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Research Article

Evaluation of Low Cost Medium for the Production of Lipids for Biodiesel and Carotenoids from Microalgae *Tetraselmis* Aff *Chuii*

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Abstract

There is a recent interest on lipids from microalgae for the production of biodiesel and high value co-products such as vitamins, essential proteins, PUFAs, antioxidants, pigments, food supplements etc. These are derived from the multifarious advantages of microalgae cultivation in comparison to vegetable cultivation; mainly due to higher biomass productivity as well as lipid productivity. *Tetraselmis*, a genus of phytoplankton, is a green algal genus within the order Chlorodendrales, mainly found in marine and fresh water bodies with high lipid content, cheaper photosynthetic mechanisms and less need for agricultural land. They are characterized by their intense green colored chloroplast, their flagellated cell bodies, the presence of a pyrenoid within the chloroplast, and a scale-produced the cal-wall. Besides the presence of potential lipids for biodiesel, produces a large variety of compounds of high commercial interest such as Poly Unsaturated Fatty Acids (PUFAs), carotenoids, food in aquaculture and biotechnology applications. In spite of the possibility of producing such high value commercial products, biomass production cost is still a bottleneck mainly for certain uses of the biomass, as for example biodiesel production. The cultivation medium represents one of the main expenses for the production of microalgael biomass and consequently is a very sensible point to be studied. On the other hand, changes in the medium cultivation need to be evaluated due to their impact on the biomass productivity and composition. Therefore, NPK (nitrogen, phosphorus, potassium) formulations containing nutrients of lower cost have been used to prepare medium cultivation for *Tetraselmis* sp. NMR (^{1}H, ^{13}C) and GC-MS techniques were used to explore the potential of the biomass for the biodiesel production as well as for carotenoids production. The results revealed that the use of medium with NPK formulation promoted only a slight decrease in the biomass, total lipids and carotenoids productivity. However, it might be overcome by using other NPK formulation concentration. The use of fertilizers led to an increase in PUFA and lowering of saturated fatty acids, which could be a problem considering the use of the oil for biodiesel production. In terms of carotenoids, lutein and zeaxanthin have been identified in the cyclohexane extract of the methanol: chloroform: water extract of the biomass, but neither β-carotene nor canthaxanthin were identified in this extract. The developed fast and cost-effective analytical strategy based on 1H NMR and GC-MS techniques has the potential for optimization of cultivation parameters to produce specific products, and thus contributes partly in the overall reduction in the cost of production of biodiesel.

Keywords: Biodiesel; Microalgae; *Tetraselmis*; Carotenoid; NMR; GC-MS

Abbreviations

DHA: Docosahexaenoic Acid; EPA: Eicosapentaenoic Acid; FAME: Fatty Acid Methyl Ester; FFA: Free Fatty Acid; GC-MS: Gas Chromatography- Mass Spectroscopy; MUFA: Monounsaturated Fatty Acid; NL: Neutral Lipid; NMR: Nuclear Magnetic Resonance; PL: Polar Lipid; PUFA: Polyunsaturated Fatty Acid; SFA: Saturated Fatty Acid; TG: Triglycerides; UFA: Unsaturated Fatty Acid

Introduction

It is a challenge for countries to increase their energy supply and at the same time keep the energy matrix "clean" in such a way that does not harm the environment, with the use of renewable fuels being a very reasonable alternative [1]. In this scenario, biomass fuels play an important role, and biodiesel is one of the most promising since it has environmental, economic and social advantages [2]. However, a disadvantage presented by biodiesel from higher plants, such as soybeans, is the huge use of arable land for its production. In this sense, microalgae have been studied as a source of lipids for biodiesel production [2,3]. The advantages of using microalgae for biodiesel production are: the facility of cultivation due to the possibility of being cultured in fresh, brackish or marine water, not requiring arable lands; biomass doubling in a short time; significant quantities of intracellular lipid and the capacity of CO_2 fixation which can be used as a strategy to control the environmental pollution [4].

Most lipids contain fatty acids and are usually classified into two categories based on the polarity of the main molecular group: neutral lipids, such as glycerides (TG) and Free Fatty Acids (FFA), which act as energy reservoirs; and polar lipids, such as phospholipids and glycolipids, which form the cell walls [5]. The proportion between these categories in the biomass depends on the species used and the nutritional and environmental conditions, for example, growth's phases, the shortage of specific nutrients and light cycles [6,7]. Moreover, the high proportion of saturated and monounsaturated fatty acids in microalgae is considered ideal from the standpoint of fuel quality [8].

However, the use of microalgae as a feedstock for biodiesel production faces some challenges such as high production cost of the biomass [2]. Once the spending with culture medium represents

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an important part of the biomass production costs, strategies for decreasing the costs with medium preparation should be employed for this purpose. In this context, the use of fertilizers could assist the composition of a low-cost culture medium. Furthermore, the extraction of commercially valuable compounds of biomass could be a strategy to turn economically feasible production of biodiesel from microalgae. Carotenoids should be a good choice since they are widely used in the food, pharmaceuticals, cosmetics and food industries, and presenting functions which is beneficial to health. In addition to the growing call for use of natural products instead of synthetics, there is an advantage of carotenoids synthesized by micro-organisms, due to being able to be obtained in a short time, regardless of the time of year [9]. Among the different species which can be used for biotechnological purposes, marine microalgae have huge advantage due to the bigger abundance of sea water compared to the freshwater required in the cultivation of freshwater microalgae. Also, the need for nutrient supplementation is decreased since sea water has reasonable amounts of nutrients and can simply be supplemented to the cultivation [10]. Agricultural fertilizers could be used as additives for sea water since they showed to be an alternative nutritional source for microalgae with similar results to conventional nutrients with lower costs [11].

Tetraselmis sp., a marine microalgae, has already shown its potential for the biodiesel and carotenoids production [12-16], but a deeper study has not been carried out using this species, including a better appreciation of lipid production, provided in this study by NMR analysis, and use of fertilizers for its cultivation. In addition, specifically this strain, *Tetraselmis* aff *chuii*, that is native from Brazil, had not been evaluated in a global way, regarding potential for biodiesel and carotenoids production.

Materials and Methods

Cell cultivation

The microalgae *Tetraselmis* aff *chuii* was donated by Prof Gleyci Moser from University of State of Rio de Janeiro and it was maintained at laboratory of Microalgae Biotechnology of National Institute of Technology. It was cultivated in 5L PET bottles with 4L of culture, under constant agitation by air injection at 3.0 L/min; cool white LED IP65 5050 of photonic flux density of 120 $\mu em^{-2}/s^{-1} \pm 10 \mu em^{-2}/s^{-1}$ and temperature maintained at 25 ± 2°C. Culture media Guillard F/2 [10] and the Guillard F/2 modified by replacement of the source of nitrate and phosphate with agricultural fertilizers in the same concentration of F/2 in terms of N and P were used. The source of nitrate was replaced by Chilean Saltpeter (Simple Mineral Fertilizer-VITAPLAN®) and phosphate source by simple Superphosphate (Mineral Fertilizer - HERINGER®). In total, six PET bottles were used in the cultivations, being that three with cell suspension in Guillard F/2 medium and half three with cell suspension in Guillard F/2 modified medium.

The cultures were monitored daily by removing 2.5 mL of the culture for Optical Density (OD) analysis in a spectrophotometer (Spectrum SP-1105) at 730 nm which were transformed into biomass concentration through regression equation. The cell count was also performed under a microscope (Olympus CX31) in order to determine the growth phase. Part of the cultivations was finished in the early stationary phase and part in the late stationary phase. Part of each culture was frozen to posterior lyophilization and lipid extraction, and part of this was filtered to carotenoid quantification.

Previously a study was conducted to obtain regression equation (Eq. 1) that correlates OD and dry mass for microalgae *Tetraselmis* aff *chuii*.

$$y=0.8747x-0.0365 \quad \text{.....Eq. 1}$$

where: y corresponds to the biomass concentration in terms of dry weight value and x corresponds to the OD value.

Extraction and quantification of total carotenoids and chlorophylls

Total carotenoids of the biomass produced were measured according to the methodology of Litchtenthaler [17]. After filtration on glass fiber membrane (Sartorius 0.7 µm to 1.2 µm), it was placed in a test tube and 10.0 mL of 100% methanol were added. The membrane was crushed and then placed in a freezer for 2 hours and the medium was filtered and centrifuged at 1372 g for 15 minutes at 4° C (Hettich centrifuge Zentrigugen - ROTIXA 50 RS). Finally, the extracts were analyzed in spectrophotometer (Thermo Scientific Genesys 10S UV-VIS) to obtain the absorbance (Abs.) at the wavelengths given in equations 2, 3 and 4. The pigment concentrations of the extracts were calculated using the equations below.

$$\text{Cl a (ext)} = 16.72^{*} \text{ Abs (665.2 nm)} - 9.16^{*} \text{ Abs (652.4 nm)} \quad \text{..... Eq. 2}$$

$$\text{Cl b(ext)} = 34.09^{*} \text{ Abs (652.4 nm)} - 15.28^{*} \text{ Abs (665.2 nm)} \quad \text{..... Eq. 3}$$

$$\text{C(ext)} = (1{,}000^{*} \text{ Abs (470 nm)} - 1.63^{*} \text{ Cl a(ext)} - 104.96^{*} \text{ Cl b(ext))/221} \quad \text{.....Eq. 4}$$

Where,

Cl a (ext) is the chlorophyll a concentration; Cl b (ext) is the chlorophyll b concentration and C (ext) is the total carotenoid concentration. The results were expressed in µ gmL^{-1} of extract. Total carotenoids in the cellular suspension was calculated taking into consideration the volume of suspension which was filtered and the volume of solvent used in the extraction; total carotenoid content in the biomass was calculated from the total carotenoids in the cellular suspension and the final biomass concentration. Total carotenoids productivity was calculated using the total carotenoids in the cellular suspension and correspondent cultivation times.

Extraction and quantification of total lipids

For the separation of the biomass from the liquid medium, the cultures were centrifuged at 1372 g, 10°C and 15 minutes (Centrifugal NEW TECHNICAL NT 825). The supernatants were discarded and the pellets were washed with modified WC medium for the removal of majority of salts until the WC medium has a very lower salt concentration than F/2 medium, and after testing it was found that there was no cell disruption (what was observed cells were washed with distilled water) [18]. After washing, cell suspensions were centrifuged again at the same conditions. The supernatants were discarded and cell pellets were lyophilized and stored in a freezer at -20°C until the lipid was analyzed.

The extraction and quantification were performed according to the Bligh and Dyer method, with some modifications [19]. The lyophilized sample (0.1 g) was ground and homogenized with 15 ml of a solvent blend of chloroform: methanol: water (2:1:0.3,v/v) and placed in an ultrasonic bath (Elma Elmasonic Analytical E30H-, 200W, 50Hz) for one hour at 30°C. The sample was filtered and the solvent was evaporated. The traces of water in the media were removed

by adding acetone and evaporation to dryness. The percentage of total lipids was determined according to the equation 5:

Lipids (%) = Mlipids/Mbiomass*100 ……Eq. 5

Where,

Mlipids is the lipid extracted mass and Mbiomass is the biomass sample used in the extraction.

Analysis of fatty acid profile

The transesterification of lipid extracted was performed according to the modified method described by Metcalfe and Schmitz [20]. The sample (10 mg) was homogenized with 1 mL methanolic KOH (1N) at 70°C. The solution was refluxed for three hours, cooled and finally refluxed with 1.5 mL to 2 mL BF3 at 70°C for 30min. In the end the trans esterified mass was extracted with heptane. The heptane layer was analyzed by GC-MS for the determination of fatty acids profile (FAME).

The analysis was carried out in a Shimadzu Gas chromatograph equipped with Quadruple Mass spectrometer using electron impact ionization. Compounds were profiled on a CP-Wax 52 CB column (30 m × 0.32 mm ID × 0.25 µm) using He as a carrier gas at 1.84 mL min^{-1}. Ramp programmed temperature conditions were 60°C for 2 min, 10°C min-1 to 200°C, 5 min to 240°C and finally hold for 7 min. Ionization voltage was 70 eV and sample injection was 1 µL. The FAMEs were identified by the use of standards and similarity using the NIST05 library, part of the GCMS solution program.

Total lipids and carotenoids extraction strategy for instrumental analyses

The total lipids were extracted in chloroform: methanol: water (2:1:0.3, v/v) blends using ultrasonic extraction procedure as described previously [21-23]. The carotenoids were extracted from the chloroform: methanol: water extracts by extracting with cyclohexane solvent, repeatedly three times. The extracts were dried after removal of cyclohexane completely.

NMR analysis

The 1H and 13C NMR analyses of lipid extracts were conducted using Bruker 500 MHz NMR Spectrometer. The samples were prepared by dissolving approximately 5 to 10 mg of microalgae extracts in 0.7 mL of CDCl3 containing internal reference TMS for 1H NMR recordings. For 13C NMR recordings, about 20 to 30 mg of the samples were taken under inverse gated parameters [22,24]. Critical analysis of the spectra was performed according to procedure described by Sarpal et al. [22].

Statistical analyses

Biomass productivity, total lipids productivity and total carotenoids productivity results were analyzed according to the statistical test Mann-Whitney to verify the significant differences between the different conditions with a significance level of 0.05. The "p-value (unilateral)" lower than 0.05 indicates significance value. This analysis was applied in two situations, one to compare the cultivation time in the same medium, and other to compare the type of medium in a same cultivation time.

Results

Biomass production

As presented in Table 1, the medium non supplemented with fertilizers and time cultivation of 16 days promoted the highest final biomass concentration of 0.592 gL^{-1} ± 0.030 gL^{-1} but the growth rate was similar to that of time cultivation of 12 days. According Mann-Whitney test, biomass productivity of the medium with fertilizers was significantly lower than that of the medium without fertilizers. Considering final biomass concentration and biomass productivity the higher cultivation time was only satisfactory for the medium without fertilizers. On the other hand, in spite of being low final biomass concentration attained with the medium with fertilizers the value was not significantly low, and it was still possible to balance the concentration of the fertilizers used in the medium in order to increase the final biomass concentration. It was possible to find similar biomass productivity for T. *chuii* (0.04 $gL^{-1}d^{-1}$) in comparison to that of *T. suecica* (0.32 $gL^{-1}d^{-1}$) cultivated in Fmedium [14,25]. However in our case both the strain and medium (F/2) used were different.

Total carotenoids production

In relation to pigments production, more specifically carotenoids, two parameters were considered, mainly content in dry biomass and volumetric productivity. As it can be observed in Figure 1, the carotenoid content (6.02 µgmg^{-1} ± 0.33 µgmg^{-1}) and carotenoid productivity (215.58 µg$mL^{-1}d^{-1}$ ± 11.86 µg$mL^{-1}d^{-1}$) were higher in F/2 with cultivation time of 12 days, and were found to diminished when cultivation time was increased from 12 for 16 days. Similar, productivity was found to decrease in case of cultivation carried out in F/2 supplemented with fertilizers. These results were more accentuated and significant in respect to carotenoid productivity. For carotenoid content, a great difference was observed between F/2 cultivated for 12 and for 16 days. However, F/2 supplemented with fertilizers resulted in the insignificant difference in comparison to cultivation in F/2 for the cultivation time of 16 days.

Similarly, total carotenoids content of 6 mgg^{-1} was reported for *T. suecica* grown in the F/2 medium and on the other hand, a higher value of 44 mgg^{-1} was attained for *T. marina* using the same medium [12,26]. It is worth to emphasize that besides the strain dependence

Table 1: Biomass final concentration and biomass productivity in biomass for cultivation in F/2 and in F/2 supplemented with fertilizers.

Cultivation medium	Time (days)	Final biomass concentration (gL^{-1})	Biomass productivity ($gL^{-1}d^{-1}$)
F/2	12	0.430 ± 0.005	0.036 ± 0.000
F/2	16	0.592 ± 0.030	0.037 ± 0.002
F/2 + Fertilizers	12	0.363 ± 0.016	0.030 ± 0.001
F/2 + Fertilizers	16	0.419 ± 0.021	0.026 ± 0.001

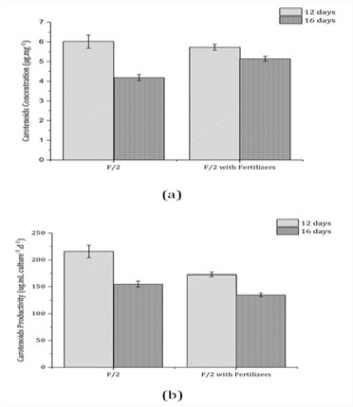

Figure 1: Total carotenoids concentration in biomass (a) and total carotenoids volumetric productivity (b) in cultivations using F/2 (control medium) and alternative medium (F/2 with fertilizers).

the total carotenoids content is dependent on the nutrient availability mainly N and P. Therefore, time cultivation, density of flux and initial concentration of these nutrients plays a very important rule on the total carotenoids content. Since the total carotenoids content in the present work was determined only after 12 and 16 days, it might be possible even using F/2 and F/2 with fertilizers media to obtain higher values at different time cultivation, as observed by Moussa et al. [26].

Total lipids production

Concerning total lipids content as mentioned in Figure 2, the values were in the range of 0.27 gg^{-1} and 0.32 gg^{-1}, which represented 27% and 32%, respectively, of lipids in the dry biomass. As evident from the graph, the difference observed was not so significant, except in the case of cultivation in F/2 supplemented with fertilizers for 16 days, which presented the smallest value. These values were highly promising since similar results (30%) were observed with *Tetraselmis* sp only under nutrient privation, more specifically nitrogen privation, being that under nitrogen repletion (2mM $NaNO_3$) the value was around 10% [13]. Similar values of 8.5% and 12.9% were observed using *T. suecica* F&M-M33 and F&M-M35, respectively [25].

Similar trend was observed with total lipid volumetric productivity, being that the values were in the range of 7.1 $mgL^{-1}d^{-1}$ and 11.1 $mgL^{-1}d^{-1}$. The higher value of total lipid content as well as of total lipid volumetric productivity was observed in case of F/2 for time cultivation of 12 days. This is exactly like observed in the total carotenoid evaluations.

Fatty acid profile by GC-MS

The results of fatty acid profile in *Tetraselmis* aff. *chuii* determined by GC-MS are presented in the Table 2.

^{1}H NMR analyses of neutral and polar lipids

The spectral features of algal oils of microalgae biomasses and vegetable oils for the nature of Neutral Lipids (NL) (triglycerides, free fatty acid) and Polar Lipids (PL) including their fatty acid profiles, and minor components such as sterols, hydrocarbons, etc., have been described in details in our earlier work [21-23]. However, these are briefly presented in order to discuss the ^{1}H NMR spectral features, particularly distinct features of unsaturated (n-3, n-6, n-9) fatty acid chains of triglycerides, and polyene chain of various carotenoid components.

The ^{1}H NMR spectra of chloroform: methanol: water extract, called as algal oils of biomasses of *Tetraselmis* aff *chuii* cultivated in F2 plus fertilizers medium (TSF2SPSE) are given in the Figure 3 and 4. The spectrum of extract of *Spirulina platensis* biomass cultivated in RM6 medium (SPRM6E) is also presented in Figure 3 for comparing the spectral features corresponding to triglycerides (TG) and carotenoids, as the features are quite prominent compared to spectral features of *Tetraselmis* aff *chuii* [23]. As shown in the Figure 3, the ^{1}H NMR spectra of SPRM6E and TSF2SPSE show signals characteristics of Triglycerides (TG) comprising of saturated and unsaturated long alkyl chain fatty acids (C16 to C22). Besides the signals due to TG, the signals corresponding to the functional groups of polar lipids are also prominently visible as marked in the Figure 3. The signals corresponding to functional groups of TG such as ester (OCH, sn2, peak no. 15; OCH_2, sn1 sn3, peak no. 12, 13), unsaturation (CH=CH; peak no. 17), carbonyl (CH_2C=O, peak no. 7), bis allylic ((-CH=CH-CH_2)n, peak no.10), allylic (CH_2CH=CH-, peak no. 6), terminal CH_3s of ω n-3 PUFAs (peak no. 2; 3 and more than 3 double bonds) and terminal CH_3 of n-6/n-9 unsaturated fatty acids components (peak no. 1), and (CH_2)n (peak no. 4) of long fatty acids alkyl chain of

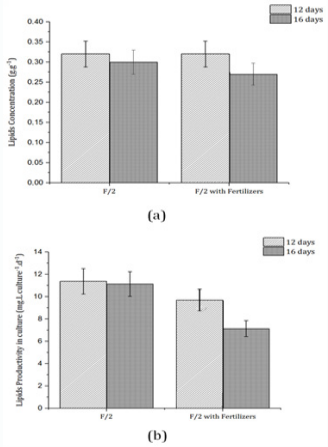

Figure 2: Total lipids concentration in biomass (a) and total lipids volumetric productivity (b) in cultivations using F/2 (control medium) and alternative medium (F/2 with fertilizers).

Table 2: Fatty acid profile of *Tetraselmis* aff. *chuii* cultivated in different media and time cultivation.

Fatty Acids	F/2 12 days	F/2 16 days	F/2 + Fertilizers 12 days	F/2 + Fertilizers 16 days
Tetradecanoic acid methyl ester	0.26			
Hexadecanoic acid methyl ester	27.54	31.82	26.3	26.12
9-Hexadecenoic acid methyl ester	6.87	10.04	8.57	8.7
7-Hexadecenoic acid methyl ester	0.39		0.58	0.35
7, 10-octadecanoic acid methyl ester	0.37		0.44	0.59
4,7,10-Hexadecatrienoic acid methyl ester	0.78		1.2	1.4
7,10,13-Hexadecatrienoic acid methyl ester	1		0.38	0.39
Octadecanoicacid methyl ester	0.96	0.95	0.81	0.64
9-Octadecanoic acid (Z) methyl ester	29.07	37.58	26.68	27.62
10-Octadecanoic acid (Z) methyl ester	4.38	4.72	3.36	3.45
7, 10 Octadedienoic acid methyl ester	0.23	1.06	3.59	3.72
9,12 Octadedienoic acid (Z, Z) methyl ester	3.93	3.05	0.5	0.58
6,9,12 Octadecatrienoic acid methyl ester	0.39			
9, 12, 15-Octadecatrienoic acid methyl ester	8.13	3.72	9.7	9.63
Eicosanoic acid methyl ester	1.63	3.15		
11- Eicosenoic acid methyl ester		1.53	0.82	0.92
5,8,11,14 Eicosatetraenoic acid methyl ester	0.4		0.91	1.03
4,7,10,13,16,19-Docosahexanoic acid methyl ester			4.2	4.24
Other compounds	13.67	2.38	11.96	10.62
Saturated (%)	30.4	35.9	27.1	26.8
Total unsaturated	55.8	61.7	60.9	62.6
Monounsaturated	40.7	53.9	40	41
Polyunsaturated	15.2	7.8	20.9	21.6
Not identified	13.7	2.4	11.9	10.6

both saturated and unsaturated fatty acids of TG and glyceroglyco/phospholipids (PL) are marked in the spectra (Figure 3 and 4) [21-23]. The signals at 4.38 to 5.2 ppm and 4.05 to 3.1 ppm were assigned to protons of OCH_2 and OCH ester groups, and CHOH and CH_2OH groups due to the glycerol part of polar lipid such as glycerophosphates and glycolipids as shown in the Figure 3. The peak no. 14 (OCH_2) and peak no. 15(OCH) are characteristic of phospholipids [22].

The presence of Poly Unsaturated Fatty Acid (PUFA) part of TG (three and more than three double bonds, ω n-3 types (C18:3, C22:6) were confirmed by the appearance of a characteristic triplet at 0.96 to 0.98 ppm due to terminal CH_3 (peak no. 2) in contrast to intense multiplets appeared at 0.7 to 0.93 ppm due to terminal CH_3 of saturated (C18:0, C16:0, C14 etc.) and unsaturated types (n-9 C18:1, n-6 C18:2) of components. The components C18:3 and C22:6 were further confirmed by expansion of the spectra as shown in Figure 4. The spectra also indicate less intense signals at 2.39 and 2.42 ppm due to carbonyl ($CH_2C=O$), and 2.84 ppm due to bis allylic (-CH=CH-CH_2)n compared to signals at 2.24 to 2.37 ppm ($CH_2C=O$) corresponding to ester group of long alkyl chain fatty acids components. These signals were assigned to C22:6. The chemical shifts of the functional groups characteristics of various components such as fatty acids including PUFAs, and carotenoids and phosphglycerolipids are compiled in the Table 3.

Discussion

Types of fatty acids including PUFAs by NMR and GC-MS

The chromatograms after the transesterification were used to determine the composition profile of fatty acid in the extracts. The major compounds identified as given in the Table 2 were 9-octadecanoic acid methyl ester (C18:1); 9,12 Octadecadienoic acid methyl ester (C18:2); 9, 12, 15-Octadecatrienoic acid methyl ester (C18:3); Hexadecanoic acid methyl ester (C16:0), Octadecanoic acid methyl ester (C18:0), 4,7,10,13,16,19-Docosahexanoic acid methyl ester (C22:6) (DHA), and other minor FAMEs constituents. It is pertinent to note that DHA was found to be present only in extracts of the F2+ Fertilizers media. The PUFA contents were also the highest in the extracts of the F/2 + Fertilizers media (20.9, 21.6%) compared to extracts of the without fertilizer media (15.2%, 7.8%).

It was possible to verify (Table 2) that the main fatty acids were hexadecanoic,9-hexadecanoic,9-octadecanoice9,12,15-octadecanoic, being that the higher percentages (31.82; 10.04 and 37.58) of the three first fatty acids were observed in the biomass from the cultivation with F/2 and 16 days of cultivation time, and for the last one it was observed 9.70% in the biomass produced in F/2 with fertilizers for 12 days. The percentage of saturated fatty acids was higher in F/2, and in general the higher percentage of unsaturated was observed in biomasses produced in media with fertilizers. In F/2 after 16 days of cultivation time in relation to other conditions, monounsaturated fatty acids were quite higher compared to PUFA. For F/2, saturated

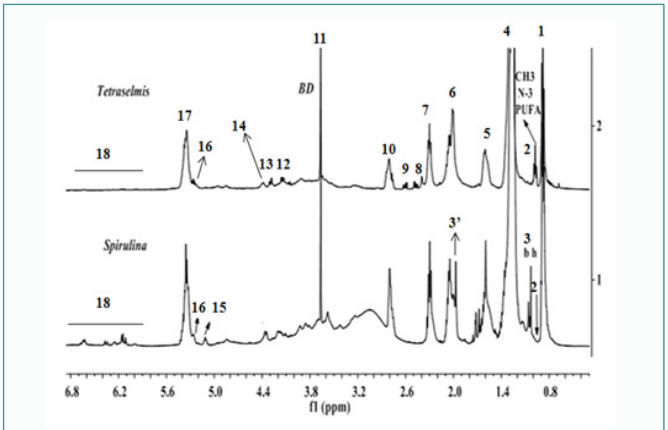

Figure 3: 500 MHz 1H NMR spectra of algal oils (TSF2SPSE) of Tetraselmis aff chuii biomass cultivated in F/2+ fertilizers medium and extract of Spirulina platensis biomass cultivated in RM6 medium (SPRM6E) [23]. Peak assignment refers to Table 3. 8=DHA, 9= Epoxy ester, 12,13,16=triglycerides, 11= (BD, biodiesel), 3,3'=bh=branched hydrocarbons (carotenoids) and 18= peaks in the region 5.6-6.8 ppm for carotenoids [23], arrow at position 2 (0.98 ppm) in spirulina showed absence of n-3 methyls.

Table 3: ^{1}H NMR characteristic chemical shift regions of functional groups of components of *Tetraselmis* aff *chuii* and *Spirulina platensis* as marked in the Figures 3 and 4.

Peak no.	Assignment	Component
1. 0.93 ppm	Terminal CH_3 (n-6, n-9)	SFA (C14:0, C116:0, C18:0) and UFA (C18:1, C18:2
2. 0.98 ppm (t)	Terminal CH_3 (n-3)	C18:3, C22:6 (PUFAs)
3. 1.05 ppm	CH_3 (b,h)	carotenoids
3 1.95 ppm	CH_3-C=CH	carotenoids
4. 1.27 ppm	(CH_2)n	Long alkyl chain of FA
5. 1.55 ppm (b)	CH_2-CH_2-C=O	Long alkyl chain of FA
6 to 6" 1.85-2.16 ppm	CH_2-CH=CH-	UFA (C18:1, C18:2, C18:3, C20:3, C22:6)
6" 1.95-2.025 ppm	CH_2-CH=CH	C18:1
6' 2.025-2.08 ppm	CH_2-CH=CH	C18:2
6 2.08-2.16 ppm	CH_2-CH=CH	C18:3+C22:5+C22:6 (PUFAs)
7. 2.16-2.42 ppm	CH_2-C=O	Alpha carbon of fatty acid/ester
8. 2.36 ppm	CH_2-C=O	C22:6 (DHA)
9. 2.42-257 ppm	CH_2O	Epoxy ester of C18:3, C22:5, C22:6
10 to 10" 2.6-2.9 ppm	(CH=CH-CH_2-)n	n=2, C18:2; n=3,C18:3; n=6, C22:6
10" 2.77 ppm	(CH=CH-CH_2-)2	C18:2
10' 2.81 ppm	(CH=CH-CH_2-)3	C18:3
10 2.84-2.85 ppm	(CH=CH-CH_2-)5 or 6	C22:6or22:5
11. 3.65 ppm	OCH_3	Methyl ester of fatty acids (biodiesel)
12, 13. 4.07-4.35 ppm (sm)	OCH_2	Triglycerides (TG)
14. 4.45 ppm	OCH_2	phosphglycerolipids
15. 5.16 ppm	OCH	phosphoglycerolipids
16. 5.26 ppm	OCH	Triglycerides (TG)
17. 5.16-5.6 ppm	CH=CH	unsaturated fatty acids/ester of TG
18. 5.8-6.8 ppm	-CH=C(CH_3)-CH=CCH_3-	Polyene chain of carotenoids

and total unsaturated fatty acids, except polyunsaturated fatty acids, were found to increase when the cultivation time was extended. However, the extension of the cultivation time promoted the increase of total unsaturated, monounsaturated and polyunsaturated fatty acids but a decrease in the saturated fatty acids when cultivated in F/2 with fertilizers. In general, these differences were quite pronounced in F/2 media. Fatty acid profiles different than in the present studies were reported with different strains of *Tetraselmis*. Pereira et al. [13] studying *Tetraselmis* sp observed a higher content of MUFA, while Moussa et al. [26] and Abiusi et al. [27] cultivating *T. marina* and *T. suecica* obtained a higher content of saturated fatt acids and PUFA, respectively. However, Moussa et al. [26] observed differences in the fatty acid profile depending on the concentration of nitrogen and phosphorous in the cultivation medium. In our work the proportion among saturated, MUFA and PUFA was maintained independently of the medium as the major class was MUFA in all the conditions tested.

The detailed ^{1}H NMR spectral analyses results as presented above has revealed that it could be possible to determine the fatty acid profile in a similar manner as described for GC-MS analyses of FAMEs of the extracts. The signals corresponding to unsaturated fatty esters (C18:1, C18:2, C18:3, C22:6) in the algal extracts of *Tetraselmis* aff *chuii* biomasses are conveniently marked in expanded spectra in the Figure 4. These extracts correspond to extraction of biomasses cultivated in different media (F2 and F2 plus fertilizers) and two different extraction procedures. The biomass cultivated in F2 was extracted in chloform: methanol: water (spectra a, TSF2E, and in the case of biomass cultivated with F2 plus fertilizers, two different extraction procedures were performed such as chloform: methanol: water extraction (spectra c, TSF2SPSE) and cyclohexane extraction of the chloform: methanol: water extracts (spectra b, TSF2SPSE'). These signals are marked as C18:1 (signal 6"), C18:2 (signals 6',10"), C18:3 & C20:3 (signals 6,10), and C22:6 (signals 6,8,10). The signals marked as 9 in the chemical shift regions of 2.42-2.66 ppm in the Figure 3, and visible more clearly in the expanded part of the spectra presented in Figure 4, were assigned to epoxy groups of linolenic or DHA or EPA types of components. The identities of epoxy types of fatty esters, were further confirmed by ^{13}C NMR and 2DNMR (HSQC-TOCSY) analyses as described previously [22]. The spectrum of *Spirulina platensis* did not show any signals at 0.98 ppm (arrow marked), thus indicate the absence of n-3 α- linolenic component (C18:3) in the extract (Figure 3). However, it contained n-6 γ-linolenic (C18:3) component as explained previously [23].

As shown in the Figure 3, the ^{1}H NMR spectra of algal extracts clearly showed very weak intensity signals in the region of 5.6 ppm to 7.0 ppm corresponding to conjugated unsaturated protons of polyene chain of carotenoids [28,29]. These signals were quite intense in case of SPRM6E compared to signals of TSF2SPSE, thereby indicated their higher amount in the extracts. The signals due to CH_3 of sterols were not observed in the regions of 0.5 ppm to 0.6ppm, as reported earlier in similar studies on microalgae extracts [22].

The sharp signal marked 11(BD) in the Figure 3 was assigned to OCH_3 group of methyl ester of fatty acids. The biodiesel might have been formed during ultrasonic solvent extraction of lipids in the presence of $CHCl_3$ and MeOH. There have not been any citation or work reported regarding the formation methyl esters without the use of acid or base catalyst during ultrasonic extraction of lipids. GC-MS analyses of the chloroform: methanol: water extracts has also confirmed methyl ester of fatty acids comprising of both saturated and unsaturated fatty acids. However, these observations need to be confirmed by way of more systematic work. The chemical shift of different functional groups observed in the various extracts is compiled in the Table 2. The observations regard to the presence of n-3, n-6 and n-9 fatty acid chains attached to glycerol back bone of TG has been confirmed by the GC-MS analyses of these extracts as described in the preceding section.

The 13C NMR analyses of the chloroform: methanol: water extracts have been carried out to confirm the presence of n-3 ϕ C18:3 and C22:6 as the signals due to each one were found to overlapped as discussed and shown in the 1H NMR spectra shown in the Figure 3, particularly in the region of 1.9 ppm to 2.015 ppm and 2.7 ppm to 2.9 ppm. As shown in the Figure 5, the 13C NMR spectral features of the extract TSF2SPSE, the unsaturated region clearly exhibited two demarcated signals due to n-3 ϕ C18:3 at 127.12 and 132.05 ppm, and n-3 ϕ C22:6 at 127.02 and 132.01 ppm. Since the spectrum of SPRM6E did not show signals at 14.29, 20.56, 127.12, 127.02, 132.06 and 132.0 ppm, this indicated the absence of ω n-3 C18:3 and C22:6 [23]. However, the spectra of SPRM6E (Figure 3) showed the presence of ω n-6 18:3 (γ- linolenic acid) as discussed in the ^{1}H NMR spectral analyses.

Characterization of carotenoids

^{1}H NMR chemical shift diverse ranges: Carotenoids are highly unsaturated hydrocarbons comprising of eight isoprenoids (branched chain unsaturated) units arranged in such a manner that the arrangement of isoprenoids units is reversed at the center of the molecule providing a perfect symmetry so that the two central methyl groups are in 1,6- positional relationship and remaining non-terminal methyl groups are in a 1,5-positional relationship. Carotenoids constitute of large polyene chain with 35to 40 carbon atoms, which is present in diverse structures of almost 750 in numbers. This polyene chain is also the feature mainly responsible for the chemical reactivity of carotenoids towards oxidizing agents and hence for any antioxidant role. Generally, carotenoids, namely trans-violaxanthin, antheraxanthin, astaxanthin, lutein, zeaxanthin, violaxanthin, neoxanthin and α and β -carotene have been found to be present in species and strains of microalgae *Tetraselmis* and these are important substitutes of antioxidants and vitamins present in plants [12,15,30]. The structure of some of the important and commonly found carotenoids with essential health effect such as α, β-carotene, astaxanthin, zeaxanthin, lycopene, lutein, etc. are mentioned in Figure 6 [28,29].

The NMR (^{1}H, ^{13}C; ^{1}D, ^{2}D) spectroscopic techniques have been widely applied for identification and characterization of macro and microalgae-based carotenoids. The general NMR spectral features of carotenids as mentioned in the literature will be discussed [31-33]. The 1H NMR characteristics spectral features of the polyene chain of every type of carotenoids as described in the Figure 6 are represented in the chemical shift range of 6.0 to 6.8 ppm (trans) and 5.0 to 7.2 ppm (cis) for -C=CH groups. The unsaturated part (C=CH-CH_3) of the ionene ring gives characteristics signals at 5.5 to 6.0 ppm (multriplets) and saturated part (CH_2) at 1.6 to 1.8 ppm (multiplets), respectively depending upon the nature and cis and trans structures of carotenoids. The signals due to CH_3 of the polyene chain exhibit singlets in the range of 1.9 ppm to 2.2 ppm characteristics of a particular carotenoid. The iso-methyls protons of the ionene rings in cis and trans position give clear cut singlet in the range of 0.85 ppm to 1.77 ppm. The chemical shift of protons at 7' and 8' positions are dependent upon the

position of double bond in the ionene ring struture, and thus can be characteristic for identification of similar types of structures such as zeaxanthin (6.10, 6.15 ppm) and lutein (5.43, 6.14 ppm) in a mixture of components. The carotenoids astaxanthin, and similar types of structures containing C=O linkage in the ionene ring, demonstrate characteristic signals due to cis and trans isomers for protons at 7, 8 and 7', 8' positions.

There are at least four numbers of cis isomers as 9-cis, 9,13-dicis, 13-cis and 13,15-dicis, and also equal numbers of trans isomers of beta-carotene. All trans isomers exhibit only one chemical shift pattern of four set of signals at 6.2 to 6.35, 6.38 to 6.4, 6.48 to 6.52 and 6.76 to 6.81ppm. The chemical shift range was extended from 6.72 to 6.82 ppm. Similarly, the cis isomer of astaxanthin can be distinguished from the trans isomer from the appearance of downfield signals of protons at positions 8, 8'at 6.13 ppm (d) and proton at position 11 at 6.9 ppm. The carotenoids without C=O groups in the ionene ring such as α and β-carotene, lutein etc. can also be distinguished from the carotene containing C=O in the ring from the distinct spectral features of protons of polyene, particularly iso methyls and C=C-CH_3 of the ionene ring. The chemical shift regions of astaxanthin start from 6.37 to 6.41 ppm, 6.5 to 6.6 ppm and 6.65 to 6.75 ppm compared to α and β-carotene, zeaxanthin and lutein at 6.1 to 6.18 ppm, 6.2 to 6.28 ppm, 6.3 to 6.4 and 6.58 to 6.65 ppm. The signals of protons of

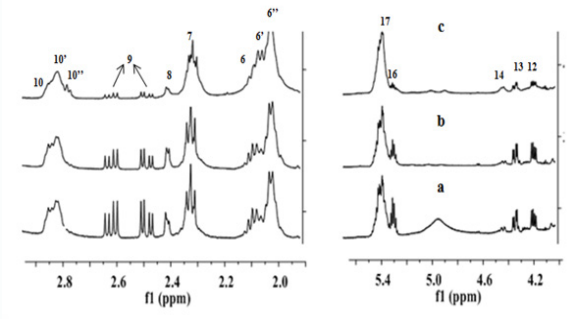

Figure 4: 500 MHz 1H NMR part spectra of algal oils of Tetraselmis aff chuii biomasses cultivated in different media, or extracted using different extraction procedures. a=TSF2E; b= TSF2SPSE', c= TSF2SPSE. Signals marked: 6"= C18:1; 6', 10"=C18:2; 6, 10'= C18:3; 6, 8, 10=C22:6; 9=Epoxy ester; 12, 13= triglycerides (OCH2, sn1, sn3); 16= OCH (sn2), 14=glycerophosphates.

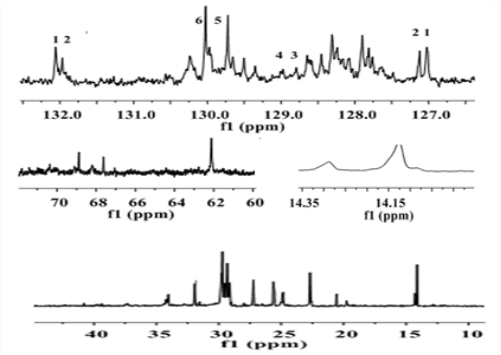

Figure 5: 125.7 MHz 13C NMR part spectra of algal oils (TSF2SPSE) of biomass of Tetraselmis aff chuii representing different chemical shift regions. Regions: 126-133 ppm= unsaturated regions (CH=CH), 14-14.35 ppm = terminal methyls of n-3 (14.28 pm), n-6 (14.1 ppm) and n-9 (14.13 ppm) fatty esters, 60-72 ppm=ester groups (OCH sn2; OCH2 sn1, sn3). Signals marked 1, 1=C22:6; 2, 2= C18:3; 3,4= C20:5; 5, 6=C18:2

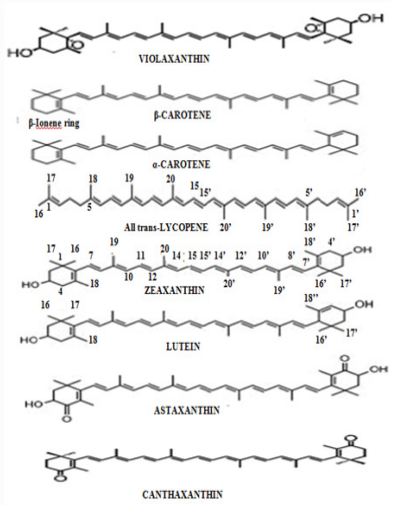

Figure 6: Structure of carotenoids generally found in Tetraselmis aff chuii, Spirulina platensis and Dunaliella salina [28,29].

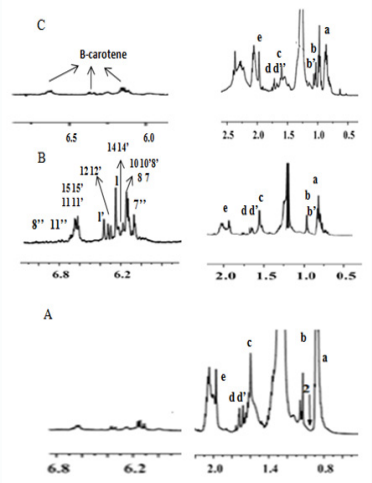

Figure 7: 500 MHz 1H NMR part spectra of extracts of (A) Spirulina platensis cultivated in RM6 medium (SPRM6E) [23], (B) Tetraselmis aff chuii biomass cultivated in F2+fertilizers (extraction of the chloroform: methanol: water extract with cyclohexane) (TSF2SPSE'), and (C) Dunaliella salina [34].

iso-dimethyl at position 16 and 17, 16' and 17' can be characteristic for their identification. The signals of iso-dimethyl of astaxanthin (1.2, 1.35 ppm), β-carotene (1.01 ppm), lutein (1.07, 0.99, 0.85 ppm) and zeaxanthin (1.07 ppm) and other similar types of carotenoids can be distinguished from their characteristic signals given in the brackets.

The part ^{1}H NMR spectrum of carotenoids enriched cyclohexane extract of *Tetraselmis* aff *chuii* (TSF2SPSE') as given in the Figure 7B shows signals corresponding to different protons of polyene chain as given in the brackets at 6.06-6.18 ppm (7, 7'), 6.1 to 6.20 ppm (8,8', 10,10'), 6.20 to 6.28 ppm (14, 14'), 6.28 to 6.33 ppm (12, 12') and 6.64 to 7.3 ppm (11, 11'). The chemical shift range and multiplets pattern more or less match with the lutein and zeaxanthin types of structures. The interpretation was also supported by appearance of signals due to iso-dimethyl (16,17,16',17') at 0.82 ppm, 0.98 ppm, and 1.02 ppm in the spectrum of the extract (marked a, b, b' in the spectra). These signals are assigned to lutein and zeaxanthin on comparison to the spectra of lutein showing signals at 0.99, 0.85, 1.07 ppm, and spectra of zeaxanthin at 1.074 ppm [28,29]. The signals due to CH_3 attached to

ionene ring at position 18 (1.68 to 1.72 ppm) marked d, d' and 18'(1.61 ppm) marked c in the spectra, and CH_3s of polyene part at position 19, 20, 19', 20' (~1.95 ppm, marked e in the spectra) matches with those of standards lutein (1.74, 1.63, 0.98, 0.85, 1.07,1.97, 1.97 ppm), and zeaxanthin (1.07, 1.74, 1.97, 1.97 ppm). The presence of signals of very weak intensities in the region of 6.82 to 7.2 ppm, marked 8" and 11", in the Figure 7B indicate the presence of cis isomers of astaxanthin or β- carotene or similar types of components in very small amount as explained in the preceding section. The spectrum of *Dunaliella* presented in the Figure 7C, which is a rich source of β- carotene, clearly shows characteristic signals in the region of 6.48 to 6.52 ppm, besides other signals in the region of 6.2 to 6.35 ppm, 6.38 to 6.4 and 6.7 to 6.8 ppm. These signals at 6.48 to 6.52 ppm are found absent in the spectra of extract of *Tetraselmis*, thus indicate the absence of β-carotene in *Tetraselmis* [30-34]. The spectrum Figure 7B due to *Tetraselmis* aff *chuii* indicates sharp singlets marked l and l', whose identity could not be known. The presence of canthaxanthin in the *Tetraselmis* is also ruled out as no downfield signals in the region of 6.8 to 7.2 ppm due to the deshielding effect of C=O in the ionene ring appeared. Similar types of components are identified in the spectrum of Figure 7A of *Spirulina platensis*.

Conclusion

Medium with fertilizers promoted lower biomass productivity which impacted the productivity in total lipids and carotenoids. However, in spite of the slight decrease in the productivity; its use can help to diminish the cost of the biomass production, and use of fertilizers can be a good choice to prepare this culture medium. The use of fertilizers led to an increase of PUFA and lowering of saturated fatty acids, which can be a serious concern when the oil is considered for the biodiesel production. The increase in the time of cultivation, in general, was not advantageous as it tended to lower productivity in biomass as well as in total lipids and carotenoids. NMR analyses of the methanol: chloroform: water extracts of the biomass produced in F/2 and F/2 with fertilizers showed similar pattern of fatty acids in comparison to GC-MS. In terms of carotenoids produced in the biomass grown using fertilizers,^{1}HNMR analyses has proved to be an convenient tool to characterize carotenoids in the cyclohexane extracts of biomass. The analyses have indicated the presence of lutein and zeaxanthin, besides possibility of the presence of astaxanthin in the extract. However, neither β-carotene nor canthaxanthin was found to be present in the extracts.

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References

1. Leung DY, Koo BC, Guo Y. Degradation of biodiesel under different storage conditions. Bioresour Technol. 2006:97(2):250-6.
2. Franco ALC, Lôbo IP, de Cruz RS, TeixeiraI MLL, de Almeida Neto JA, Menezes RS. Biodiesel de microalgas: avanços e desafios. Quim Nova. 2013;36(3):437-48.
3. Mata TM, Martins AA, Caetano NS. Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews. 2010;14:217-32.
4. Chisti Y. Biodiesel from microalgae. Biotechnol Adv. 2007;25(3):294-306.
5. Halim R, Danquah MK, Webley PA. Extraction of oil from microalgae for biodiesel production: A review". Biotechnol Adv. 2012;30(3):709-32.
6. Shifrin NS, Chisholm SW, Lund Univ. Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light dark cycles. J Phycol. 1981;17:374-84.
7. Sukenik A, Carmeli Y. Lipid synthesis and fatty acid composition in Nannochloropsis sp (Eustigmatophyceae) grown in light-dark cycle. J Phycol. 1990;26:463-9.
8. Demirbas A. Progress and recent trends in biodiesel fuels". Energy Convers Manag. 2010;50(1):14-34.
9. Eunice V, Pihetra OT, Lídia T, Helen T, Geciane T, Jamile Z, et al. Produção de carotenoides: microrganismos comofonte de pigmentos naturais. Quim Nova. 2009;32:2429-36.
10. Guillard RRL. Culture of phytoplankton for feeding marine invertebrates. Culture of Marine Invertebrates Animals. Plenum Publishing. 1975;29-60.
11. Lam MK, Lee KT. Potential of using organic fertilizer to cultivate Chlorella vulgaris for biodiesel production. Appl Energy. 2012;94:303-8.
12. Ahmed F, Fanning K, Netzel M, Turner W, Li Y, Schenk PM. Profiling of carotenoids and antioxidant capacity of microalgae from subtropical coastal and brackish waters. Food Chem. 2014;165:300-6.
13. Pereira H, Gangadhar KN, Schulze PSC, Santos T, Sousa CB, Schueler LM, et al. Isolation of a euryhaline microalgal strain, Tetraselmis sp. CTP4, as a robust feedstock for biodiesel production. Sci Rep. 2016;6:35663.
14. San Pedro A, González-López CV, Acién FG, Molina-Grima E. Marine microalgae selection and culture conditions optimization for biodiesel production. Bioresour Technol. 2013;134:353-61.
15. Clementina S, Christian G, Ida O, Genoveffa N, Elvira L, Adele C, et al. The green microalga Tetraselmis suecica reduces oxidative stress and induces repairing mechanisms in human cells. Sci Rep. 2017;7:41215.
16. Ita W, Muhammad Z, Hermien PK, Ragil S, Yann H, Vincent L, et al. Antioxidant activity of three microalgae Dunaliella salina, Tetraselmis chuii and Isochrysis galbana clone Tahiti. IOP Conf. Ser.: Earth Environ. Sci. 2017;55:012067.
17. Litchtenthaler HK. Chlorophylls and carotenoids: pigment photosynthetic biomembranes. Methods Enzymol. 1987;148:350-82.
18. Guillard RR, Lorenzen CJ. Yellow-green algae with chlorophyllide. J Phycol. 1972;8:10-14.
19. Bligh EG, Dyer WJ. A rapid method for total lipid extraction and purification". Can J Biochem Physiol. 1959;37(8):911-7.
20. Metcalfe LD, Schmitz AA. The rapid preparation of fatty acid estsaers for gas chromatographic analysis. Anal Chem. 1961;33:363-4.
21. Sarpal AS, Teixeira CM, Silva PR, da Costa Monteiro TV, da Silva JI, da Cunha VS, et al. NMR techniques for determination of lipid content in microalgal biomass and their use in monitoring the cultivation with biodiesel potential. Appl Microbiol Biotechnol. 2016;100(5):2471-85.
22. Sarpal AS, Teixeira CM, Silva PR, Lima GM, Silva SR, Monteiro TV, et al. Determination of lipid content of oleaginous microalgal biomass by NMR spectroscopic and GC-MS techniques. Anal Bioanal Chem. 2015;407(13):3799-816.
23. Sarpal AS, Ingrid CR Costa, Claudia MLL Teixeira, Diego Filocomo, Renata Candido, Paulo RM Silva, et al. Investigation of Biodiesel Potential of Biomasses of Microalgae Chlorella, Spirulina and Tetraselmis by NMR and GC-MS Techniques. J Biotechnol Biomater. 2016;6:220.
24. Sarpal AS, Silva PRM, Martins JL, Amaral JJ, Monnerat MM, CunhaRomeu J VS, et al. Biodiesel potential of oleaginous yeast biomass by NMR spectroscopic techniques. Energy Fuels. 2014;28:3766-77.
25. Rodolfi L, Chini Zittelli G, Bassi N, Padovani G, Biondi N, Bonini G, et al. Microalgae for Oil: Strain Selection, Induction of Lipid Synthesis and Outdoor Mass Cultivation in a Low-Cost Photobioreactor. Biotechnol Bioeng. 2009;102(1):100-12.
26. Dahmen-Ben MI, Chtourou H, Karray F, Sayadi S, Dhouib A. Nitrogen or phosphorus repletion strategies for enhancing lipid or carotenoid production from *Tetraselmis marina*. Bioresour Technol. 2017;238:325-332.
27. Ab Abiusi F, Sampietro G, Marturano G, Biondi N, Rodolfi L, D'Ottavio M, et al.

Growth, photosynthetic efficiency and biochemical composition of Tetraselmis suecica F&M-M33 grown with LEDs of different colors. Biotechnol Bioeng. 2014;111(5):956-64.

28. LaFountain AM, Pacheco C, Prum RO, Frank HA. Nuclear magnetic resonance analysis of carotenoids from the burgundy plumage of the Pompadour Cotinga (*Xipholena punicea*). Arch Biochem Biophys. 2013;539(2):133-41.

29. Rachel EK, Jessica LC, Morgan JC, Steven JS. Analysis Methods of Carotenoids. Wiley. 2012.

30. Ahmed F, Fanning K, Netzel M, Schenk PM. Induced carotenoid accumulation in Dunaliella salina and Tetraselmis suecica by plant hormones and UV-C radiation. Appl Microbiol Biotechnol. 2015;99(22):9407-16.

31. Englert G, Terje B, Jensen SL.1D and 2D NMR study of some allenic carotenoids of the fucoxanthin series". Magn Reson Chem. 1990;28:519-28.

32. Piero P, Renato T, Erminio M, Sergio P, Roberto R, Bjarne JK. Lipid extracts from different algal species: 1H and 13C-NMR spectroscopic studies as a new tool to screen differences in the composition of fatty acids, sterols and carotenoids. J Appl Phycol. 1992;4:315-22.

33. Deli J, Gonda S, Nagy LZS, Szabo I, Marton K, Vasas G, et al. Carotenoid composition of three bloom-forming algae species. Food Res Int. 2014;65:15-223.

34. Sarpal AS. Biodiesel and product potential of microalgae *Dunaliella salina* by NMR techniques. Adv Microbiol Biotechnol. 2018;11:555812.