Chlorella vulgaris, a fortune for the Biodiesel and Fatty Acid Production

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ABSTRACT

The world is in a combat with pollution and depleting natural resource and there is a need to protect our resources by replacing non-renewable with renewable sources. The main objective of this study is to extract biodiesel from dense growing algae that obstructs the aquatic life and clogs water by its vast growth. In this study, *Chlorella vulgaris*, a single celled (2 to 10µm diameter); flagellate organism having huge importance in aquaculture and environmental sciences is selected. It is commercial because of its rapid reproduction. Utilizing of accumulated lipids as biodiesel is an incredible alternative to fossil fuels which avoids the dependence of non-renewable resources. The algae have been cultivated in three different media for maximum optimization selected as TAP, N8 and Bg11. This is quantitatively analysed for the amount of proteins and carbohydrates by UV-visible spectrophotometer and was utilised for the extraction of PUFA's (poly unsaturated fatty acids) and production of biodiesel. Based on the retention time by chromatographic technique the highest percentage of PUFA'S was estimated as 33.23% in Bg11 media. The biodiesel was produced by transesterification where the Bg11 and TAP media showed highest viscosity values.

Keywords: PUFA, *Chlorella vulgaris*, Transesterification, Biodiesel, Lipid

1. INTRODUCTION

Microalgae are small-sized organisms found in fresh and saline waters, in both benthic and littoral habitats, and also throughout the ocean waters as phytoplankton, while the larger macro algae (seaweeds) occupy the littoral zone [1,2]. Microalgae range in size from about 5 µm (*Chlorella*) to more than 100 µm (*spirulina*) [3]. The immense chemical diversity of microalgae provides numerous applications in the food, feed and pharmaceutical industries [4].

1.1 *Chlorella vulgaris*

The first photosynthetic microbe to be isolated and grown in pure culture was the freshwater microalgae *Chlorella vulgaris*. It is spherical, unicellular eukaryotic green algae that present a thick cell wall (100–200 nm) as its main characteristic. This cell wall provides mechanical and chemical protection, and its relation to heavy metals resistance is mostly used microorganisms for waste treatment. It has a size of 2-8 microns which makes it possible to be observed under a microscope. *Chlorella* is largely used for nutritional and therapeutic purposes [5].

1.2 Lipids and fatty acids

Lipids are the substance of biological origin that are soluble inorganic solvents such as chloroform and ethanol and are sparingly soluble in water. Lipids get converted to glycerol and three fatty acids by esterification reaction [6]. Fatty acids are long chain carboxylic acids (typically 16 or more carbon atoms) which may or may not contain carbon-carbon double bonds [7]. The number of carbon atoms is mostly even numbered and usually unbranched. Oleic acid is the most abundant fatty acid in nature. Fatty acids have a wide range of commercial applications like production of food products, soaps, detergents, and cosmetics. Fatty acids, particularly omega-3 fatty acids, are also commonly sold as dietary supplements.

1.3 Biodiesel

Modern biodiesel fuel is an outcome of converting vegetable oils into compounds called fatty acid methyl esters. Biodiesel is a renewable, biodegradable fuel manufactured domestically from vegetable oils, animal fats, or recycled restaurant grease [8]. Biodiesel is compatible with existing diesel engines and distribution infrastructure. However, it is usually blended with Petro diesel (10%-30%) since most engines cannot run on pure Biodiesel without modification. Biodiesel blends can also be used as heating oil [9][10].

2. MATERIALS AND METHODS

2.1 Collection and Cultivation of Algae:

Chlorella vulgaris was purchased from Culture Collection of Algae at JNTU Hyderabad Telangana, India. The cells were maintained in three different media's viz. Bg-11, N-8, TAP [11]. The algae were incubated for 18 days and cell count is 1.25 x 10^8cells/ml [12]. The cells were grown in 250ml Erlenmeyer flasks on an orbital shaker set at 150 rpm at room temperature. Optimization conditions for the growth are 18 hrs light using fluorescent lamps, temperature maintained at 37^0C. The pH of the media is 7.0. Disinfected air was provided by an air motor apparatus (Hailee V-35) as aeration at the rate of 136 cm^3 min-1 for 7hrs. The lipids were extracted from inoculated algae by using different soxhlet and solvent extractions. Biodiesel was separation by using different solvents.

2.2 Total protein estimation:

To a volume of algae growth, double the volume of lysis buffer (31.5g/l TrisHCl, 3.7g/l EDTA, and 29g/l NaCl, 77g/l Ammonium Acetate, 1% SDS) was added and incubated at 45^0C for half an hour. To the supernatant, equal volume of Reagent-I [48:1:1 of 2% sodium carbonate in 0.1 N NaOH: 1% sodium potassium tatarate: 0.5% $CuSO_4.5H_2O$] added to the collected supernatant, equal volume of Reagent-II was added to the above mixture and

incubated in dark place for 20 min. Finally, OD values were taken at 660nmusing UV-Vis-Spectrophotometer.

2.3 Lipid Extraction:

Lipid extraction was done by utilizing Bligh and Dyer with few alterations. The algae sample was soxhlet extracted with hexane: isopropanol (3:2) solvent with temperature maintained at 80^0C for about 70 cycles. Later, the extract was isolated and treated with a mixture of chloroform, methanol and water (5:5:2). Finally, the lower chloroform layer was collected to extract the lipids and dried in a water bath at 80°C for 10 min. The extracted lipids were allowed to PUFA and Biodiesel production.

2.4 Fatty Acid estimation:

The fatty acid composition of the oils was determined by gas chromatography (GC) as fatty acid methyl esters (FAME), IUPAC (1987). Chromatographic analysis was performed in Shimadzu GC-2010 chromatograph using a DB-23 fused silica capillary column (30m, 0.25mm), 0.25 µm film thicknesses. Helium was used as a carrier gas which is operated at a flow rate of 1.00ml/min. The column temperature was isothermal at 190ºC where in the injector and detector temperatures were 230ºC and 240ºC, respectively. FAME was identified by comparison of their retention times with those of the reference standards.

The extracted lipids were treated with 0.4gm NaOH in 20ml methanol, incubated for 10 min at 80°C. 1:5 ratio of the boron trifluoride and extract were added and refluxed for 10 min at 80°C (mixing). After cooling, 700µl of 1 bromo-tetra-decane and 300µl of n-heptane, 1 ml saturated NaCl solution was added and mixed well. The upper fatty acid layer was carefully collected and analysed by Gas chromatography.

2.5 Transesterification:

Trans-esterification is the path toward exchanging the normal social occasion R″ of an ester with the common get together R′ of an alcohol [23]. These reactions are often catalyzed by the development of an acid or base impetus. The reaction can in like manner be refined with the help of synthetic compounds (biocatalysts) particularly lipases Strong acids catalyse the reaction by giving a proton to the carbonyl social event, therefore making it an all the more ground-breaking electrophile, however bases catalyze the reaction by removing a proton from the alcohol, subsequently making it more nucleophilic. Esters with greater alkoxy social occasions can be created utilizing methyl or ethyl esters in high flawlessness by warming the mix of ester, Acid/base, and broad alcohol and vanishing the little alcohol to drive amicability. Through Transesterification, biodiesel and PUFA's were extracted. Transesterification is a chemical reaction used for the conversion of triglycerides (fatty acids), contained in oils used as biodiesel. Biodiesel produced by the process of Transesterification has a much layer viscosity, making it capable of replacing petroleum diesel in diesel engines.

2.6 Viscometer:

For estimation of consistency, the temperature of substrate being estimated must be precisely controlled since slight temperatures may prompt change stamped changes of thickness. The single-step technique was completed with 4.5 grams of dried green growth raced into the

vessel, in which the impetus, methanol and were added to permit the extraction of lipid and the trans-esterification to happen all the while. To contrast its execution and the customary two-advance biodiesel creation, a chose condition was utilized for the two strategies, which was transesterified at 14% of impetus, 6:1 (v/w) proportion of liquor to biomass and the response time of 2 h. Two layers will be shaped. In the base layer glycerols will be available and upper layer biodiesel. The biodiesel was sifted through by a No. 5 Whatman's paper and store it for advance GC examination.

3. RESULTS:

3.1 Cultivation of algae:

Figure 1 Cultivation of algae

Chlorella vulgaries was cultivated in three different media's viz. BG-11, N-8 and TAP medium (Figure 1). The growth was observed in 19 days under different physico-chemical conditions

3.2 Total estimation of carbohydrates:

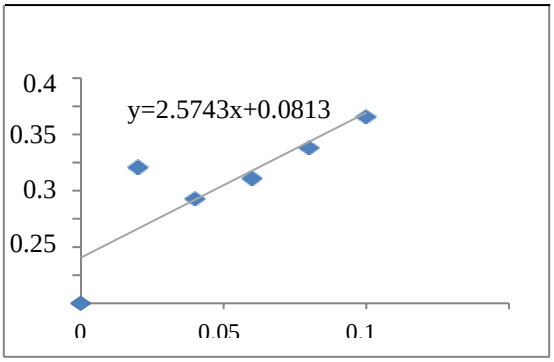

Figure 2: Standard graph for carbohydrates

The D-glucose was taken as standard for estimating carbohydrates where the x-axis indicates concentration of D-glucose and y-axis indicates as absorbance values at 620nm.

The algae and Hydrochloric acid were taken for which 1M sodium carbonate, 5% phenol and 96% H_2SO_4 were added and incubated in dark place for 20min. Carbohydrates were estimated by using Anthrone's test with D-Glucose as standard and OD values were taken at 490nm using UV-visible spectrophotometer (Table 1).

Table 1 Total Estimation of Carbohydrates

S.no	Conc. Of D-glucose (mg/ml)	Vol of D-glucose (ml)	Volume of Distilled water (ml)	Volume of Reagent-I (ml)	Incubation time 20 mins	Volume of Reagent- II (ml)	Incubation Time 20 mins	OD at 490nm
1	0.00	0.00	0.10	1.00		3.00		0.000
2.	0.02	0.02	0.08	1.00		3.00		0.044
3.	0.04	0.04	0.06	1.00		3.00		0.060
4.	0.06	0.06	0.04	1.00		3.00		0.065
5.	0.08	0.08	0.02	1.00		3.00		0.069
6.	0.10	0.10	0.00	1.00		3.00		0.082
UNKNOWN D1	0.422	0.00	0.18	1.00		3.00		0.11
D3	0.415	0.02	0.16	1.00		3.00		0.10
D5	0.376	0.04	0.14	1.00		3.00		0.09
D7	0.361	0.06	0.12	1.00		3.00		0.08
D9	0.336	0.08	0.10	1.00		3.00		0.08
D11	0.330	0.10	0.08	1.00		3.00		0.08
D13	0.310	0.12	0.06	1.00		3.00		0.07
D15	0.302	0.14	0.04	1.00		3.00		0.07
D17	0.295	0.16	0.02	1.00		3.00		0.07
D19	0.158	0.18	0.00	1.00		3.00		0.03

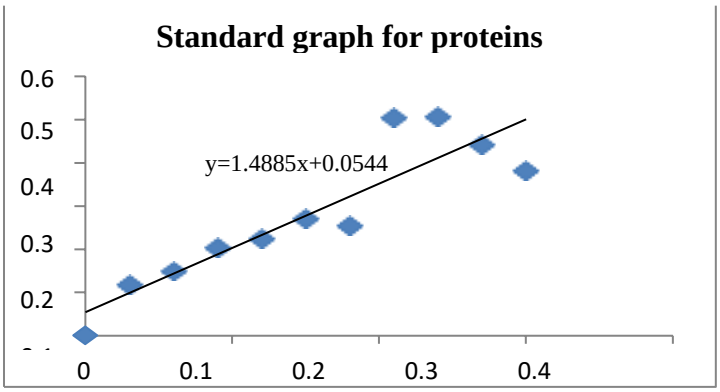

Figure 3: Standard graph for proteins

3.3 Estimation of total proteins:

The BSA was taken as standard for estimating proteins where the x-axis indicates as concentration of BSA and y-axis indicates as absorbance at 595nm.

Table 2 Total Estimation of Proteins

S.no	Conc. of BSA (mg/ml)	Vol of BSA (ml)	Volume of Distilled water (ml)	Volume of Reagent-I (ml)	Incubation Time 5 mins	Volume of Reagent-II (ml)	Incubation Time 20 min	OD at 660nm
1	0.00	0.00	0.10	4.5		0.5		0.000
2.	0.02	0.02	0.08	4.5		0.5		0.198
3.	0.04	0.04	0.06	4.5		0.5		0.246
4.	0.06	0.06	0.04	4.5		0.5		1.246
5.	0.08	0.08	0.02	4.5		0.5		1.011
6.	0.10	0.10	0.00	4.5		0.5		0.865
UNKNOWN D1	0.060	0.00	0.18	4.5		0.5		0.03
D3	0.068	0.02	0.16	4.5		0.5		0.04
D5	0.087	0.04	0.14	4.5		0.5		0.05
D7	0.125	0.06	0.12	4.5		0.5		0.07
D9	0.142	0.08	0.10	4.5		0.5		0.08
D11	0.252	0.10	0.08	4.5		0.5		0.14
D13	0.356	0.12	0.06	4.5		0.5		0.20
D15	0.377	0.14	0.04	4.5		0.5		0.21
D17	0.400	0.16	0.02	4.5		0.5		0.22
D19	0.455	0.18	0.00	4.5		0.5		0.25

The algae growth was treated with lysis buffer and incubated at 45^0C for half an hour. To the supernatant, equal amount of Reagent-I and Reagent-II was added and incubated in dark place for 20 min. OD values were taken at 660nm by using UV-Vis-Spectrophotometer (Table 1)

3.4 Extraction of lipids:

Table 3: Lipids values at final day.

Lipids	Volume of lipids	% of Lipids obtained
TAP media	68ml	13.6%
Bg11 media	60ml	12%
N8 media	57ml	11.14%

Lipid extraction was done by utilizing Bligh and Dyer with few alterations and observed different lipid volumes extracted from different Medias.

3.5 Fatty acids estimation:

Table 4: Fatty acids values on the final day.

Fatty acids	Volume of fatty acids	% of fatty acids obtained
TAP media	34ml	6.8%
Bg11	30ml	6%
N8	28ml	5.7%

The extracted lipids were treated by using different chemicals and after cooling, 700µl of 1 bromo-tetra-decane and 300µl of n-heptane, 1 ml saturated NaCl solution was added and mixed well. The upper fatty acid layer was carefully collected and analyzed by Gas chromatography.

3.5.1 Tap media:

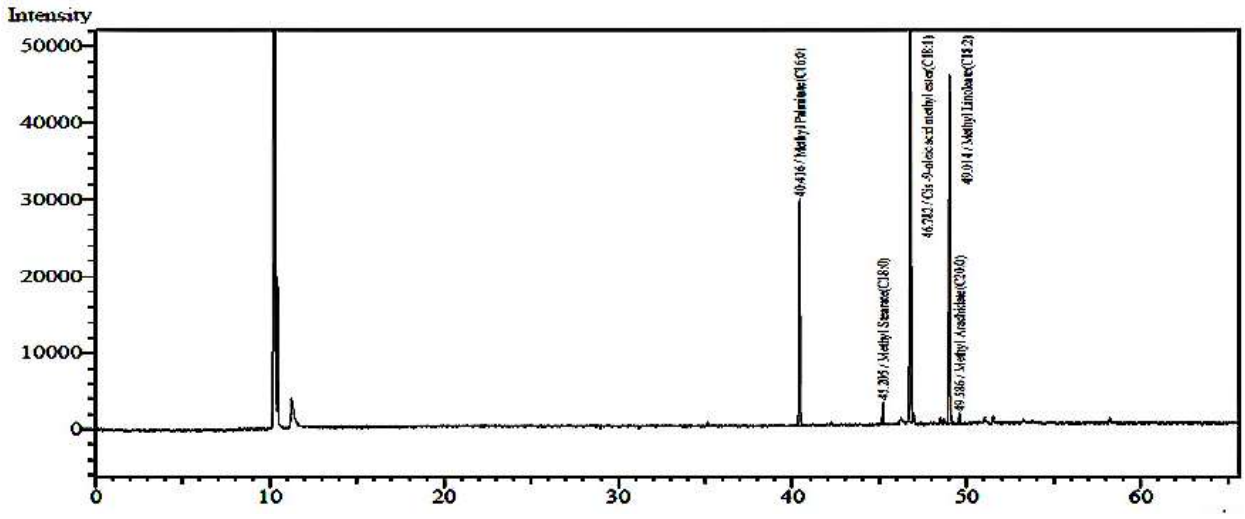

Figure 4: TAP Media

The fatty acid compounds present in the TAP Media with their area, height and area percentages are listed below in (Table 4)

Table 5: TAP Media

Peak	Ret. time	Compound Name	Area	Height	Area %
1	40.416	Methyl palmitate (C16:0)	134863	29483	21.1865
2	45.205	Methyl stearate (C18:0)	12858	2849	2.0199
3	46.782	Cis-9-oleic acid methyl ester (C18:1)	273757	56902	43.0061
4	49.014	Methyl linoleate (C18:2)	209319	45521	32.8832
5	49.586	Methyl Arachidate (C20:0)	5756	1382	0.9043
Total			636553	136137	100.000

The GC analysis of fatty acids in TAP media has recorded a polyunsaturated fatty acid- Methyl Linoleate (C18:2) with a concentration of 32.88%, Monounsaturated fatty acid- Cis-9-oleic acid methyl ester (C18:1) with a concentration of 43.01%, saturated fatty acids- Methyl Palmitate (C16.0), Methyl Stearate (C18.0), Methyl arachidate (C20:0) with the concentration of 22.09%

3.5.2 N_8 Media:

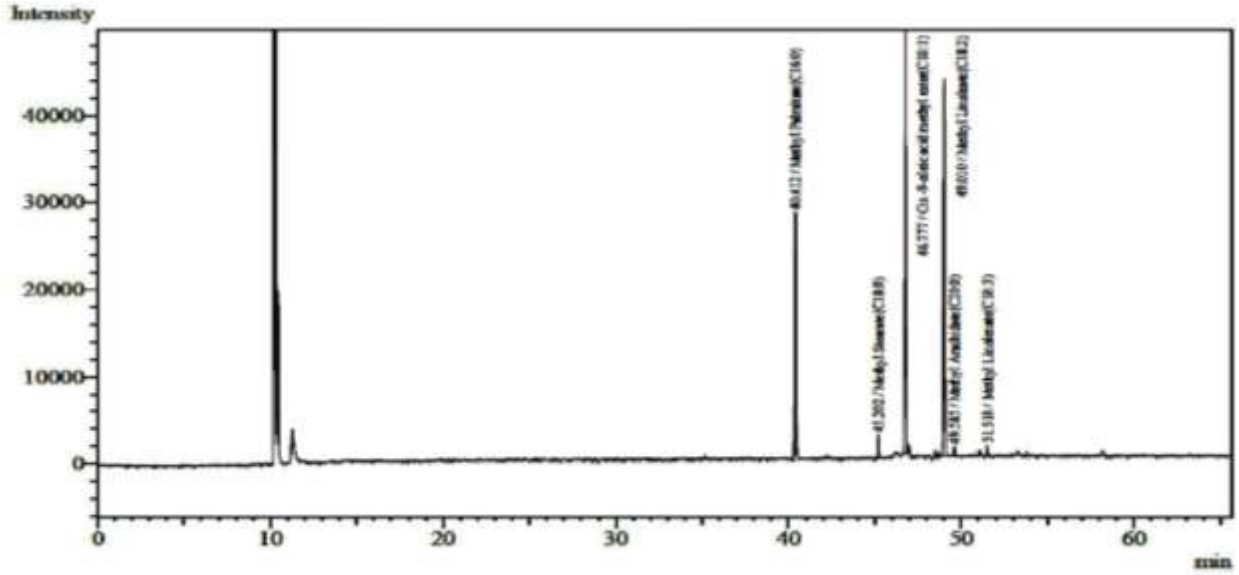

Figure 5: N_8 Media

The fatty acid compounds present in the N8 Media with their area, height and area percentages are listed below in (Table 5)

Table 6: N_8 media

Peak	Ret. time	Compound Name	Area	Height	Area %
1	40.412	Methyl palmitate (C16: 0)	133353	28324	21.7303
2	45.202	Methyl stearate (C18:0)	12419	2630	2.0237
3	46.777	Cis-9-oleic acid methyl ester (C18:1)	260463	54374	42.4434
4	49.010	Methyl linoleate (C18:2)	197208	43222	32.1358
5	49.585	Methyl Arachidate (C20:0)	4129	1078	0.6729
6	51.518	Methyl Linolenate (C18:3)	6099	1279	0.9939
Total			613671	130907	100.000

The GC analysis of fatty acids in N_8 media has recorded a polyunsaturated fatty acid- Methyl Linoleate (C18:2) and Methyl Linolenate (C18:3) with a concentration of 33.12%, Monounsaturated fatty acid- Cis-9-oleic acid methyl ester (C18:1) with a concentration of 42.44%, saturated fatty acids-Methyl Palmitate (C16:0), Methyl Stearate (C18:0), Methyl arachidate (C20:0) with the concentration of 23.19%.

3.5.3 Bg 11 Media:

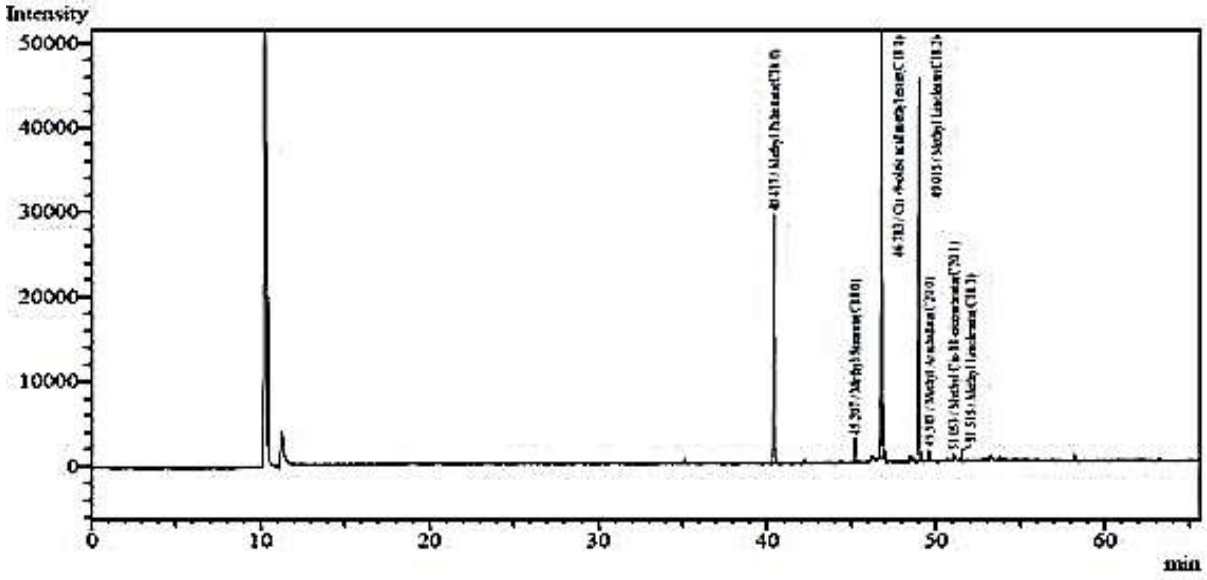

Figure 6: Bg11 Media

The fatty acid compounds present in the Bg11 Media with their area, height and area percentages are listed below in (Table 6)

Table 7: Bg11 Media

Peak	Ret. time	Compound name	Area	Height	Area %
1	40.417	Methyl Palmitate (C16:0)	137641	29428	21.2586
2	45.207	Methyl Stearate (C18:0)	13016	2884	2.0104
3	46.783	Cis-9-oleic acid methyl ester (C18:1)	272443	56663	42.0787
4	49.015	Methyl linoleate (C18:2)	209095	45101	32.2946
5	49.585	Methyl Arachidate (C20:0)	6185	1394	0.9553
6	51.053	Methyl cis-11-eicosanate (C20:1)	3002	710	0.4637
7	51.515	Methyl Linolenate (C18:3)	6078	1266	0.9388
Total			647460	137446	100.000

The GC analysis of fatty acids in BG11 media has recorded a polyunsaturated fatty acid-Methyl Linoleate (C18:2) and Methyl Linolenate (C18:3) with a concentration of 33.23% , Monounsaturated fatty acid- Cis-9-oleic acid methyl ester(C18:1) and Methyl csi-11-eicosanate (C20:1) with a concentration of 42.54% ,saturated fatty acids-Methyl Palmitate(C16:0), Methyl Stearate(C18:0), Methyl arachidate(C20:0) with the concentration of 24.05%.

Table 8: Fatty acid profile

s.no	TEST PARAMETER	Batch no/ lot no	Test method/ equipment name	Unit of measurement	Test result
Fatty acid profile					
1.	Saturated fatty acids	TAP	AQAC996.06-19thEdn	%	22.09
	Mono unsaturated fatty acids			%	43.01
	Poly unsaturated fatty acids			%	32.88
	Trans fatty acids			%	<0.2
2.	Saturated fatty acids	N8		%	23.19
	Mono unsaturated fatty acids			%	42.44
	Poly unsaturated fatty acids			%	33.12
	Trans fatty acids			%	<0.2
3.	Saturated fatty acids	Bg11		%	24.05
	Mono unsaturated fatty acids			%	42.54
	Poly unsaturated fatty acids			%	33.23
	Trans fatty acids			%	<0.2

Based on the retention time tabulated by gas chromatography the highest percentage of PUFA (poly unsaturated fatty acids) was estimated as 33.23 % in Bg11 media.

3.6 Biodiesel extraction:

3.6.1 Transesterification:

Trans-esterification is the reaction which often catalyzed by the development of an acid or base impetus. Through transesterification, biodiesel and PUFA's were extracted. This is a chemical reaction used for the conversion of triglycerides (fatty acids), contained in oils used as biodiesel. Biodiesel produced by the process of transesterification has a much layer viscosity, making it capable of replacing petroleum diesel in diesel engines.

Figure 7: Biodiesel formation

3.6.2 Estimation of Viscosity:

Table 9: Chlorella *vulgaris* algae biodiesel viscosity values at 100 rpm.

Parameters	Bg-11 media	N-8 media	TAP media
Viscosity	4.40	4.14	4.40
RPM	100	100	100
Temperature	29.4	29.7	28.6
Torque	6.6	6.6	6.6
Spindle	61	61	61

The single-step technique was completed with 4.5 grams of dried green growth raced into the vessel, in which the impetus, methanol and were added to permit the extraction of lipid. Whereas the viscosity of *C. vulgaris* at rpm 100 results 4.40 in Bg-11 media , 4.14 in N8 media and 4.40 in TAP media.

4. Discussion

Chlorella vulgaris is easily grown algae that grows in any environment without any specific conditions. It can produce high lipid content when grown in nutrient rich media when compared to other species. It is very efficient in producing biofuels which helps the mankind to solve the problem of dependence on fossil fuels and hence save the non-renewable resources present underneath the earth. It is also used for the production of unsaturated fatty acids (Essential for brain development) where the daily intake recommended by American heart association is 13 grams per day on a 2,000 calorie daily diet.

Chlorella vulgaris was purchased from Culture Collection of Algae at JNTU Hyderabad Telangana, India and was inoculated in three Medias Viz. TAP, Bg11, and N8 media and observed for growth for about 19 days. The protein and carbohydrates were quantitatively estimated by Lowry's and anthrone's test using UV spectrophotometer (LABINDIA (UV3000+). The BSA is taken as standard for estimating the proteins in the algae samples of different media at 660nm using UV-Vis Spectrophotometer (LABINDIA (UV3000+). The standard graph was plotted by taking concentration values on X-axis and absorbance values on Y-axis (Figure 3). Similarly, the D- Glucose is taken as standard for estimating the carbohydrates in the algae samples of different media at 490nm using UV-Vis Spectrophotometer (LABINDIA (UV3000+). The standard graph was plotted for carbohydrates (Figure 2). The proteins gradually increased from 0.04 to 0.25 (Table 2) and the carbohydrates gradually decreased from 0.11 to 0.03 (Table 1). These variations can be assumed that the carbohydrates present were utilized by the algae for its growth and development which led to the formation of biomass and protein.

Lipid extraction was done by utilizing Bligh and Dyer with few alterations. The lower chloroform layer was collected using acid treatment and solvent extraction methods. Percentage of lipids extracted from TAP media, Bg11 media and N8 media are 13.6%, 12% and 11.14% respectively (Table 3).

The extracted lipids were treated to separate and purify fatty acids. The crude lipids were incubated in water bath at 90^0C for 5 minutes. To this, 7mM of sodium hydroxide pellets

were then added to 20 ml methanol and incubated in water bath at 90^0C for 5 minutes. Then, 1ml of boron trifluoride was added and incubated in water bath at 90^0C for 5 minutes. Later, 300ul of n-hexane and 700ul of 1-bromotetradecane was added and finally 1ml saturated NaCl solution was added and mixed thoroughly. After mixing, two layers were observed, from which the upper layer is collected as fatty acids.

The collected fatty acids from different media's- TAP, N8, and Bg11 were analysed by Gas chromatography (GC) as fatty acid methyl esters (FAME), IUPAC (1987). Chromatographic analysis was performed in Shimadzu GC-2010 chromatograph using a DB-23 fused silica capillary column (30m, 0.25mm), 0.25 µm film thicknesses. Helium was used as a carrier gas which is operated at a flow rate of 1.00ml/min. The column temperature was isothermal at 190ºC where in the injector and detector temperatures were 230ºC and 240ºC, respectively. FAME was identified by comparison of their retention times with those of the reference standards.

Based on the retention times of poly unsaturated fatty acids- Methyl Linoleate (49.015) and Methyl Linolenate (51.515) tabulated by gas chromatography, the highest percentage of PUFA's (poly unsaturated fatty acids) was estimated in Bg11 media i.e., 33.23 % (Table 8).

The Biodiesel which was produced by the process of Transesterification showed optimal amounts of viscosity. Viscosity of biodiesel from different media such as Bg11, N8 and TAP media are 4.40, 4.14 and 4.40cp respectively (Table 9). The highest viscosity values were recorded in Bg11 and TAP media

5. Conclusion

Chlorella vulgaris, an ubiquitous algae which grows dense profuselyin aquatic habitats. It can produce high lipid content when grown in nutrition rich media when compared to other species. It is efficient in producing biofuels which helps the mankind to solve the problem of dependence on fossil fuels and hence save the non-renewable resources present underneath the earth. It is also used for the production of unsaturated fatty acids, which are essential fatty acids required for the brain and neuronal development, lowers the risk of insulin resistance and chronic diseases.

This is being cultivated in different media viz. TAP, N8 and B11, was utilised for the extraction of PUFA's and production of biodiesel. The extracted PUFA's triumphed over the disadvantages in fish oil viz., potential contamination, unpleasant odours and multifarious purification. The produced biodiesel could replace the process of depletion of non-renewable resources and change the face of the earth by halting carbon dioxide emissions.

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