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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF MICROALGAE FOR BIODIESEL PRODUCTION

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ABSTRACT

Samples of unicellular microalgae were collected from different locations of Pune city of Maharashtra state, India (Latitude 18.5203° N, Longitude 73.8567° E). The microalgae isolate was selected based on its morphology and ease of cultivation at our test conditions. By further microscopic analysis this culture was identified as strain of *Chlorella vulgaris*. On carrying out media optimization using different media's it was seen that *Chlorella* showed best growth in Modified Chu's 10 medium. The isolated organism was further cultured and examined for morphological features, chlorophyll content, total carbohydrate content and total protein content. Biomass estimation at various stages of growth was done. Nile red staining for lipid determination indicated that lipid bodies are present in *Chlorella vulgaris*. Lipid estimation was done by single step method and it was seen that maximum lipid accumulation was obtained at 25th day of growth. Further it was seen that highest lipid accumulated at 8th concentration of nitrogen limitation. Biodiesel was extracted by direct trans esterification method. Cow dung can be used as a cheap source for large scale production of microalgae.

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INTRODUCTION

Fossil fuel depletion and its effect on environment is an important parameter to increase global warming. Therefore depending on fossil fuel for energy demands is unsustainable. Renewable energy has recently been receiving more attention due to its environmental benefits along with the fact that it is derived from renewable energy sources such as plant oils. Investigation of novel and renewable fuel alternatives has become essential, owing to the world's excessive demand for energy, oil crisis, and continuous increase in oil prices. Biofuel have been produced using many traditional oil crops like rapeseed, corn, sugarcane, soybean (Sharma and Tandon, 2013) etc. But they have many demerits such as low yield, requirement of lot of water and space to grow. On the other hand, for production of enough biofuels food supplies are threatened and native biodiversity is affected (Greenwell 2009). Algae are photosynthetic microbes ranging in size from single-celled microalgae to multicellular microalgae. It does not compete with other food crops even if it is grown in infertile land. Algae can create biomass using solar energy and CO_2 to lower carbon emission. Algae are used in trans esterification for production of neutral storage lipids in the form of triglycerides, long-chain fatty acids and biodiesel production. The demand for sustainable biofuels that are not derived from fossil oil reserves has increased in the last few years, and this trend seems set to continue for microalgae. Microalgae, have seeked global attention in recent years for the valuable natural products they produce (Greenwell *et al.*, 2009). Microalgae like *Dunaliella salina* are mass cultured for the production of β-carotene. Understanding of algal physiology and developments in bioprocess engineering have opened the way for current initiatives to mass culture microalgae for bioenergy applications (Muller Fuga *et al.,* 2003). Microalgae are rich in lipids; they produce many different kinds of lipids like tri- and diglycerides, phosphor and glycolipids, hydrocarbons which can be used for producing biodiesel (Chisti 2007; Hu *et al*). Biodiesel are esters of fatty acids known as aminoalkyl. It is produced from triglycerides by trans esterification (Demirbas, 2003). Biodiesel refers to a diesel-equivalent processed fuel derived from biological sources. Biofuel can be made from processed organic oils and fats. Due to its lower toxicity, biodiesels will play an important role in meeting future fuel demands (Sastry *et al*. 2006). Many species of microalgae grow very rapidly and many micro algal species can be induced to accumulate sufficient quantities of lipids, often greater than approximately 60% of their biomass.

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MATERIALS AND METHODS

Sample collection: Samples of unicellular microalgae were collected from different locations of Pune city of Maharashtra state, India (Latitude 18.5203° N, Longitude 73.8567° E under aseptic conditions in sterile air tight bottles. After collection they were maintained at 4°C for further studies.

Isolation of microalgae: Isolation of microalgae was done by serial dilution method. Different microalgae were compared and the dominant in the present study i.e. *Chlorella vulgaris* was used for the further work as it grew faster compared to other microalgae. *Chlorella* species present from the algal samples was isolated using Chu's medium. Different dilutions such as 1/10, 1/100 were prepared. Then 0.1 ml of each dilution was spread on Chu's agar medium and kept under the laboratory conditions. Discrete green colonies appeared, after 10 days. Pure colonies were isolated and transferred to Chu's medium and kept under laboratory conditions. Then, they were subcultured and maintained. The algae were identified microscopically. The culture was purified by repeated plating and by observation under microscope.

Morphological and microscopic identification of the isolate: *Chlorella vulgaris* was isolated successfully from the algal samples and was identified based on morphological characteristics i.e. cell shape, size, flagella length, presence or absence of vacuoles, presence or absence of granules and lipid, they were identified.

Media optimization-To find out the best culture medium, the anoxic cultures of algae were subjected to 4 different media of different chemical compositions and pH.

1. Chu's medium
2. Modified Chu's-10 medium
3. Juller's medium
4. BG-11 medium

Three test tube sets of each medium containing 10 ml of medium and 1×10^3 cells/ml freshly grown cultures were subjected to different medium and there growth was checked using 2 parameters optical density (OD) and cell count (CC). Optical density was recorded by using colorimeter at 640 nm and cell count was done using haemocytometer according to (Rekha Sharma *et al.,* 2011). Impact of pH and temperature were also checked on its growth in modified Chu's 10 media.

Different carbon source-To see the effect of various carbon sources on growth of *Chlorella vulgaris*, it was subjected to organic carbon sources like acetic acid, citric acid and glucose, inorganic carbon like sodium acetate and sodium carbonate. Three sets of flasks, each containing 50 ml of modified Chu's medium with different carbon source and the culture were prepared. The growth was followed by taking the dry cell weight (El-Sayed *et al.,* 2013). The effect of different concentrations of sodium carbonate was also checked on growth.

Biomass estimation at various stages of growth-For biomass estimation the O.D. was taken at 660 nm and dry cell weight was calculated after every 10 days. 5ml sample was filtered over a pre weighed sterile Whatman filter paper no1. After filtration, paper was dried for 30 minutes at 105°C in the oven and then kept in desiccator till room temperature and then re-weighed. The difference between weights gave the net dry weight of the grown microalgae. The dry weight was calculated as g/l. (El-Sayed *et al*., 2013)

Total carbohydrate and protein estimation: The *C.vulgaris* culture was grown in Chu's 10 media for analysis of carbohydrate and protein. After every 10 days 10ml of culture was taken and centrifuged at 5000 rpm for 5 minutes. Pellet was stored at -20°C until analysis. Determination of intracellular carbohydrates was done using phenol- sulphuric acid method using glucose as standard (Lui, 1973). Intracellular proteins were determined according to the standard protocols of Bradford using Bovine serum albumin (BSA) as standard (1976). (Mathias a. Chia *et al*., 2013)

Chlorophyll estimation: The *C. vulgaris* was grown in Chu's medium for Chlorophyll estimation. After 10 days 10 ml of *C. vulgaris* culture was centrifuged for 5 minutes at 5000 rpm at 30°C followed by washing the pellet with distilled water. The pellet was homogenized with 5 ml acetone and was kept in water bath for 30 minutes at 60°C. It was again centrifuged for 5 min at 5000 rpm. The supernatant which contained the pooled extracts was then collected and the absorbance was measured at 470nm, 663nm and 645 nm, against acetone which was used as blank, chlorophyll (a + b) was measured using Lichtenthaler equations (Lichtenthaler, 1987) (Dayananda *et al*., 2007).

Lipid estimation using FTIR: To determine the effect of nitrogen on lipid accumulation, modified Chu's 10 media was taken with different concentrations of nitrogen like 0mg, 4mg, 8mg, 12mg, 16mg and 20 mg.10 ml from each concentration was taken in each tube and 1×10^4cells/ml were inoculated and incubated for 10 days. Lipid accumulation was checked using FTIR, in which a 0.5 ml sample was taken from each tube mixed, centrifuged. The supernatant was removed and the cells re-suspended in approximately 100 ml of distilled water. 30µl was then deposited on an 96 well microplate and oven-dried at 40ºC for 30 min (Andrew P. Dean *et al*., 2010).

Lipid extraction from *Chlorella*: Single step method: Three extraction methods devised by Folch *et al.* (1957), Bligh and Dyer (1959) and Selstam and Öquist (1985) were used. The Selstam and Öquist (1985) protocol was modified to a faster single-step procedure. Here, extraction of total lipids from green microalgae was done. In the procedure, 1.5 gram of fresh microalgal paste was mixed with 8 ml of a 2:1 chloroform-methanol (v/v). The biomass was suspended by vigorous shaking of the tube for few seconds and then 2 ml of a 0.73% NaCl water solution was added. Phase was separated after centrifugation at 5000 rpm for 5 minutes and the lower phase was recovered for analysis in a pre-weighed crucible and dried. The crucible was weighed.

Observation of increasing lipid content in *Chlorella:* *Chlorella vulgaris* was inoculated into Chu's medium and was allowed to grow. Lipid estimation by single step method of lipid extraction was carried out at an interval of every 10 days. The lipid that was obtained was dried and weighed to find out dryweight. As number of days increased an increase of lipid content was seen until the nutrients in the Chu's medium began to deplete.

Comparison of lipid content in Chu's Nitrogen limiting medium and nitrogen non limiting medium: A nitrogen

deficient (N2 limiting) and non-deficient (Chu's medium) was inoculated with $1x10^8$cells /ml and was allowed to grow. After sufficient growth was obtained lipid extraction of nitrogen deficient Chu's medium and nitrogen non deficient Chu's medium was performed using single step method of lipid extraction. Lipid that was extracted was dried and weighed. The values were then used for further calculations.

Screening of lipids extracted by thin layer chromatography: Screening for lipids was carried out by TLC. TLC plates were developed in chamber of a solvent system that consisted of hexane: diethyl ether: acetic acid in a ratio of 18:2:1. The TLC plates were then removed from the chamber and air dried. The iodine vapor (iodine crystals) was used to visualize the separated lipid compounds. The lipid compounds were detected by brown spots against a white background. Brown spots were observed after continuous exposure to iodine vapor.

Nile red staining: Intracellular lipid droplets present in *Chlorella vulgaris* were detected by Nile red [9-(Diethyl amino) -5H benzo [α] phenoxazin- 5-one] staining .(0.5 ml) Micro algal cells were centrifuged at 5000 rpm for 10 min. The pellet was washed with distilled water (0.5 ml) several times. 1:100 v/v nile red solution was added to cell suspensions and incubated for 10 min. After washing once, stained micro algal cells were observed by fluorescent microscopy (Tadashi Matsunaga, 2009). Fluorescence spectra were determined with a Fluoromax 4 spectrophotometer with a 450 watt xenon lamp. Fluorescence was measured after 30 minutes at 460 nm.

Biodiesel production by direct trans esterification method: Biodiesel was produced from microalgae by direct trans esterification method. The wet biomass was collected by taking the media containing algae and centrifuging it at 8000 rpm for 15 min. 20 gram of wet biomass was obtained. The wet biomass was put in round bottom flask. 56.66 ml methanol was added and mixed properly. 10 ml sulfuric acid and 66.66 ml chloroform was added and mixed. The reaction mixture was heated at 90ºC for 1 hour on a hot plate to maintain the temperature while continuously stirring. After 1 hour the flask was kept for cooling at room temperature. 50ml water was added to the flask and mixed. This was centrifuged at 3000 rpm for 10 min for separation of crude diesel. The organic layer containing the biodiesel was collected and weighed.

Use of cow dung as medium for cultivation microalgae on large scale: 10 g of dried cow dung was taken and added to 100 ml of distilled water. It was shaken to make suspension. It was centrifuged at 3000 rpm. The supernatant was collected in flask, the pH was adjusted to 7 and media was autoclaved. The sample was added to the cow dung medium and checked for growth. The growth was checked by measuring the optical density.

Comparison of lipid content extracted from Chu's medium with that of cow dung medium: Chu's medium and Cow dung medium were prepared and 1.108/ml cells were inoculated in both the medium. After 15 days lipid was extracted by single step method and the extracted lipid content was measured

RESULTS

Isolation and identification: Samples were obtained from different water bodies of Pune city and cultured in Chu's medium and were grown by exposing it to direct sunlight. Morphological and physiological characters such as color, shape, unicellular nature of the isolate resembles closely with genus *Chlorella vulgaris. C. vulgaris* is a green alga belonging to the division Chlorophyta and class Trebouxiophyceae.

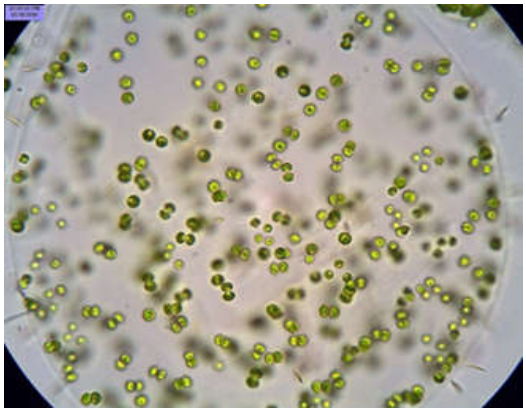
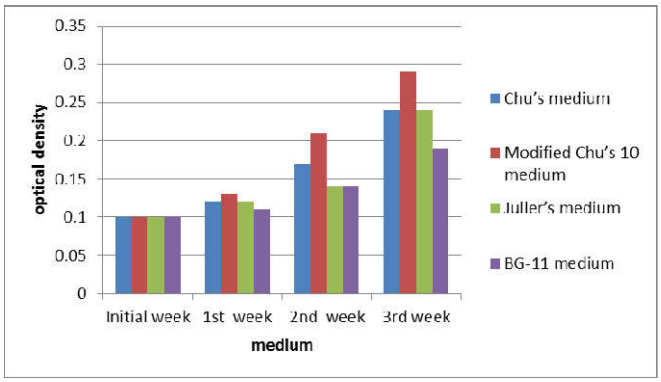


Figure : Microscopic image of *Chlorella vulgaris*. Fastest growing algal species was selected for further work

Media selection for maximum growth

Estimation of growth was done by taking the optical density and the cell count of *C. vulgaris* in different media for 3 weeks. Among all the four media, modified Chu-10 medium shows maximum growth followed by Chu's medium, Juller's medium and minimum growth was seen in BG-11 medium. Effect of pH and temperature was also checked using modified Chu's 10 media. The growth was monitored by taking the O.D at 660 nm.*C.vulgaris* showed best growth at 28 ±3 ºC and pH 6.



Graph of effect of different media on growth

Table 1. Effect of various media on the growth of *C. vulgaris* (O.D) at 660nm

Media	Week 0	1st week	2nd week	3rd week
Chu's medium	0.10±0.2	0.12±0.2	0.17±0.2	0.24±0.2
Modified Chu's 10 medium	0.10±0.2	0.13±0.2	0.21±0.2	0.29±0.2
Juller's medium	0.10±0.2	0.12±0.2	0.14±0.2	0.24±0.2
BG-11 medium	0.10±0.2	0.11±0.2	0.14±0.2	0.19±0.2

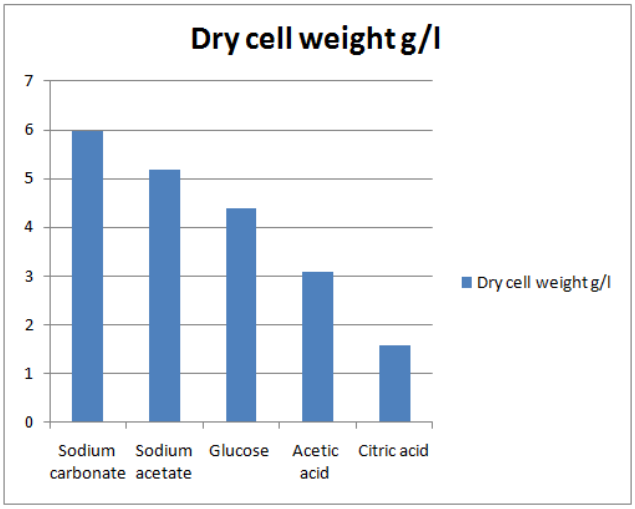
Table 2. Effect of various media on the growth of *C. vulgaris* (cell count)

Media	Initial week	1st week	2nd week	3rd week
Chu's medium	100×10^4	105×10^4	132×10^4	173×10^4
Modified Chu's medium	100×10^4	125×10^4	184×10^4	238×10^4
Juller's medium	100×10^4	101×10^4	126×10^4	176×10^4
BG-11 medium	100×10^4	97×10^4	124×10^4	134×10^4

Carbon source-The algae needs carbon for photosynthesis. About 50% of the algal biomass is made up of carbon and is therefore needed to a large extent for its growth (Becker, 1994). Dry weight of the *Chlorella vulgaris* varied according to the different carbon sources. Maximum growth was seen in medium containing inorganic carbon source sodium carbonate followed by, sodium acetate and glucose. The cell morphology was intact and chlorophyll pigment color was normal. There was very less growth seen in medium containing carbon sources like citric acid and acetic acid. It seems to be toxic to the algae as the cell morphology changed and the chlorophyll pigment seemed to be bleached.

Table 3. Effect of various carbon sources on growth

Carbon source	Dry cell weight g/l
Sodium carbonate	6.0
Sodium acetate	5.2
Glucose	4.4
Acetic acid	3.1
Citric acid	1.6



Graph of effect of various carbon sources on growth

Effect of various concentrations of carbon on growth According to the above results, *C. vulgaris* grew better in carbon source containing sodium carbonate compared to other carbon sources. Therefore, sodium carbonate was used for further study. To study the effect of concentration of sodium carbonate the media was made having different concentrations of sodium carbonate 0mg/l, 0.5mg/l, 1.0mg/l, 1.5mg/l and 2.0mg/l respectively.

Table 4. Effect of concentration of sodium carbonate on growth (O.D)

Concentration of sodium carbonate (mg/l)	Growth O.D at 660nm
0	0.11
0.5	0.16
1.0	0.23
1.5	0.28
2.0	0.47

Effect of concentration of sodium carbonate on growth (O.D)

Biomass estimation-The growth was monitored by taking the O.D of algae and calculating its dry weight. O.D and the dry cell weight are directly proportional to each other.

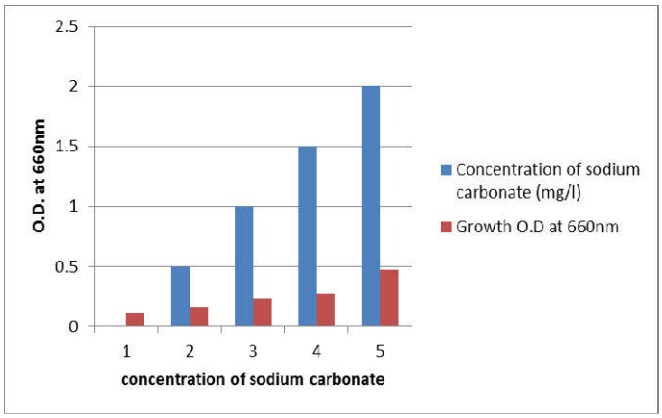
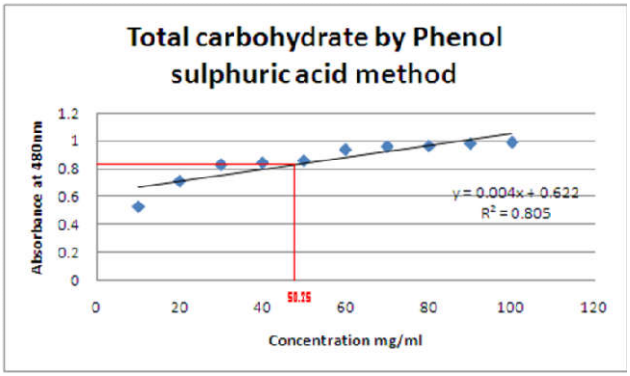


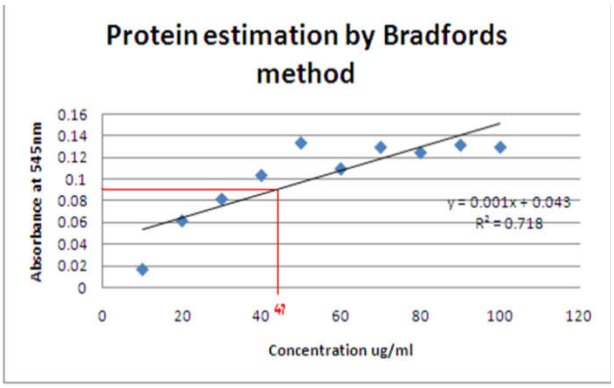
Table 5. Biomass estimation

Days	Optical density at 660nm	Dry cell weight g/l
0 day	0.10	0.5
10 days	0.14	2.3
20 days	0.21	4.2
30 days	0.29	4.1

Estimation of total carbohydrate and total protein: For biochemical composition, the isolated *C.vulgaris* was characterized for carbohydrates and protein content. Protein and carbohydrate content was estimated for three weeks. There is a relation between protein and lipid production. When the protein content decreases the lipid content increases. Algal growth showed highest concentration of protein and carbohydrate in the second week as it had reached the exponential phase. Using a standard graph the concentration of carbohydrate using phenol sulphuric method in *Chlorella vulgaris* was found to be 50.25mg/ml (graph A) and the concentration of total protein using Bradford's method was found to be 47µg/ml (graph B)



Graph A



Graph B

Chlorophyll estimation: Chlorophyll estimation was done by Lichtentaler and Wellburn method. The absorbance properties of pigments facilitate the qualitative and quantitative analysis. Chlorophyll estimation was carried out and calculated by Lichantentaler equations.

Formula: Chlorophyll a= 11.75 A662-2.350 A645 =7.353µ/g

Chlorophyll b= 18.61 A645-3.960 A662==10.993µ/g-By Lichtentaler & Wellburn (1985)

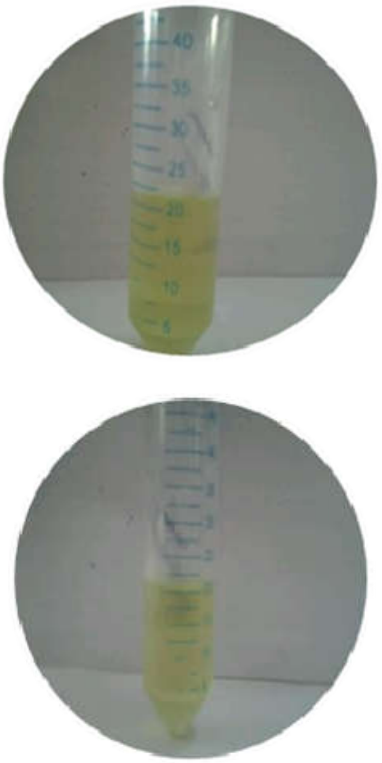


Figure : Chlorophyll estimation

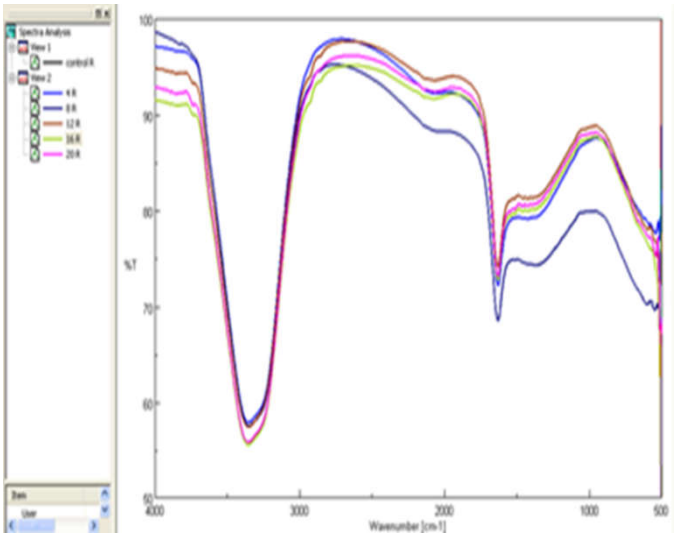
Lipid estimation- Effect of nitrogen starvation on lipid accumulation: In the algal cells nitrogen accounts for 7%–20% of cell dry weight. Major effects of nitrogen deficiency in algal culture include the enhanced biosynthesis and accumulation of lipids and triglycerides with a reduction in protein content. This, in turn, results in a higher lipid/protein ratio at the expense of growth rate. Detection of lipid is done using FTIR method (Andrew P. Dean 2010). Maximum lipid was seen at 8 mg/l in our study.

Lipid extraction by single step method: The lipid content was found out by the single step method which is faster than the Bligh and Dyer method and Flotch method. Total lipids are extracted by this method. It is an easy method and can be performed within 2 hours compared to the traditional methods. All the lipid extraction were done by this method and the calculations were done as follow

Lipid extraction from *Chlorella*: Single step method

Calculations of Lipid extraction:

Weight of empty Falcon tube (W1) =28.16
Weight of falcon tube with biomass (W2) =32.66
The amount of biomass= W2-W1



Graph of FTIR

The amount of biomass =4.5 g

Weight of empty crucible (W3) =44.16g
Weight of crucible after adding the lipid and drying it (W4) =46.26g
The amount of lipid content =W4-W3

The amount of lipid content =2.1 g

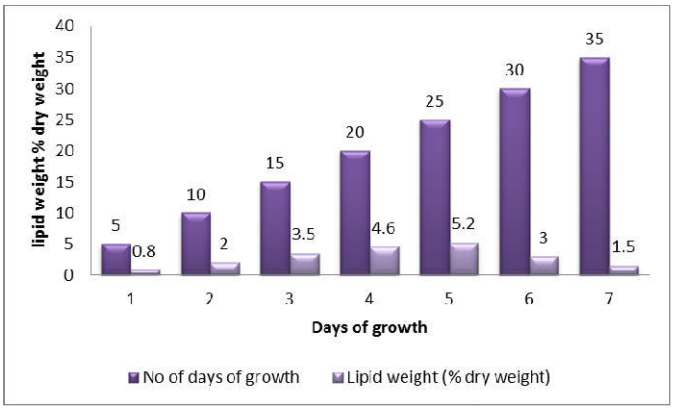
Figure : The extracted lipid in crucible

Observation of increasing lipid content in *Chlorella*: The lipid was extracted after every 5 days and estimated. There was increase in the lipid content. The maximum lipid content was observed on the 25 th day and after that it reduced.

Table 6. Increase in amount of lipid content

No of days of growth	Lipid weight (% dryweight)
5	0.8
10	2
15	3.5
20	4.6
25	5.2
30	3
35	1.5

Comparison of lipid content in Chu's Nitrogen limiting medium and nitrogen non limiting medium: Nitrogen limitation increases the amount of lipid content. The lipid obtained in nitrogen limiting medium is more the non-limiting medium by 0.02 g. Thus proving that nitrogen limitation causes increase in lipid content.



Change in lipid content with increase in number of days

Table 7. Effect of Nitrogen limitation on lipid content

Medium	Lipid content
Chu's medium Nitrogen non limiting	4.88g
Chu's medium Nitrogen limiting	4.90g

Screening of algal lipid by thin layer chromatography: TLC of extract showed three components i.e triglcerol, diacyl glycerol and monoacyl glycerol.

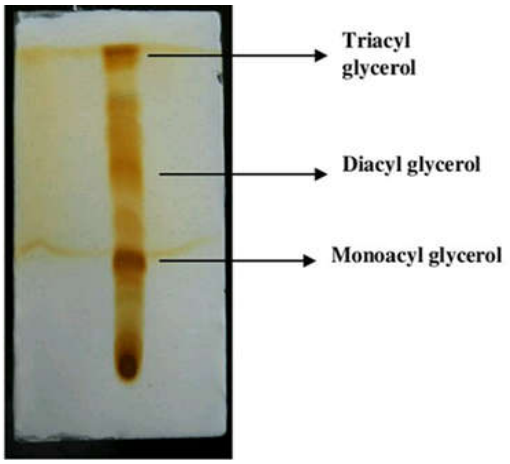


Figure: Fractionation of algal lipids by TLC showed presence of mono, di, triacyl glycerol

Nile red staining :

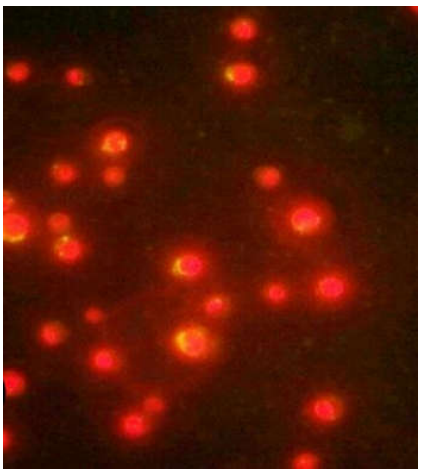


Figure: Nile red staining of *Chlorella vulgaris*: Red fluorescence is emitted by chlorophyll (lipid granules appear red)

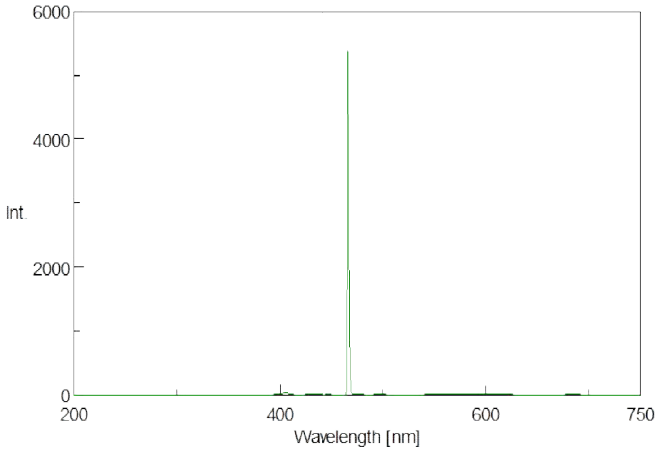


Figure: Emission spectrum of *Chlorella vulgaris* with Nile red

Comparison of lipid content extracted from Chu's medium with that of cow dung medium: The lipid content in cow dung medium is more than Chu's medium as the growth of algae is faster in cow dung medium. The increase in lipid content is 0.11g

Table 8. Comparison of lipid content in Chu's medium and cow dung medium

Medium	Lipid content
Chu's medium	4.7g
Cow dung medium	4.81g

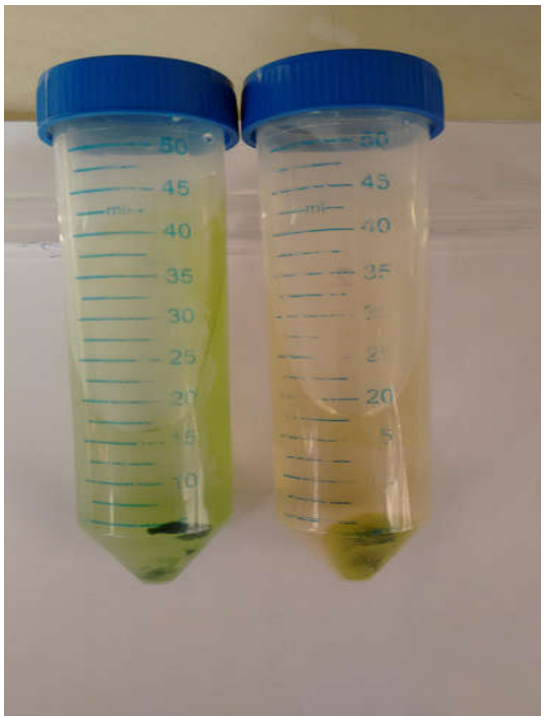


Figure : Falcon tubes containing a) Chu's medium b) Cow dung medium

Biodiesel extraction by direct transestrification method: The biodiesel was extracted successfully. From 20 g of algal biomass 10 ml of crude biodiesel was extracted. The amount of biodiesel was calculated by taking the weight of empty falcon tube and then the falcon with biodiesel. Thus biodiesel was successfully extracted from microalgae by direct transestrification method.

Figure : Falcon tube containing extracted Biodiesel

Comparison of biodiesel with standard: The extracted biodiesel was compared to standard diesel to find out its efficiency. Two physical parameters checked were color, odor.

Table 9. Comparison of standard Diesel with extracted biodiesel

Biodiesel	Standard	Test
Color	yellow	blackish yellow
Odor	Strong smell of gasoline	Mild

DISCUSSION

Chlorella vulgaris was selected for the further work of our project as it was the fastest growing amongst all the other microalgae. The isolated alga was further subjected to different parameters for the efficient production of biodiesel.

Different media- *C.vulgaris* was grown in four different media, which varied in their chemical composition and pH. Among the four media modified Chu's 10 showed the best growth. Similar observations were also reported by (Rakesh Sharma *et al.,* 2011). Thus *C. vulgaris* grows fastest in modified Chu's 10 media therefore this media can be used for culturing it.

Different carbon source- *C.vulgaris* showed better growth in medium with sodium carbonate compared to other carbon sources. In some carbon sources like citric acid the cells seems to be bleached. Our results are in harmony with the results obtained by Jegan and Mukund 2013. Alongwith the carbon sources the concentration of sodium carbonate, at which *C.vulgaris* gave maximum growth were similar with their results. However, *C.vulgaris* showed best growth in medium containing sodium acetate as carbon source according to El-Sayed (2012), which did not match with our results.

Biochemical Composition: The protein production by *Chlorella vulgaris* in modified Chu's 10 estimated in the second week was (47µg/ml) higher than those obtained by Bertoldi *et al.* (2008) Our results for protein content in Chu's are about two times higher than those reported by Mathias A Chia (2013), who showed 7.0 $mg.L^{-1}$ protein production. The highest carbohydrate production by *C.vulgaris* in the present condition was obtained in second week in Chu's medium (50.25 mg/ml) which was less than that obtained by Habib *et al.* (2003).

Chlorophyll estimation: Chlorophyll a and b production by cells was calculated during the exponential phase and the values obtained in our study are lower than those obtained by Chinnasamy *et al*. (2009) i.e. 50µg/ml.

Lipid estimation-In our study maximum lipid was seen at 8 mg/l which was not in harmony with Andrew Dean as maximum lipid was seen at 3mg/algae grown in nitrogen-depleted cultures. The culture tends to divert their photosynthetically fixed carbon to carbohydrate synthesis. To ensure high lipid productivity attempts should be made to increase lipid concentration by limiting available nitrogen.. The interest in the use of algal lipid-derived biofuels, biodiesel in particular is widely used in research on algal lipids and lipid metabolism. (Chisti, 2007; Hu *et al*., 2008) When lipid content was determined in Chu's nitrogen limiting and Chu's non limiting medium it was found that lipid was increased by 0.02g.

Comparison of media and lipid content: When *Cholera* was grown in Cow dung medium it grew faster and the biomass yield was more than Chu's medium. Thus it can be used for cultivation of algae. Cheap source for large scale cultivation of microalgae can be cow dung. This reduces the cost of growing and maintaining the algae in commercial media formulations. This will also help in bringing down the cost of biodiesel production. Our results were not in harmony with Agwa *et al* (2012) were they got maximum yield in poultry waste. However it did match with Shweta Jain results. The physical properties of biodiesel extracted were not found to be correlated to the standard Biodiesel (Rekha, 2012). This might be because of the impurities in the extracted biodiesel. It needs further purification. However we have been successful in extraction which can have a good market potential once it is purified.

Conclusion: Microalgae are considered as third generation biofuel. It is being counted for CO_2 sequestration. With high growth rate and lipid content, there is better probability for it as renewable fuel source and atmospheric air improvement. It could be a potential source for biofuel. The influence of media composition on the growth rate of microalgae *Chlorella vulgaris* was also studied. The study shows that FTIR analysis can be used to find out differences in lipid and carbohydrate content in the algal cells in response to an induction treatment. This states that FTIR can be used for rapid monitoring of lipid accumulation in microalgae at multiple stages of growth following lipid induction. Therefore microalgae has applications for algal biofuel production. This study shows that cultivation and biomass yield of microalgae can be done using animal waste like cow dung. Biomass yield of microalgae is more in cow dung medium as compared to commercial media. Therefore cultivation of algae is easier and cheaper. *C. vulgaris* seems to be a good candidate for biodiesel production, as it grows faster and has high lipid content than other microalgae.

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