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Fatty Acids Extraction from Algae- Chlorella Vulgaris

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Abstract: **Chlorella is a single-celled, spherical shaped, flagellate organism which is about 2 to 10um diameter. It contains green photosynthetic pigments viz. chlorophyll a and chlorophyll b in its chloroplast. Through photosynthesis, it multiplies rapidly by utilizing only carbon dioxide, water, sunlight, and a small number of minerals to reproduce. Chlorella transforms inorganic matter to organic matter by using natural light via photosynthesis, at suitable growing conditions. It contains expert proteins, chlorophyll, dietary fiber, vitamins, minerals, enzymes, nucleic acids and phytonutrients that are advantageous to the humans. Growth and harvest of chlorella vulgaris in the closed and open pond system was done for the production of fatty acids. Growth was optimized at different parameters like temperature, pH, light, intensity, aeration, and medium in both open and closed pond system. For closed pond system chlorella vulgaris was cultivated in three different media like Bg11, TAP, N8. In both open and closed pond system, lipids were extracted using lipid extraction methods and the fatty acids were extracted by using methylation or esterification method.**

Key words- Biochemical test, Chlorella vulgaris, Fatty acids, Lipid extraction method, Methylation.

I. INTRODUCTION

Microalgae can produce various commercial byproducts such as fats, oils, sugars. and functional bioactive compounds. Some are heterotrophic organisms and the other are photosynthetic organisms where photosynthetic organisms can be considered as plants in the world[1]. The algae have chlorophyll that can manufacture their food through photosynthesis. But some are can be considered as cyanobacteria because they have a prokaryotic cell structure[2].

Microalgae are an extremely important species. They produce more oxygen than compared to all other plants in the world. An alga is an important food source for many animals such as little shrimps and huge whales[3]. One interesting information is found out with recent research by using algae to produce biofuels they are Biodiesel, butanol, gasoline, methane, ethanol, jetfuel, vegetable oil and fatty acids.

A. Chlorella Vulgaris:

The name "Chlorella" was derived from the Latin words 'Chloro' for green and 'Ella' for small. It has a size of 2-8 microns which makes it possible to be observed under a microscope[4]. The chlorella cell is equal to human red blood cells but differs in shape, where chlorella is spherical and the human red blood cell is disc-shaped[5]. Every 17-24 hours, chlorella produces four new cells. There are 25,000 species of algae which are plain plants without roots, stems, branches, and leaves. Chlorella vulgaris contains a high content of chlorophyll. It can manufacture its own food through the process of photosynthesis. It produces more oxygen than compared to other plants in the world. High amount of lipids are present in chlorella[6].

B. Lipids:

Lipids are the substance of biological origin that are soluble in organic solvents such as chloroform and ethanol and are only sparingly soluble, if at all, in water[7]. They are a heterogeneous group of compounds related to the fatty acids either actual or potential and chemically they are esters of fatty acids and some alcohols[8]. Hence they are easily separated from other biological materials by extraction into organic solvents and may be further fractionated by such techniques as, adsorption chromatography, thin layer chromatography, and reversed phase chromatography[9].

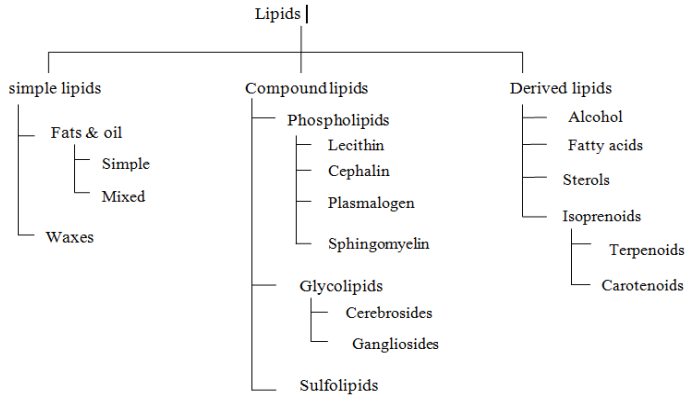

Fig 1 Classification of lipids

C. Fatty acids:

Fatty acids are carboxylic acids with long-chain hydrocarbon side groups. They are rarely free; rather occur in esterified form as the major component of the various lipids[10].

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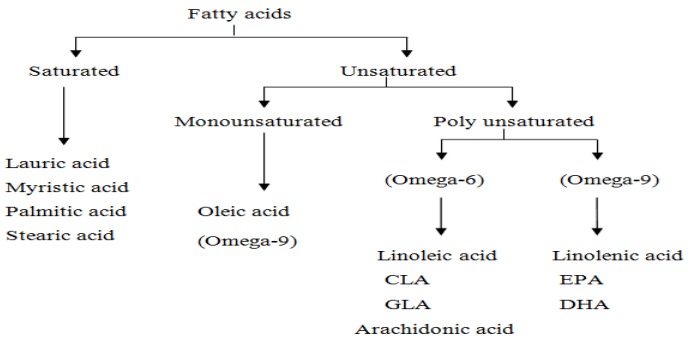

Fig. 2 Classification of fatty acids

II. MATERIALS AND METHODS:

A. Cultivation of algae in closed pond: 3ml of algae is inoculated to 500ml of (Bg11, N8, and TAP) media observe and measure the growth from alternative days by using UV-Visible Spectrophotometer[11].

Cultivation of algae in open pond: 10ml of algae is inoculated to 1.75 lit of TAP media observe and measure the growth from alternative days by using UV-Vis-Spectrophotometer [12].

- Physical and chemical conditions:

Closed pond: Optimized P_H is 7.1, aeration is 7hr, and light is 14hr, for Bg11, TAP, and N8 Medias.

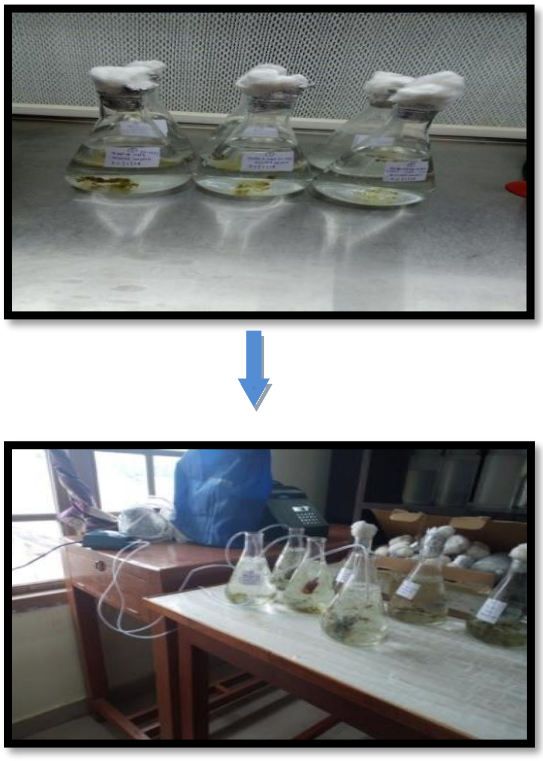

Fig. 3 Cultivation of algae in closed pond and aeration to closed pond and open pond

Open pond: The culture chlorella vulgaris is inoculated in to open pond, nature aeration and sodium nitrate-0.066gm, sodium carbonate-0.096gm, ferric chloride-0.002gm, magnesium sulfate-0.082gm, EDTA-0.074gm, potassium nitrate-0.054gm, calcium chloride dehydrate-0.04gm

N8 media: Potassium nitrate- 0.06gm, dipottasium hydro orthophosphate- 0.046gm, sodium nitrate-0.048gm, citric acid -0.052gm, sodium carbonate- 0.054gm, ferric sulphate- 0.018gm.

B. Phytochemical test:

- Primary metabolites:

Benedicts test: Take 1ml extract and add a small amount of Benedict's reagent, heating up to 5 min in water bath at 90^0C it appears brick red, green, and yellow, and orange, specify presence of carbohydrates[14].

Fehling test:

Take 1ml extract and add a small amount of Fehling reagent, heating up to 5 min in water bath at 60^0C it appears red precipitate, specify the presence of proteins and amino acids[15].

Molisch test:

Take 1ml extract and add a small amount of Molisch reagent, handle slightly 45^0C angle and add H_2SO_4 drop wise it appears purple to violet colour ring, specify the presence of proteins and amino acids[16].

Ninhydrin test:

Take 1ml of extract and add few drops of ninhydrin reagent, heating up to 5min in water bath at 45^0C it appears blue or violet color, indicate the presence of proteins and amino acids[17].

- Secondary metabolites:

Wagner's test:

Take 1ml extract and add few drops of Wagner's reagent formation of reddish brown precipitate, indicate the presence of alkaloids[18].

Dragendroffs test:

Take 1ml extract and add few drops of dragendroffs reagent formation of oranges brown precipitate, indicate the presence of alkaloids[19].

Hager's test:

Take 1ml extract and add few drops of Hager's reagent formation of yellow coloured precipitate, indicate the presence of alkolids[20].

Gelatin test:

Take 1ml extract and add a small amount of gelatin reagent formation of white precipitate, specify the presence of alkaloids.

Phenols test:

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Take 1ml extract and add a small amount of phenols reagent formation of intense colour, specify the presence of alkaloids[21].

Anthrones test for carbohydrates estimation:

Algae: HCl is added in the ratio of 1:2.5, add 1 molar of sodium carbonate until the smell will disappears, centrifuge 10,000 rpm for 5 min, add 1ml of 5% phenol and 3ml of 96% sulfuric acid, kept in dark place for 20min and take OD values at 490nm by using UV-Vis-Spectrophotometer[22].

C. Lowry's method for protein estimation:

Add double amount of Lysis buffer to the algae growth and incubate at 45^0C for half an hour add small amount of SDS and centrifuge at maximum rpm for 5min. to the supernatant add equal amount of Reagent-I [48:1:1 of 2% sodium carbonate in 0.1 N NaOH: 1% sodium potassium tatarate:0.5% $Cuso_4.5H_2O$] to the collected supernatant, add equal amount of Reagent-II to the above mixture and incubate in dark place for 20 min. take OD values at 660nm by using UV-Vis-Spectrophotometer[23].

D. Pigment estimation:

About 10 ml of the sample is taken and is centrifuged at 5000rpm for 5 min. The supernatant was discarded and washed with distilled H2O. The pellet is mixed with 10 ml of 90% ethanol and again it is centrifuged. The procedure from step1 to step4 is repeated until the pellet becomes colorless. The sample was measured at 663nm by using 90% ethanol as blank in UV-VIS-Spectrophotometer. The amount of chlorophyll is derived by using the following formula[24]

(Absorbance at 663nm x 12.63 x volume of ethanol) / sample

E. Lipid extraction method:

Fill the algae solution in centrifuge tubes. Centrifuge 2 times for 15 min at 1500 rpm. After centrifugation, discard the upper layer of liquid. Add 1.25 ml of hexane and again centrifuge twice for 15 min at 1500 rpm. After that add 1ml of chloroform and again centrifuge twice for 15 min at 1500 rpm. Pour the algae solution into a conical flask. Incubate in a water bath for 30 min at 85°C. Collect the lipids with the help of pipette /separating funnel [25].

F. Methylation /esterification of fatty acids:

The lipids are dried under a water bath at 80°C for 10 min. 0.4gm NaOH in 20ml methanol is added into the lipids. Incubate in a water bath for 10 min at 80°C. 5ml of the extract is added into each one of the screw-capped test tubes. 1ml of boron trifluoride is added into the test tube. The extract is refluxed for 10 min in a water bath with stirring at 80°C. After cooling, 700ul of 1 bromo-tetra-decane and 300ul of n-heptane is added. 1 ml saturated NaCl solution is added into the test tube and shaken well. The upper layer is carefully collected with the help of a pipette. Finally, PUFA (polyunsaturated fatty acids) are collected.

F. Methylation /esterification of fatty acids:

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G. Fatty Acid estimation by Gas Chromatography:

The fatty acid composition of the oils was determined by gas chromatography (GC) as fatty acid methyl esters (FAME). FAME was prepared in accordance with the official method of the 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 thickness, Agilent j and W, USA). Helium is 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.

III. RESULTS

A. Open pond:

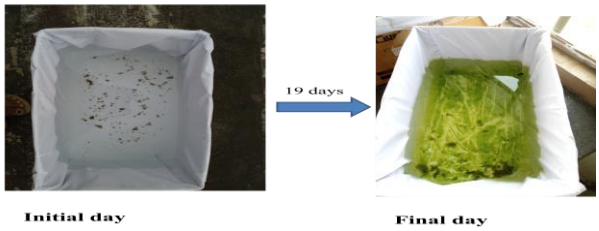

Fig. 4 Open pond cultivation

B. Closed pond:

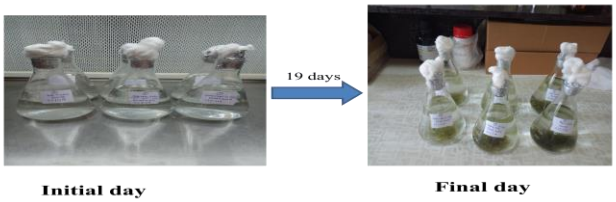

Fig. 5 Closed pond Cultivation

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C. Prepared Algae extract by using soxhlet:

Fig. 6 Extraction of secondary metabolites using soxhlet extraction

G. Phytochemical test:

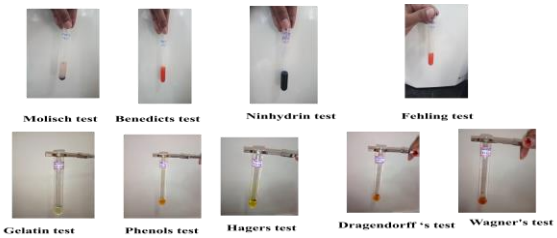
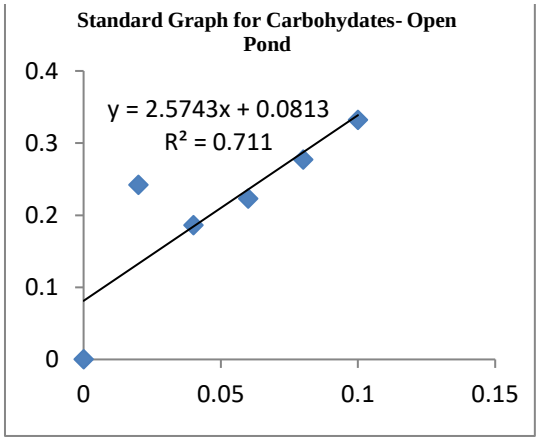


Fig. 3 Phytochemical tests for primary and secondary metabolites

H. Standard graph for carbohydrates



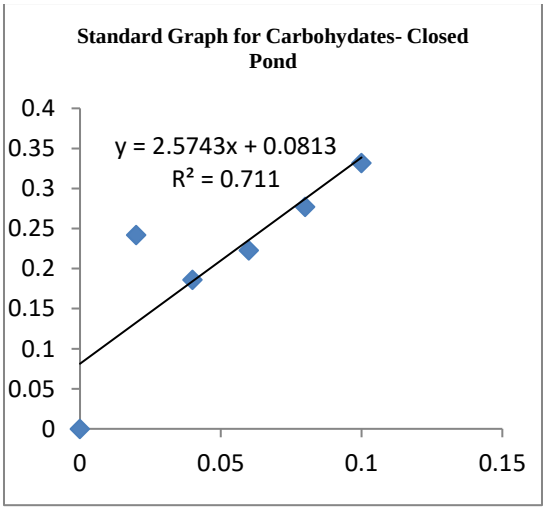
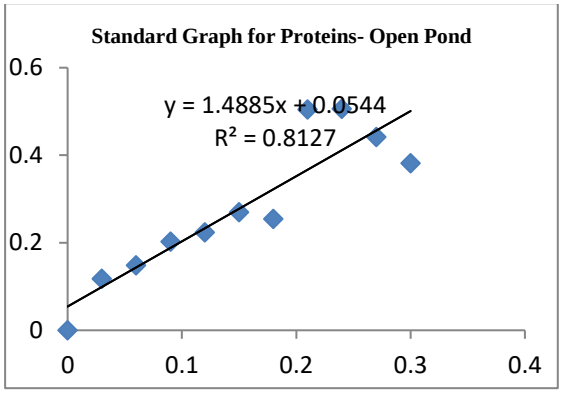


Fig. 7 Standard graph for carbohydrates in open and closed pond respectively

The standard graphs were plotted for the carbohydrates in open and closed pond using D- Glucose as a standard for the estimation of carbohydrates in algae samples using UV VIS spectrophotometer at 490nm. The concentrations were plotted on X axis and absorbance on y axis

I. Standard graph for proteins:



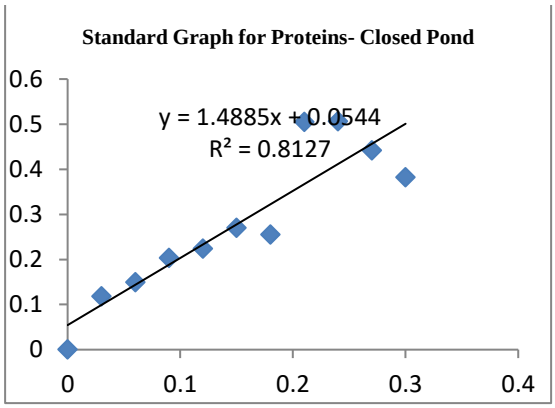


Fig. 8 Protein standard graphs for open and closed ponds

The standard graphs were plotted for the Proteins in open and closed pond using BSA (BOVINE SERUM ALBUMIN) as a standard for the estimation of proteins in algae samples using UV VIS spectrophotometer at 660nm. The concentrations were plotted on X axis and absorbance on y axis

Table 1: Carbohydrate and Protein estimation values in open pond

Open pond	Biomass values at 670nm	Carbohydrates at 490nm		Protein at 660nm	
		Abs	conc.	Abs	conc.
Day1	0.034	1.583	0.41	0.091	0.05
Day3	0.203	0.617	0.16	0.099	0.06
Day5	0.246	0.487	0.12	0.132	0.07
Day7	0.286	0.372	0.09	0.139	0.08
Day9	0.331	0.334	0.08	0.147	0.08

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Day11	0.366	0.323	0.08	0.246	0.14
Day13	0.415	0.302	0.07	0.381	0.21
Day15	0.504	0.289	0.07	0.481	0.27
Day17	0.699	0.280	0.07	0.540	0.30
Day19	0.856	0.268	0.06	0.629	0.35
Lipid	30.8 ml	6.16%			
Fatty acids	17ml	3.4%			

Table 2: Carbohydrates and protein estimation values in closed pond

CLOSED POND	**Biomass values at 670nm**	**Carbohydrate at 490nm**		**Protein at 660nm**	
		Abs	**Conc.**	**Abs**	**Conc.**
Tap media day1	0.202	0.422	0.11	0.060	0.03
day3	0.208	0.415	0.10	0.068	0.04
day5	0.235	0.376	0.09	0.087	0.05
day7	0.307	0.361	0.08	0.125	0.07
day9	0.385	0.336	0.08	0.142	0.08
day11	0.517	0.330	0.08	0.252	0.14
day13	0.525	0.310	0.07	0.356	0.20
day15	0.538	0.302	0.07	0.377	0.21
day17	0.544	0.295	0.07	0.400	0.22
day19	0.709	0.158	0.03	0.455	0.25

Table 3: Lipids and fatty acid values at final day in closed Pond

Lipids:		
Tap media	68ml	13.6%
Bg11 media	60ml	12%
N8 media	57ml	11.14%
Fatty acids:		
Tap media	34ml	6.8%
Bg11 media	30ml	6%
N8 media	28.5ml	5.7%

Table 4: Pigment estimation values in closed and open pond

Closed pond	**Pigment estimation values at final day**
TAP media	0.285438
Bg11 media	0.269019
N8 media	0.26523
Open pond	**Pigment estimation values at final day**
TAP media	0.453417

Lipids are extracted from algae:

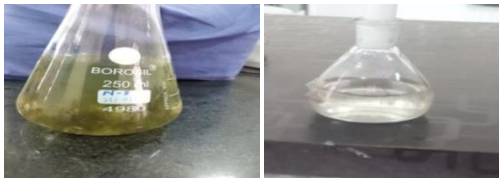


Fig. 9 Extraction of lipids and fatty acids from the algae sample

Tap media (open pond):

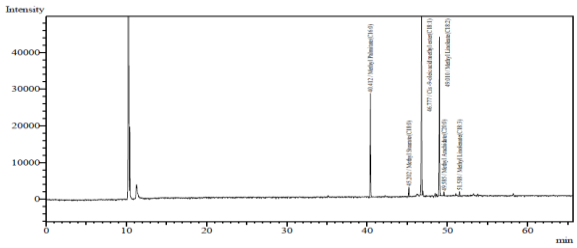
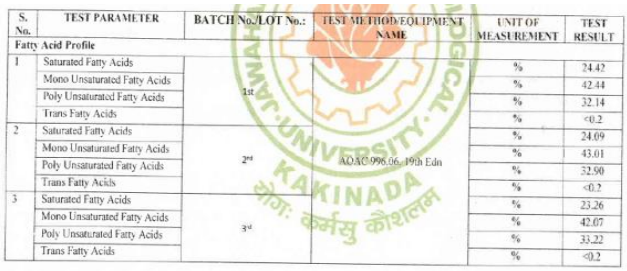


Fig. 10 indicates the fatty acid compounds present in the TAP Media (open pond) with their area, height and area.

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	1st		%	24.42
	Mono Unsaturated Fatty Acids			%	42.44
	Poly Unsaturated Fatty Acids			%	32.14
	Trans Fatty Acids			%	<0.2
2	Saturated Fatty Acids	2nd	AOAC 996.06, 19th Edn	%	24.09
	Mono Unsaturated Fatty Acids			%	43.01
	Poly Unsaturated Fatty Acids			%	32.90
	Trans Fatty Acids			%	<0.2
3	Saturated Fatty Acids	3rd		%	23.26
	Mono Unsaturated Fatty Acids			%	42.07
	Poly Unsaturated Fatty Acids			%	33.22
	Trans Fatty Acids			%	<0.2

Fig. 11 Fatty acid profile by using Gas chromatography

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4 Discussions:

Open pond

In an open pond, the alga was inoculated and growth was observed after 19 days.

Closed pond

In an closed pond, the alga was inoculated and growth was observed after 19 days. It is explained Fig 7.

Algae extract by using soxhlet

The algae was collected from the conical flask and transferred into the falcon tubes. The pellet is obtained by centrifugation for 30min at 1500 rpm. 1.25ml of hexane and 1ml of chloroform was added to the pellet and again centrifuged. The algae supernatant was collected from the falcon tubes with the help of micropipette and was poured into the petri plates. The plates were then kept in the MS oven for 15 minutes at 50^0C.

Finally the algae powder was collected and was utilized in Soxhlet to obtain algae extract[23].

Phytochemical test for primary and secondary metabolites

The algae extract is used to test for the presence of Phytochemical. In Molisch test, the sample reacts with Molisch reagent and colour of the sample changed to purple thus, this indicates the presence of carbohydrates. In benedicts test, the color of the sample changed to brick red on reaction of plant extract with Benedict's reagent. Thus, this indicates the presence of carbohydrates. In ninhydrin test, the color of the sample changed to violet color on reaction of plant extract with ninhydrin reagent. Thus, this indicates the presence of proteins and amino acids. In Fehling test, the color of the sample changed to red precipitate color on reaction of plant extract with Fehling's A and B reagents. Thus, this indicates the presence of proteins and aminoacids[24].

In gelatin test, the color of the sample changed to white precipitate. Thus, this indicates the presence of alkaloids. In phenols test, the color of the sample changed to intense color. Thus, this indicates the presence of alkaloids. In Hager's test, the sample forms yellow colored precipitate on reaction of plant extract with Hager's reagent. Thus, this indicates the presence of alkaloids. In dragendroffs test, the sample forms orange brown precipitate on reaction of plant extract with dragendroffs reagent. Thus, this indicates the presence of alkaloids. In Wagner's test, the color of the sample changed to reddish brown on reaction of plant extract with Wagner's reagent. Thus, this indicates the presence of alkaloids.

Carbohydrates and protein estimation values in open pond

In open pond, sample readings were read on alternative days using UV-Vis-Spectrophotometer. From the result obtained, it is observed that the biomass and protein readings gradually increased and the carbohydrates gradually decreased. This can be interpreted as the carbohydrates present are utilized by the algae for its growth and development leading to the formation of biomass and protein. After 19 days, lipids and fatty acids content was estimated.

Carbohydrates and protein estimation values in closed pond

In closed pond, sample readings were read on alternative days using UV-Vis-Spectrophotometer. From the result obtained, it is observed that the biomass and protein readings gradually increased and the carbohydrates gradually decreased. This can be interpreted as the carbohydrates present are utilized by the algae for its growth and development leading to the formation of biomass and protein. After 19 days, lipids and fatty acids content was estimated.

Lipids and fatty acid values at final day in closed Pond

After 19 days lipids and fatty acids content was estimated in closed pond and highest percentage of lipids and fatty acids are obtained from Tap medium. i.e. 13.6% and 6.8% respectively.

Lipids are extracted from algae

The algae is collected from the conical flask and transferred into falcon tubes. The pellet is obtained by centrifugation for 30min at 1500 rpm. 1.25ml of hexane and 1ml of chloroform was added to the pellet and again centrifuged. The algae pellet is transferred to a conical flask and incubated in a water bath for 10 min at 90^0C. The Lipids which float in the conical flask were collected with the help of micropipette.

Extract the fatty acids from lipids

The lipid samples were taken in different test tubes and were kept in water bath at 90^0C for 5 minutes. Then 0.3gm of sodium hydroxide pellets were added to 20 ml methanol and the mixture was added to each of the test tubes. The tubes were again kept in the water bath at 90^0C for 5 minutes. From the sample, 5ml of extract were transferred to another set of test tubes. To these, add 1ml of boron trifluoride to each test tube. The tubes were again kept in water bath at 90^0C for 5 minutes. Then to each tube 300ul of n-hexane and 700ul of 1-bromotetradecane was added, finally 1ml saturated NaCl solution was added and the contents in the test tube were mixed thoroughly. After mixing, two layers were formed. The upper layer in the test tubes were collected as it contains fatty acids.

Fatty acid profile

The 1st batch is TAP media (closed pond). In these saturated fatty acids are 24.42%, monounsaturated fatty acids are 42.44%, polyunsaturated fatty acids are 32.14%, overall 99.26% fatty acids are present, The 2nd batch is TAP media (open pond). In these saturated fatty acids are 24.09%, monounsaturated fatty acids are 43.01%, polyunsaturated fatty acids are 32.90%, overall 100% fatty acids are present, The 3rd batch is Bg11 media (closed pond). In these saturated fatty acids are 23.26%, monounsaturated fatty acids are 42.07%, polyunsaturated fatty acids are 33.22%, overall 98.55% fatty acids are present, Higher amounts of fatty acids are present in open pond when compared to the closed pond. The TAP media has recorded the highest fatty acid concentration i.e. 99.26%.

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The fatty acids composition is estimated by the Gas Chromatography as FAME (fatty Acid Methyl Esters). Based on the retention times tabulated from gas chromatography, the PUFA (Poly Unsaturated Fatty Acids) were estimated as 32.14% in Tap medium of open pond, 32.90% in Tap medium of closed pond, and 33.22% in Bg11 medium of closed pond. Hence, it can be concluded that Bg11 medium cultivated in closed pond has the maximum PUFA's (Poly Unsaturated Fatty Acids) when compared to all other medium.

5 CONCLUSIONS:

Chlorella vulgaris can easily grow in any environment without any specific conditions. It can produce high lipid content when grown in nutrition 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. The daily intake recommended by American heart association is 13 grams per day on a 2,000 calorie daily diet. Omega-3 fatty acids are critical for brain

Pigment estimation values in closed and open pond

At final day, pigment values are estimated in both closed and open pond and from the results it can be inferred that the pigments are 71% more in open pond when compared to closed pond.

development and function. May boost heart health, promote healthy infant development and prevent mental decline in older adults[25].

Chlorella vulgaris is grown in both open ponds (outdoor) and closed pond (in vitro) as a comparison between the outer environment and laboratory environment. Three medias Viz. TAP, Bg11, N8 media chosen for the closed pond cultivation against the tap media in open pond. The growth characteristics were done timely to choose the best suitable media. By the characterization, it was concluded that the chlorella vulgaris grows best in open pond when compared to closed pond.

The fatty acids were extracted from the extracted lipids by the methylation process from all the above media's (open and closed). The samples were analyzed by the gas chromatography. The results shows that the open pond has 100% fatty acids and the closed pond TAP media has 99.26%, Bg11 media has 98.55% and N8 media has 96% fatty acids. By this, it is concluded that higher amounts of fatty acids are present in open pond when compared to the closed pond. The TAP media has recorded the highest fatty acid concentration i.e. 99.26%.

The fatty acids composition is estimated by the Gas Chromatography as FAME (fatty Acid Methyl Esters). Based on the retention times tabulated from gas chromatography, the PUFA (Poly Unsaturated Fatty Acids) were estimated as 32.14% in Tap medium of open pond, 32.90% in Tap medium of closed pond, and 33.22% in Bg11 medium of closed pond. Hence, it can be concluded that Bg11 medium cultivated in closed pond has the maximum PUFA's (Poly Unsaturated Fatty Acids) when compared to all other mediums. The PUFA's extracted from chlorella vulgaris has triumphed over the disadvantages in fish oil viz., potential contamination, unpleasant odors and multifarious purification.

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