Current Trends in Biotechnology and Pharmacy
Vol. 19(1) 2116-2129, January 2025, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2025.1.2

A Study on *Chlorella* Biomass as a Vegan Source for Omega-3 Fatty Acids and Dietary Proteins

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Abstract

Microalgae are one of the less-explored, nutritional treasures of the marine biosphere. With the emergence of algal technology, research has been shifting slowly towards exploring the nutraceutical values of these microorganisms, and microalgae like *Chlorella vulgaris* are gaining high market value. In addition, with the increasing demand for vegan source of omega-3 fatty acids, the *Chlorella vulgaris* biomass may be considered as a potential alternative. In this study optimization of various parameters such as culturing conditions, media composition, pH, RPM, inoculum percentage, carbon source, concentration of glucose, and salt to enhance the yield has been carried out. The best results were obtained at pH 7 with an inoculum percentage of 5 and the addition of 1.5 gm of NaCl and glucose enhanced the yield of biomass, protein, and lipid in *Chlorella vulgaris*. Optimized conditions gave a maximum yield of biomass, lipids, and protein. Among the lipids, omega-3 fatty acids have high nutraceutical value. In the fatty acid methyl esters, DHA and EPA were found to be 2.9% and 0.55 %. Estimation of omega-3 fatty acids was done using TLC and GC-MS. The Omega-3 fatty acids and dietary proteins extracted from *Chlorella vulgaris* can serve as an alternate vegan source for nutritional supplements.

Keywords: *Chlorella vulgaris*, Optimisation, Lipids, Omega-3 -Fatty acids, Protein.

Introduction

Omega-3 fatty acids are a category of fatty acids that the human body is unable to produce on its own. These are necessary fats that must be obtained from external sources. Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) are the two forms of omega-3 that are majorly obtained from fish Biomass. Plants contain omega-3 fatty acids in the form of alpha-linolenic acid (ALA). Due to the abundance of non-vegetarian sources of omega-3 fatty acids and the scarcity of vegan alternatives, omega-3 fatty acid supplements derived from fish oil are not practical for vegetarians, who are unable to meet their daily needs for omega-3 fattyacids. Since fishes spawn in a specific season, the biomass of fish is not available year-round for the production of omega-3 fatty acids. Fish from contaminated waterways may have accumulated mercury, which can cause cancer[1]. Fish and fish oil allergies exist in certain people. Omega-3 fatty acid supplements derived from fish oil are not feasible for such individuals[2]. Furthermore, some people have been known to develop gastritis and indigestion after taking fish oil.

Microalgae hold great potential for the sustainable production of high-value chemicals, feed, and biofuels. Microalgae, such as *Spirulina* and *Chlorella*, are possible sources of proteins and lipids that can be utilized to make functional meals that enhance human health [3]. When

compared to other microalgae, *Chlorella vulgaris* has a high market value, with a market scope analogous to *Spirulina (Arthrospira platensis).* However, the added advantage of *Chlorella vulgaris* over Spirulina is that it does not have a cytotoxic effect on healthy cells. The Spirulina produce a compound called microcystin which is toxic to liver cells [4]. Most of the applications of *C. vulgaris* have been on its metabolism of lipids, proteins, and carbohydrates[5]. They are commercially important as they can produce a variety of essential and non-essential amino acids [6]. Apart from its nutritional value *Chlorella vulgaris* has also been known for its medical applications like cardioprotective properties [7], immunomodulatory effects [8], anticancer properties [9] and antidiabetic properties [10]. Protein makes up to 45-58% of the dry weight of the *Chlorella* biomass of which 20% of the protein is bound to the cell wall supporting the structural integrity of the cell and also serves as a transporter. The total protein content may vary 12-120 kDa of which the majority lies in the range of 39-75 kDa. Lipids constitute around 5-40% of the *Chlorella vulgaris* dry weight. The lipids are present in the form of glycolipids, waxes, hydrocarbons, fatty acids, and phospholipids. These are synthesized in the chloroplasts and directed towards the cell membrane and cell wall [11]. Concerning the type of fatty acids in *C. vulgaris*, it has been noted that 70.18% are saturated fatty acids (SFA),16.85% are monounsaturated fatty acids (MUFA) and 8.72% are polyunsaturated fatty acids (PUFA) hence, it can be considered as a storehouse of various fatty acids [12].

Microalgae can be explored as a solution for the problems related to the production of Omega-3 fatty acids from fish biomass. In comparison to higher-level plants, the growth of microalgae is quicker, they can grow in harsh conditions and give better metabolite yield[13]. Omega 3 fatty acids are produced in higher amounts in the microalgae *Chlorella vulgaris*[14]. Microalgal biomass is available throughout the year and it takes only a few days to produce the culture. The micrometer size and its unicellular nature contribute to a high surface-to-volume ratio giving efficient nutrient utilization and metabolite production[15]. It is a better alternative for people with allergies to fish and fish oil. In addition, there is no wastage in this process, as the by-products can be used as poultry/cattle feed, as they have high nutritional value.

In this study, the optimization of physical parameters and media for maximal production of lipids and proteins has been carried out through sustainable and cost-efficient processes using *Chlorella vulgaris*. The lipids and proteins that come as a byproduct in culturing processes are of high nutraceutical and pharmaceutical significance.

Materials and Methods

Revival and culturing of the microalgal strain

The pure culture of *Chlorella vulgaris* was obtained from Biopol Biosciences, Bangalore. The microalgae was revived in the BG11 media for further experiments. To find the optimal growth of the microalgae a comparative study was made between the growth, using an incubator shaker with 200 rpm at room temperature and plant tissue culture setup [16]. 200 ml of BG11 media was autoclaved and 50µl of inoculum was added to both the media under aseptic conditions and kept for incubation in their respective conditions. Once the microalgal growth was observed they were added with 40% glycerol and cryopreserved at -80ºC and used for further experiments.

Optimization studies

To enhance the yield, various parameters such as the composition of media, pH, RPM, inoculum percentage, carbon source, concentration of glucose, and salt have been optimized. The optimization experiments were done in triplicates.

Magnetic stirrer vs static condition

One ml of the culture was added to one litre of BG-11 media and kept on a magnetic stirrer with 150 rpm, at room temperature under light. Similarly, the culture was kept in static condition. The OD at 680nm was noted for every 24 hours. Microscopy was carried out at 100x to check for contamination. Wet cell weight, Dry cell weight, protein, and lipid analysis were carried out on day 9 [17].

Inoculum % optimization

Inoculum percent of 1,5 and 10, was added to BG11 media and made up to 250 ml to study the effect on yield. OD at 680nm was noted every 24 hours. Microscopy was carried out to check for contamination. Wet cell weight was measured on day 10. The pellets were dried for 24 hours at 60ºC, Dry cell weight was recorded and stored at 4ºC.

pH optimization

The initial pH of the BG11 media was set to 7,7.5,8,8.5 and 9 under aseptic conditions. The OD at 680 nm was noted for every 24 hours. The wet cell weight, dry cell weight, lipid, and protein estimations were made on day 6 [18].

Carbon source

Acetic acid and glucose were selected as carbon sources for the analysis. Bottles containing 1g/l glucose were added with BG11 media and 0.06 gm NPK and another set of bottles containing 1g/l acetic acid was added with BG11 media and 0.06gm NPK.OD at 680 nm and DCW were noted every 24 hours [19].

Growth kinetics studies for BG11 and NPK (Selection of nitrogen source)

100 ml of solution was prepared with 95 ml of BG11 media and 5 ml of inoculum. Each day optical density at 680 nm, wet cell weight, dry cell weight, protein content, and lipid content were analysed. Similar steps were carried out for NPK media which has an NPK concentration of 0.6gm/l [20].

Glucose concentration

To the BG11 media, various concentrations of glucose (1gm/l,1.5gm/l,2gm/l) were added. Estimation of OD at 680 nm, WCW, and DCW every 24 hours for 7 days was carried out. lipid and protein content was estimated for the culture on day 7 [21].

RPM optimisation

One litre of the prepared media was subjected to a magnetic stirrer at room temperature at 200 rpm. OD at 680 nm was noted down every 24 hours. The WCW, DCW, and Protein were estimated on day 7. Similarly, the experiment was repeated for 250 rpm and 300 rpm [22].

Salt stress

Various concentration of NaCl (0,1,1.5,2gm/l) was added to BG11 media. OD at 680 nm, WCW &DCW were recorded every 24 hours. Lipid and protein content were estimated on day 7 [23].

Scale up production of culture under optimized conditions

950 ml of media was prepared and 50 ml of inoculum was added to the media followed by the addition of 1.5 gm of glucose. OD at 680 nm was noted every 24 hours. The WCW, DCW, lipids, and protein were estimated only on Day 7 [24].

Analysis of omega-3 fatty acid

Confirmation by TLC

For standard preparation: Content from 1 tablet of omega-3 fatty acid soft gelatine capsule (OMEGALARK3) was dissolved in 10 ml of chloroform.

For the sample preparation, the Modified Bligh & Dyer method was used for the extraction of lipids from dried algal biomass, where 50 mg of lipid samples were mixed with a solution of water, chloroform, and methanol (0.8:1:1 v/v/v).

4 mL of a water: chloroform solution (1:1, v/v) was added after the vortex. After being vortexed, the sample was centrifuged for ten minutes at 5,000 rpm. The mobile phase was prepared using ether: benzene in the ratio 2:2.

The TLC procedure was carried out by spotting the silica gel TLC plate and the movement of the mobile phase was marked. The bands formed were observed under a UV transilluminator and marked [25].

Estimation by GC MS

The extracted lipids were measured using Gas Chromatography Mass Spectrometry[26]. Using standards (Sigma), individual FAMEs were quantified and identified. Unidentified FAMEs were estimated using the averaged RF factor [27].

Results and Discussion

Revival and culturing of the strain

The pure culture of *Chlorella vulgaris* was revived in the BG11 media. The OD at 680nm and biomass analysis on Day 8 confirmed that the plant tissue culture condition gave a yield of 0.023g DCW with an absorbance of 0.264 in comparison to the Shaker incubator. Microscopic studies revealed that there is no contamination in the culture with an appreciable level of microalgal growth.

Optimization studies

Magnetic vs static stirrer condition.

Absorbance at 680 nm showed that a stationary phase was attained on day 7 in both cultures (Figure 1). In dry biomass estimation, the higher yield was derived in the magnetic stirrer condition(0.17±0.02g/l) rather than the static condition(0.14±0.01g/l). The protein content was higher in the static condition (357.27±2.72 mg/g) when compared to the magnetic stirrer condition (319±2.67mg/g). The lipid content was higher in the magnetic stirrer condition(9.89±0.32mg/g) in comparison to the static condition (7.10±0.28mg/g) (Figure 2).

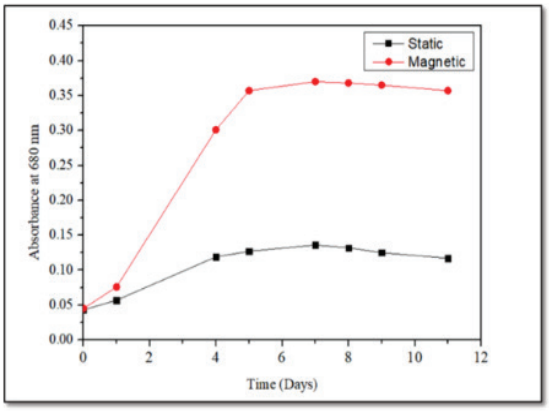

Figure 1: Growth curve for absorbance at 680 nm for static and magnetic stirrer condition.

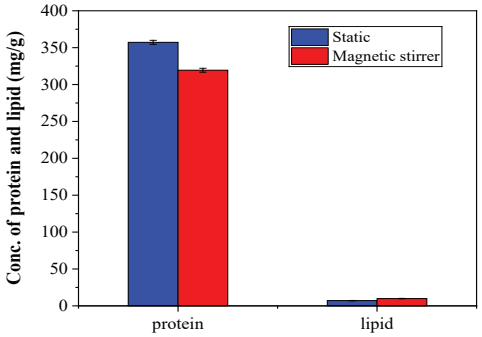

Figure 2: Protein and Lipid estimation for cultures in static vs magnetic stirrer condition.

Optimization of Inoculum

From the absorbance studies, it was observed that maximum growth was achieved by culture with an inoculum percentage of 5% on day 10 (Figure 3). However, the culture with an inoculum percentage of 10 % showed a good increase in OD initially. But started to decrease sharply from day 7 to day 10 indicating the nutrient depletion in the culture. Inoculum concentration of 5 gives the maximum yield of biomass and lipid (590.02±4.25 mg/gm) in comparison to the 1% and 10% inoculum (Table 1). From the protein analysis, it was observed that an inoculum percentage of 10 gives a maximum yield of protein (93.75±1.74 mg/g).

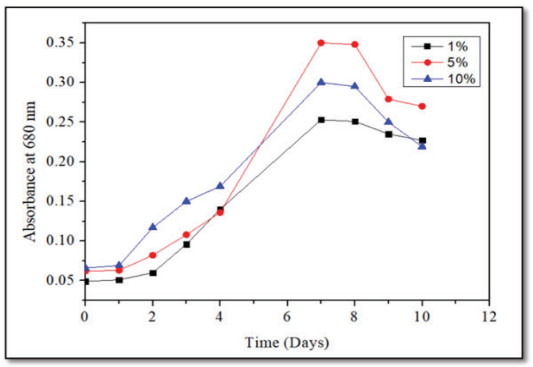

Figure 3: Growth curve for 1%,5%and 10% inoculum concentrations

pH optimization

From the absorbance studies, it was found that pH 7.5,8,9 had almost achieved the stationary phase on day 5 while pH 7 and pH 8.5 were still in the exponential phase (Figure 4). From the biomass estimation, the maximum yield of dry biomass (0.022±0.002gm/l) and lipid (509.73±3.860mg/g) was obtained from culture with an initial pH of 7 in comparison to culture with an initial pH of 7.5,8,8.5,9 (Table 1). However maximum yield of protein was obtained in culture with an initial pH of 7.5(126.57±3.521mg/g.

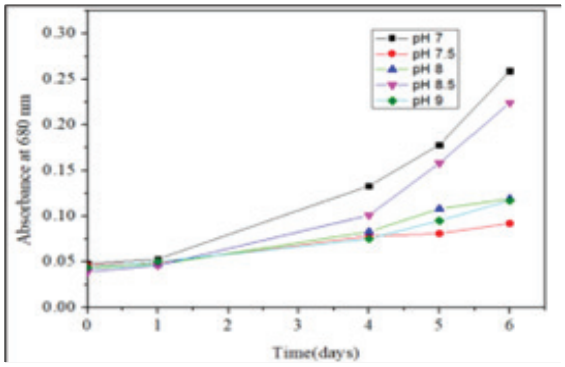

Figure 4: Growth curve for cultures of different initial pH

Growth kinetics studies (BG11)

For every 24 hours, the absorbance at 680 nm was observed till day 7. The maximum OD reading was obtained on day 7 and the absorbance was found to be linear (Figure 6). On biomass estimation, it was noted that biomass growth was exponential till day 6 however the growth declined from day 6 to day 7. The maximum biomass (4.00 g/l) (Figure 7) and protein (11.21mg/g) (Figure 5) were obtained on day 7. Specific growth and doubling time were also found to be maximum at Day 7. The maximum lipid was obtained on Day 4 (172.171±1.807 mg/g) for the cultures grown in the BG 11 media (Figure 5). The absorbance was noted at 530 nm and the lipid content was calculated based on the lipid standards.

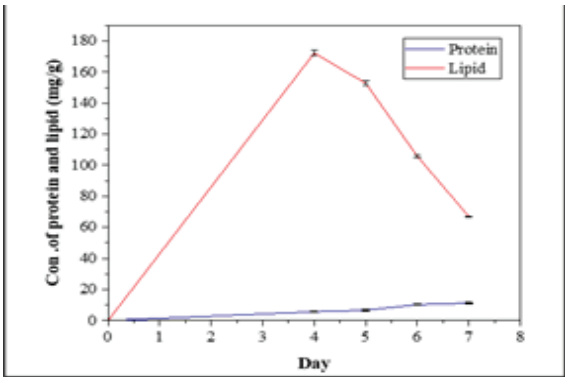

Figure 5: Protein and Lipid estimation for BG11 media culture.

NPK growth kinetics

According to biomass estimation, the absorbance at 680 nm was noted every 24 hours till Day 7, and the Growth curve was plotted (Figure 6). The graph was found to be linear, and a sharp increase in the absorbance from Day 6 to 7 indicates the culture was still in the exponential phase on day 7. The maximum biomass (0.30±0.565 gm/l) was obtained on day 7 for the NPK (Figure 7). The protein and lipid estimation was carried out for dry biomass and the growth curve was plotted. The maximum protein and lipid were obtained on day 4 and the decline of protein and lipid content was observed from day 5 to 7 (Figure 8). Absorbance was noted at 530 nm, However, the yield of biomass and lipid in NPK media was low in comparison to the BG11 media, hence BG11 is a better media for enhancing biomass growth.

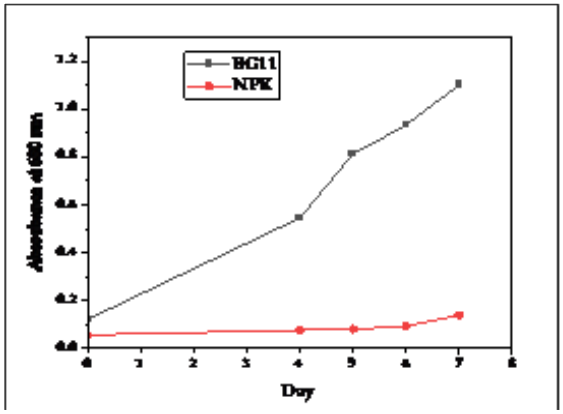

Figure 6: Absorbance growth curve for BG11 and NPK media culture.

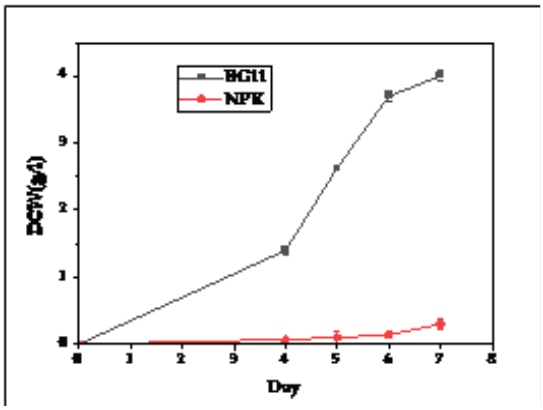

Figure 7: Biomass growth curve for BG11 and NPK media.

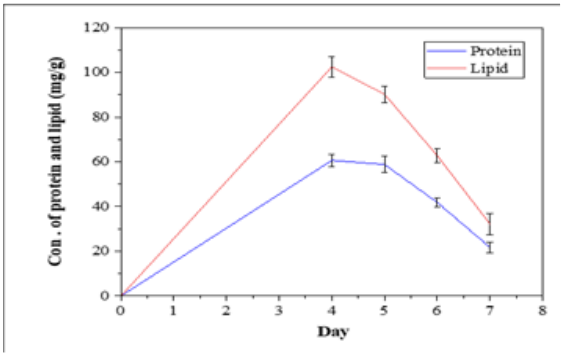

Figure 8: Protein and Lipid growth curve for NPK media.

Selection of carbon source

The growth was observed with different carbon sources. The Dry cell weight was also noted every 24 hours. From the absorbance at 680 nm (Figure 9) and dry cell estimation, it was found that glucose along with BG11 gave an appreciable amount of biomass (2.85g/l). In contrast, NPK along with acetic acid or glucose and BG11 along with acetic acid gave no biomass yield (Figure 10). Hence, glucose was selected for further studies as a carbon source.

Glucose concentration.

On day 7 the culture was harvested and the biomass estimations were carried out along with protein and lipid estimation. The maximum biomass and lipid yield was obtained in the culture with 1.5 g/l of glucose concentration in comparison to the culture with a glucose concentration of 1 g/l or 2 g/l. From the protein estimation, it was found that culture with 2g/l glucose concentration gave a maximum yield of protein.

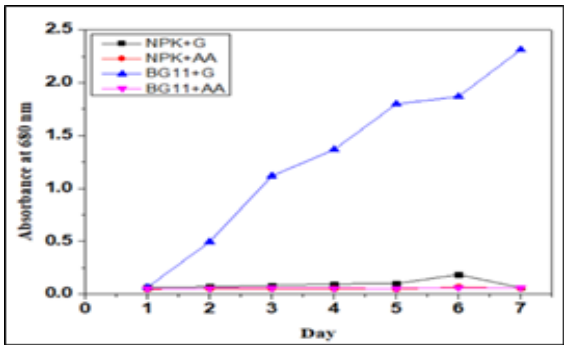

Figure 9: Growth curve for various carbon sources Based on absorbance at 680 nm.

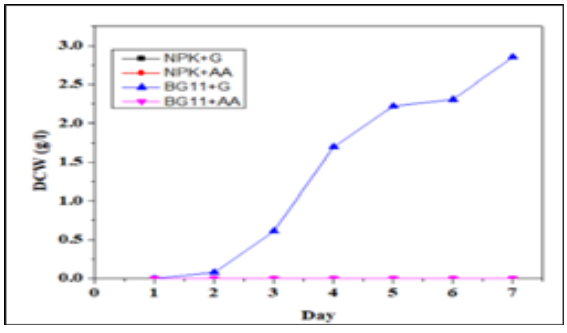

Figure 10: Dry cell weight estimation for various carbon sources.

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RPM optimization

The biomass was harvested on day 7. The maximum yield of biomass (1.125±0.061g/l) and lipid was found in the culture subjected to 250 RPM (Table 1). At 300 rpm the yield of protein was found to be maximum.

Salt concentration

The cultures were harvested on day 7. The maximum yield of biomass was obtained for culture with an initial NaCl concentration of 0 g/l in comparison to 1.1.5,2g/l (Table 1). Maximum yield of protein and lipid was obtained in the culture with 1.5 g/l NaCl concentration in comparison to the culture with 0,1,2 g/l NaCl.

Table 1: Optimization studies of protein and lipid

		Protein (mg/gm)	Lipid content (mg/g)
Inoculum %	1%	56.58±1.45	585.15±1.96
	5%	50.18±2.37	590.02±4.25
	10%	93.75±1.74	169.50±2.12
pH	7	20.68±1.442	509.73±3.860
	7.5	126.57±3.521	499.92±2.672
	8	55.22±3.336	235.33±3.634
	8.5	15.97±2.729	436.84±1.499
	9	49.02±3.280	132.66±4.228
RPM	200	17.96±0.537	164.51±4.002
	250	25.51±2.319	286.25±3.66
	300	31.54±2.969	191.27±4.963
Concentration of glucose (gm/l)	1	21.03±3.73	259.16±2.418
	1.5	47.1±4.05	320.84±4.313
	2	66.65±3.450	288.94±2.927
Concentration of NaCl (gm/l)	0	26.46±0.791	139.30±3.874
	1	23.77±1.852	261.65±4.737
	1.5	62.37±1.152	420.80±3.323
	2	17.87±0.311	279.06±4.313

A high yield of dry cell weight was achieved at the Optimal conditions of pH 7,250 RPM, inoculum concentration of 5, with 0 and 1.5 g/l concentrations of NaCl and glucose (Figure.11). Similarly, the maximum yield of lipid was obtained at pH 7, inoculum concentration of 5%, RPM of 250, with salt and glucose concentration of 1.5mg/l (Figure 12).

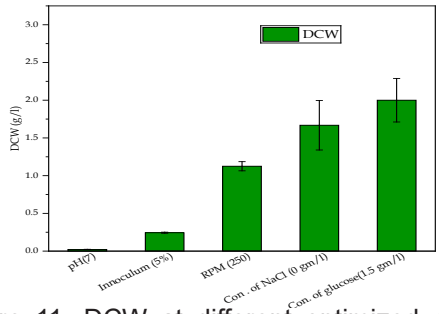

Figure 11: DCW at different optimized conditions

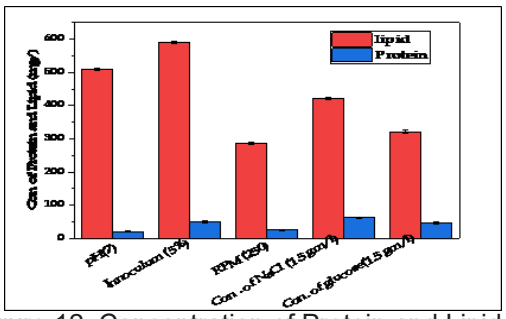

Figure 12: Concentration of Protein and Lipid at different optimized conditions

For the scale-up, pH 7, inoculum concentration of 5%, and salt, and glucose concentration of 1.5 g/l, were chosen as optimal conditions to maximize the yield of dry biomass and lipid with protein as a byproduct.

Scale-up production of culture under optimized values

In scale-up production, the culture was subjected to optimized conditions (pH 7,250 rpm,1.5gm glucose on the 1st day, and 1.5g/l NaCl on the 6th day evening). The cell was harvested on day 7, The obtained contents of dry biomass, protein, and lipid are 4.25±0.395g/l, 53.23±4.327mg/g, and 621.85±4.412 mg/g.

In comparison to the initial BG11 media culture in the optimized scale-up culture the dry biomass was found to be increased by 1.08 times (Figure 13) lipid by 8.11 times and protein by 11.18 times (Figure 14).

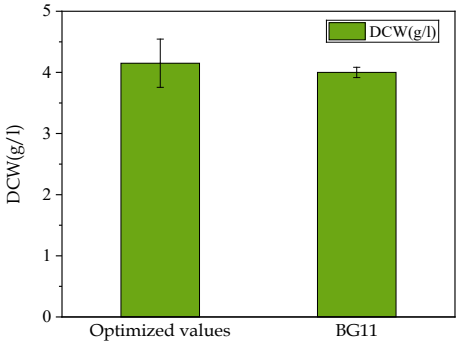

Figure 13: Biomass estimation comparison between BG11 media and optimized condition value.

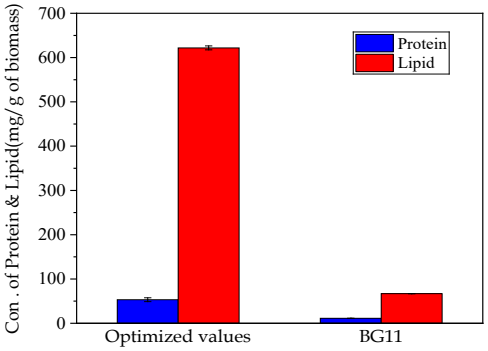

Figure 14: Protein and lipid estimation comparison between BG11 media and optimized condition value.

TLC for qualitative determination of omega-3 fatty acid

The presence of omega-3 fatty acids (DHA) was confirmed by TLC. Among the Mobile phases, ether: benzene at the ratio 2:2 gave results for the microalgae sample. The Rf value for the standard was 0.204 and 0.194 for the test sample. Which were similar to the Rf value of DHA.

Quantitative estimation of omega-3 fatty acids

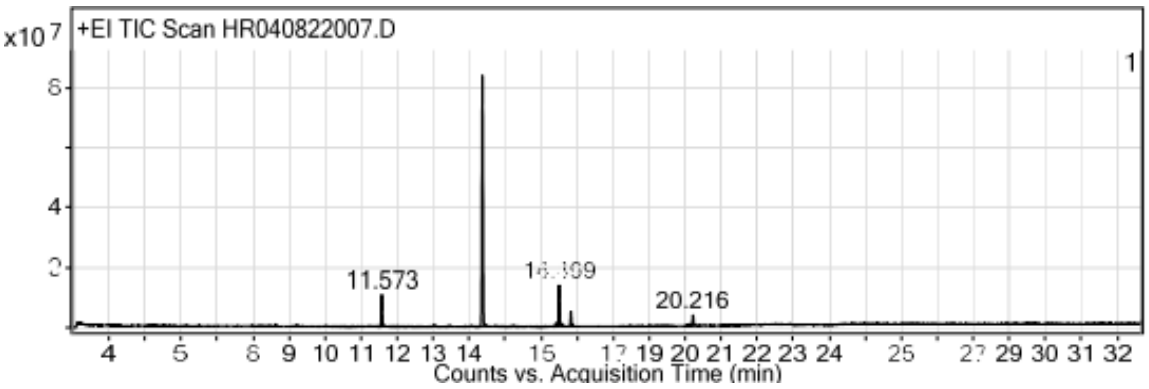

Figure 15: Results of GC-MS for fatty acid methyl esters of Chlorella vulgaris culture under optimized conditions.

Table 2: Identified fatty acids and percentage in fatty methyl esters.

Peak	Start	RT	End	Compound Name	Area	Area Sum Percent
1	11.522	11.573	11.659	Myrisitc acid	22446899.55	8.39
2	14.315	14.369	14.472	Palmaitic acid	193693525	72.43
3	16.375	16.419	16.45	Alpha-Linolenic Acid	2271729.18	0.85
4	16.453	16.499	16.546	Linoleic acid	27909906.71	10.44
5	16.546	16.567	16.603	Arachidonic Acid	1334195.15	0.5
6	16.782	16.826	16.902	Stearic Acid	9787733.04	3.66
7	20.031	20.061	20.094	Docosapentanic Acid	735398.01	0.27
8	20.108	20.146	20.177	Eicosapentanic Acid	1481883.42	0.55
9	20.177	20.216	20.287	Docosahexanic Acid	7765264.58	2.9

There were around 9 identified fatty acids such as Myristic acid, palmitic acid, Alpha-linolenic acid, linoleic acid, arachidonic acid, stearic acid, docosapentanoic acid, eicosapentanoic acid, docosahexanoic acid. In the fatty methyl esters, if we consider the omega 3 fatty acids the DHA was found to be 2. 9%, EPA was found to be 0.55 % overall the amount of omega 3 fatty acids was found to be 0.33% and omega 6 fatty acids were found to be 0.85% concerning the biomass (Figure 15, Table 2).

Discussion

Many studies conducted on microalgae are carried out conducted in photo bioreactors with CO_2 supply. Photo bioreactors being an expensive option led to this study, we investigated sustainable and effective alternatives for microalgae production. The options that were explored were the orbital shaker and plant tissue culture setup condition. From this study, we found that the plant tissue culture setup gave a better biomass yield in comparison to the shaker incubator. In the incubator shaker, the insufficiency of light might have led to the hindrance. Microalgae development depends on agitation[28], and the best RPM for maximal biomass and lipid output was discovered using a magnetic stirrer. In our studies, 250 RPM on magnetic spinner is the optimal RPM for the maximal yield of biomass (approximately 1.125g/l) and lipid (28.25% to biomass). This is the first attempt to optimize RPM for *Chlorella vulgaris* cultivation in a magnetic stirrer. In the previous study, where the RPM of the shaker incubator was optimized to 150, the biomass yield was 0.499mg/l, and the lipid yield was 15.98% of dry biomass was less in comparison to the yields of biomass and lipid at 250 rpm magnetic stirrer condition in the present study[29].

The best inoculum to use in Chlorella vulgaris cultures to maximize biomass, protein, and lipid output is 5%. When the 10% initial inoculum culture reached the nutrition depletion phase, it grew more slowly than the 5%, as reported by others [30]. Optimal temperature range for microalgal cultures is 25–35°C[13,18]. Previous studies show that 25ºC was an ideal temperature for growing *Chlorella vulgaris*[31]. pH 7 is ideal for microalgae to produce their maximum biomass and lipid output, which is in line with findings from earlier research[32–34].

Different types of media have been used in culturing of *Chlorella vulgaris* in algal research. BG11 media was found to be the optimal media in comparison to the bold basal media, Fog's medium, and M4N media[35]. In the present study, NPK and BG11 media are compared for cultivating *Chlorella vulgaris.* The

best yields of protein, lipids, and biomass of 4g/l biomass yield on day 7 which was higher compared to the previous study (approximately 1.64g/l on day 15) are obtained with BG11 medium[36]. On day 10, the proportion of lipids to biomass was 7.66%, highlighting the significance of the nutrition depletion phase in the formation of lipids. In the NPK media since the nitrogen content is high which results in a lower amount of lipid in comparison to the BG11 media. The productivity of lipids and biomass is similarly influenced by nitrogen concentration, demonstrated high lipid content over time with BG11 media. This emphasizes how important nitrogen depletion is to the lipid synthesis process in *Chlorella vulgaris.*

Glucose is the most effective carbon source for increasing biomass and lipids[36]. On the other hand, the risk of contamination was emphasized[37]. Therefore, experiments on glucose optimization were carried out to determine the ideal concentration for maximizing the yield of fat, protein, and biomass. 5g/l of glucose was found to give a maximum yield of biomass (1.39g/l) and lipid (19.29%) of biomass weight[38]. BG11 and 1.5 gm/l of glucose are the ideal concentrations with a maximum yield of biomass (approximately 2gm/l) and lipid (approximately 32.08%), varying on the specific culture conditions [36].

Under the conditions of salt stress, it was discovered that there was a reduction in biomass at the first addition of salt; yet, there was a notable rise in lipid (66.16%), which was higher than the previous report of (24%) [39]. Therefore, to reduce the possibility of salt impeding biomass growth and to increase the amount of lipids in biomass, salt was injected on the sixth day of the large-scale production of microalgae under optimal conditions.

This investigation revealed that, as compared to an orbital shaker incubator, a plant tissue culture setup was the best setting for cultivating *Chlorella vulgaris.* The goal of the study is to enhance *Chlorella vulgaris* biomass, protein, and lipid output by combining optimum conditions. Better biomass, lipid, and protein yields than individual optimized parameters are shown in the results. According to previous studies, 16.15% of lipids were obtained in the optimization studies concerning biomass[22]. 62.1% lipid was produced in this scale-up study, with yields akin to those of genetically modified strains for maximal lipid output [23]. *Chlorella vulgaris* can be employed as a source of production for DHA (2.9%) and EPA (0.55%) according to estimates of omega-3 fatty acids. This makes the *Chlorella vulgaris* a suitable option for producing omega-3 fatty acids.

Conclusion.

This study demonstrated that microalgae can be successfully cultivated in a laboratory environment with the presence of light. The ideal conditions for the growth of *C. vulgaris* biomass are pH 7 and 5% inoculum. BG11 media turned out to be the best choice when contrasted with NPK media. Maximum yield of biomass and lipid was found at 250 rpm with 1.5 g/l of glucose and NaCl. We developed a sustainable method to increase the production of protein, lipid, and omega-3 fatty acids in *Chlorella vulgaris* by combining various approaches (salt stress, glucose, and agitation). The large-scale studies combining all the optimized parameters into a single strategy demonstrated an increase in the yield of biomass, lipid, and protein. In addition to being fed to the poultry, the protein byproducts can be utilized in the production of snack bars that serve as nutritional supplements. However, maximizing agitation on a large scale and raising the yield sustainably and economically are the real challenges.

Statements and Declarations

Funding

The authors declare that no funding was received for conducting this study.

Conflict of interest /Competing interests

The authors declare that they have no

relevant financial or non-financial interests to disclose.

Authors' contribution

Ananya N Nayak, Akash S and Dhamodhar Prakash conceived and designed the research and did Data acquisition. Ananya N Nayak, Akash S, Renju Raju did data analysis. Ananya N Nayak and Renju Raju drafted the manuscript work. Dhamodhar Prakash supervised the research work and revised the manuscript. All authors have read and approved the manuscript.

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