising hydrocarbons, saturated and unsaturated fatty acids were detected. The fatty acid profile separated 26 components containing hydrocarbons (about 1.80%), fatty acids (about 50.03%). Hydrocarbons included n-Decane, n-Undecane, n-Dodecane, n-Tridecane and n-Tetradecane. Although, overall less FAME was recorded, PUFA part of FAME was higher than SFA and hydrocarbons. Mainly C16:4, C17:0, C19:2, C19:3 and C22:1 was recorded. Major fatty acids were methyl hexadecanoate (palmitate) (15.52%), methyl 9, 12, 15-octadecatrienoate (linolenate) (11.57%), linoleic acid methyl ester (6.37%), methyl 5, 9, 12-octadecatrienoate (3.53%), methyl methacryloctadecyl ester (2.28%), methyl cis-8, 11, 14-eicosatrienoate (2.19%), methyl 9, 12, 15-octadecatrienoate (linolenate) (1.50%) and methyl 4, 7, 10, 13-hexadecadienoate (1.22%).

Table: 5 Profile of purified lipids of green microalgae *Scenedesmus* fw-28

S. No.	Chemical name	Shorthand designation	Area (in percentage)
1.	n- Decane	C10:0	0.25
2.	n- Undecane	C11:0	0.42
3.	n- Dodecane	C12:0	0.39
4.	n- Tridecane	C13:0	0.36
5	n- Tetradecane	C14:0	0.21
6.	9,11- Dimethyl tetra cyclotetradecane	C14:0	0.17
7.	Methyl tetra decanoate (Mysteric acid methyl ester)	C15:0	0.20
8.	Methyl dodecanoate (Laurate) (Lauric acid methyl ester)	C13:0	0.08
9	Methyl pentadecanoate (Pentadecanoic acid)	C16:0	0.13
10	Methyl 4, 7, 10- hexadecatrienoate (Gamolenate)	C17:3	0.70
11.	Methyl 4, 7, 10,13- hexadecadienoate	C16:4	1.22
12.	Methyl 7, 10- hexadecadienoate	C16:2	0.59
13.	Methyl 9- hexadecanoate (Palmitoleate)	C17:1	0.58
14.	Methyl hexadecanoate (Palmitate)	C17:0	15.52
15.	Methyl heptadecanoate (Margarate)	C18:0	0.60
16.	Sesquisabinene	C15:2	0.98
17.	Methyl 5, 9, 12- Octadecatrienoate	C19:3	3.53
18.	Methyl 9, 12, 15- Octadecatrienoate (Methyl linolenate)	C19:3	1.50
19.	Methyl 9, 12- Octadecadienoate (Linoleic acid methyl ester)	C19:2	6.37
20.	Methyl 9, 12, 15- Octadecatrienoate (Linolenate)	C19:3	11.57
21.	Methyl Octadecanoate (Stearate)	C19:0	1.28
22	Methyl 9, 12 Octadecadienoate (Linolenate)	C19:2	0.90
23.	Methyl cis- 8,11,14-Eicosatrienoate	C21:3	2.19
24	Methyl 9,12, 15- octadecatrienoate	C19:3	0.42
25.	Methyl methacryloctadecyl ester	C22:1	2.28
26.	Methyl tetracosanoate (Lignocerate)	C25:0	0.37

Discussion

In support of our isolated potent microalgal isolate *Scenedesmus* fw-28, a recent study has shown that two of the isolates of *Scenedesmus dimorphus* performed better than other isolates with respect to important growth parameters and lipid content of~ 30% of dry biomass (Gour et al., 2016). The authors also found that *Scenedesmus dimorphus* was more suitable candidate for biodiesel feedstock as it also contained five important fatty acids that of oleic acid, saturated fatty acids (53.04%), monounsaturated fatty acids (MUFA) (23.81%) and polyunsaturated fatty acids (PUFA) (19.69 %) Gour et al., (2016). Hoekman et al., (2012) and Knothe (2006) studied biodiesel standards and its properties and suggested that good quality biodiesel should contain relatively low concentrations of long chain saturated FAME as well as polyunsaturated FAME for low temperature operability and oxidative stability. Ma et al., (2014) studied the fatty acid profile of 9 *Nannochloropsis* sp. and suggested that microalgal biodiesel should not be only high lipid and TAGs producing, but also suitable composition of fatty acids should be there like saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs). In case of our isolate, saturated fatty acids were 19.98%, unsaturated fatty acids were 32.83%, monounsaturated fatty acids were 2.86% and polyunsaturated fatty acids 29.97% were recorded. ANOVA test carried for growth period versus isolates showed significant results in columns factor as well as row factor. *Scenedesmus* was kept as control for both biomass (g/L on dry wt. basis) and lipid % of dry weight (DW).

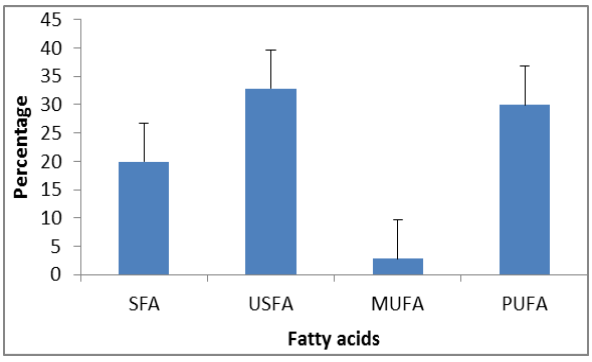

Fig: 4 Percentage of different fatty acids in lipids

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

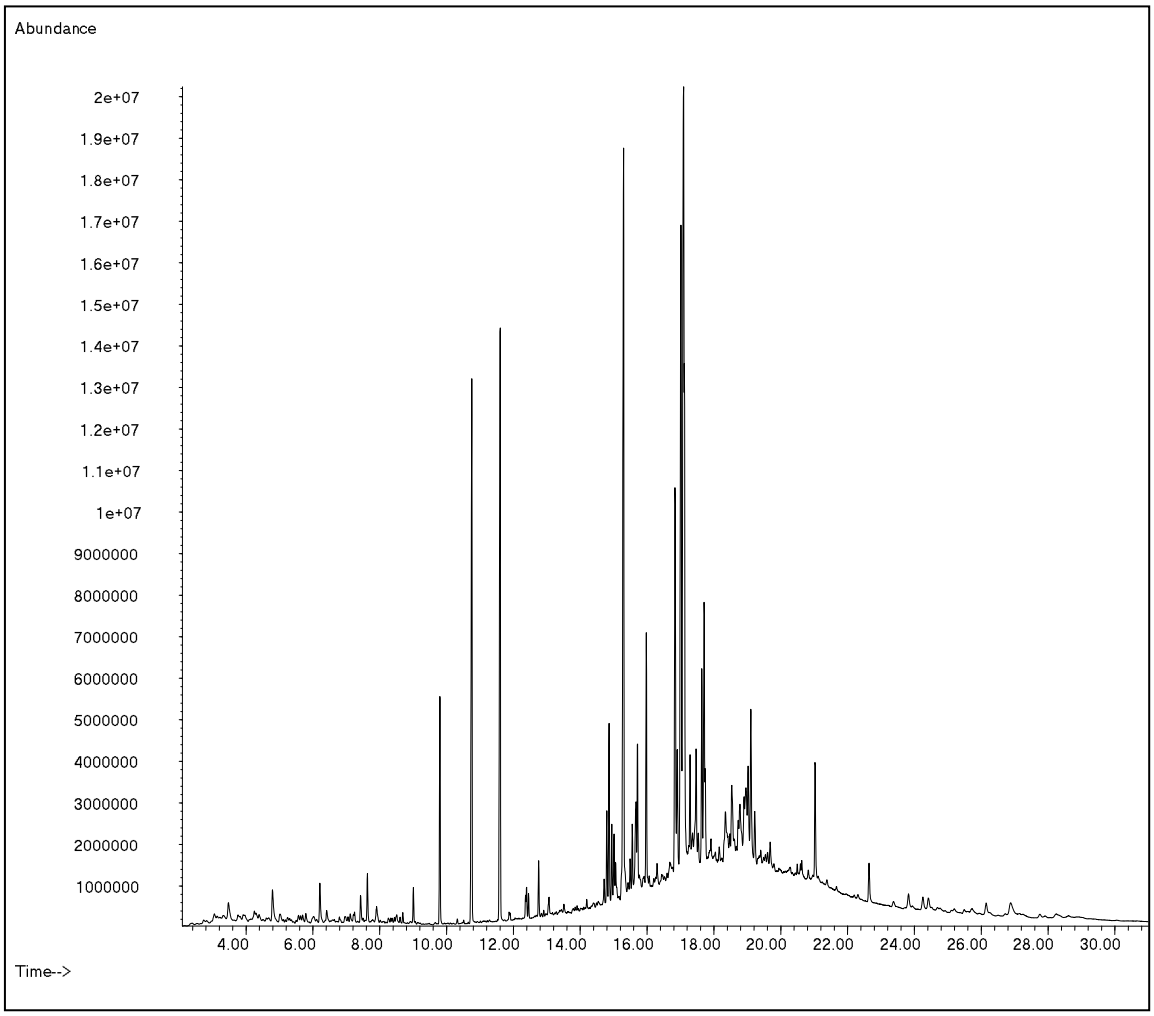

Fig: 5 Chromatogram of lipids profile of selected microalgae *Scenedesmus* fw-28

Conclusion

This study demonstrated ability and suitability of selected microalgal strain to use as biofuel as a potential and renewable alternative source to the fossil fuels which are highly pressed by the ever increasing global demand of fuels. Not only the microalgae have higher lipids content (high yield of biofuel), they have environmental benefits, they are the renewable source of energy, they give higher production in less inputs and area and can also remove greenhouse gases (GHG) from environment. We have identified an oleaginous species (*Scenedesmus* fw-28) out of large number of microalgal species, which are high lipid accumulating green algae. Approximately 50% of its dry biomass is lipid. Large amount fatty acids found in lipid profile of this isolate makes it as more potential feedstock for biofuel purpose. Major fatty acids found in this strain were methyl hexadecanoate (palmitate) (15.52%), methyl 9, 12, 15-octadecatrienoate (linolenate) (11.57%), linoleic acid methyl ester (6.37%), methyl 5, 9, 12-octadecatrienoate (3.53%), methyl methacryloctadecyl ester (2.28%), methyl cis-8, 11, 14-eicosatrienoate (2.19%), methyl 9, 12, 15-octadecatrienoate (linolenate) (1.50%) and methyl 4, 7, 10, 13-hexadecadienoate (1.22%).

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Conflict of interest

The authors declare that, they have no conflict of interest in the publication.

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