The International Journal of Engineering and Science (IJES)
|| Volume || 7 || Issue || 6 Ver. I || Pages || PP 88-99 || 2018 ||
ISSN (e): 2319 – 1813 ISSN (p): 23-19 – 1805

Enhancement of Lipid Accumulation in *Chlorella* Sp. Farming Culture Held in Open Photobioreactors

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Highlights

- A new strain of *Chlorella* was isolated from a dam located in Paraíba, Brazil.
- The growth conditions for optimal biomass accumulation were established for *Chlorella* sp. grown in open photobioreactors (raceway ponds) in a semiarid region of Brazil.
- Decreased levels of NPK and an additional carbon source in the culture medium enhanced the lipid production and starch accumulation in *Chlorella* sp.
- The modified medium changed the profile of the fatty acid methyl esters produced by *Chlorella* sp.

----------ABSTRACT----------
Microalgae are recognized as source for renewable and environmental-friendly alternative to fossil fuels. Chlorella sp. are algae capable of accumulating lipids in specific growth conditions. In this study, it was established the growth conditions required for a new strain of Chlorella to accumulate biomass and lipids. The new strain, designated as Chlorella sp., was grown in open photobioreactors located in a semiarid region of Brazil, using nutrient conditions with high levels of nitrogen, phosphorus and potassium (NPK), leading to accumulation of biomass. In addition, the new strain of Chlorella was characterized in terms of physicochemical properties and starch accumulation. Decreasing the levels of NPK and supplementing the medium with an additional source of carbon enhanced the levels of starch and lipid accumulation and modified the profile of fatty acid methyl esters (FAMEs) produced by Chlorella sp. These results revealed possibilities in developing affordable and scalable microalgal lipids for biofuel production.
KEYWORDS: *Chlorella* sp. Lipids Fatty acid methyl esters Biofuels Photobioreactors

Date of Submission: 01-06-2018 Date of acceptance: 16-06-2018

I. INTRODUCTION

In the context of global climate change, the demand for renewable energy is increasing to replace fossil fuels. Microalgae have been broadly used for the development of large-scale production of renewable fuels. These organisms use photosynthesis to generate biomass from light and carbon dioxide. Oleaginous microalgae are environmental-friendly bioreactors for lipid production, which can be used for biodiesel generation, a carbon neutral alternative to petroleum fuels [1]. Microalgae are very attractive especially because of their high capacity of solar energy absorption and conversion to bioproducts, a process that is usually more efficient and faster when compared to other cultures [2]. In addition, microalgal culture can be performed in marginal lands, using nutrient-rich wastewater as medium [3]. Algae essentially require light, carbon source as CO_2 for autotrophic metabolism, growth medium and nutrients for reproduction.

Microalgae can be cultured in open or closed bioreactors. Cultures grown in open reactors are usually performed in raceway ponds. Raceways are mainly built in concrete and are made of a closed loop recirculation

channel, where the mixing and circulation are produced by a paddlewheel [1]. Therein, microalgae culture is pumped in a cyclic way, being directly illuminated from sunlight. The closed systems comprise more sophisticated and expensive reactors, such as tubular photobioreactors, made of plastic or glass oriented horizontally or vertically, where the sunlight is captured. These tubes are supplied with nutrients, sensors CO_2 and pumps. These bioreactors allow high growth rates and purity levels of microalgae biomass.

The nutrients required for microalgal growth are mainly nitrogen (N) and phosphorus (P). Nutrients such as phosphorus must be supplied in excess because of the phosphate complexes formed with metal ions, which leads to P depletion [1]. Nutrients are the most cost-involving requirement for biomass growth because their production involves energy utilization and natural resources. The current methods for algal cultivation utilize large quantities of nutrients, which can lead to overall imbalance of energy and environmental benefits. Thus, the optimization of algal growth using minimum nutrients is desirable [4]. In addition to nutrients, light is also an important factor that affects algal growth rates. Some studies revealed that the lipid content of the microalgae is strongly affected by different light intensities [5], [6], [7], [4].

Here, we use a new strain of an oleaginous *Chlorella* to study the lipid accumulation in these microalgae. We isolated and purified the new strain that was collected from a dam located in the farm Tamanduá, State of Paraiba, Brazil. This new strain was characterized in terms of physicochemical properties using different methods. Since the nutrient requirements are essential for growth, we tested different nutrient composition of the medium for optimal biomass and lipid accumulation. The algae were cultured in raceway ponds located in a region of Brazil (Paraiba) where the sunlight remains for large periods, as the days are longer throughout the year. It was verified that the optimal nutrient composition differs for biomass and lipid accumulation, with less nitrogen favoring the increase of lipid contents. In addition, the fatty acid composition of the new *Chlorella* sp. was analyzed, demonstrating that these important value-added products can be obtained from the new strain in optimal conditions.

II. MATERIALS AND METHODS

2.1. Establishment of Chlorella sp. and raceway culture conditions

A general schematic representation of the processes conducted in the present study is presented in a supplemental figure present in online version of this manuscript. A new strain of *Chlorella* was collected, isolated and purified from a dam located at Tamanduá´s farm, in the semiarid region of Paraiba, Brazil and designated as *Chlorella* sp.. The algae were precultured in a medium previously established by [8] for *Chorella ellipsoidea* (CT, control treatment medium; Table 1). The pre-culture was grown to the exponential growth phase in glass photobioreactors and a volume of 8% (v/v) of pre-culture was added to 900 mL of CT medium. The culture was kept in a growth chamber at 25°C, 12 klux and under 12 h light/12 h dark photoperiod for 15 days. The culture was initiated by inoculation of 339 mg/L of the pre-culture in raceway ponds built in concrete and consisting of 1.98 m width, 22 m length and 0.50 m depth, with total capacity for 10 m^3 of MC medium. The culture was supplemented with 0.04% $NaHCO_3$ (w/v) as additional carbon source [9], [10] and aerated using a pump (Aeromack CRE-01) with continuous flux of 1.4 $m^3.min^{-1}$ at 0.12 bar. The pH, temperature and light conditions were constantly monitored throughout the experiments that were performed in triplicate. Samples were collected aseptically in a time-course manner from 0-16 days for biomass growth, lipid analysis and determination of physicochemical parameters. After culture establishment, different nutrient compositions (T1 to T5) were tested to enhance lipid accumulation, according to Table 1.

2.2. Microalgae growth curve analysis

Aliquots (1 L) of each triplicate were collected every 2 days from the culture medium and submitted to centrifugation at 5000 x *g* for 15 min. The fresh weight (FW) was determined and the biomass was dried in the oven at 40°C for 24 h. After, the dry weight (DW) was determined and total biomass was calculated according to the formula FW-DW/DW.

2.3. Total lipid and sugar contents

The total lipid content was performed every 2 days in 1 L aliquots according to [11]. Briefly, the dried algae biomass was mixed with 6 mL of hexane and sonicated under 40 Khz for 30 min. at 20°C. The samples were centrifuged at 1000 x *g* for 10 min. and the organic phase was collected and transferred to a new tube. This procedure was repeated 3 times and the samples were evaporated under reduced pressure and dried in the oven at 60°C to constant weight. The total lipid content was represented as percentage of dry weight. Total sugar content of dry biomass was performed by the DNS method according to [12].

2.4. Physicochemical analysis of Chlorella sp.

After 16 days of culturing, the microalgae biomass was harvested by flocculation with 0.5 g/L of $Al_2(SO_4)_3.18\ H_2O$ during 30 min, followed by decantation, according to [13]. After, the cells were filtered through nylon (325 mesh), dried in oven at 40°C for grinding. The algae powder was used to determine the physicochemical parameters of *Chlorella* sp. grown in different culture conditions. The parameters analyzed were moisture, ashes, water activity, total proteins, chlorophyll, pheophytin and amino acid and mineral profiles. The methods used to measure each one of these parameters can be found at the Supplementary Data available in online version of this manuscript .

2.5. Starch analysis

The starch extraction was carried out according to [14]. Briefly, 100 g of dry biomass was homogenized in water (1:2; w/v) for 5 min. using a blender. After, the mixture biomass:water was filtered through a sieve (200 mesh) and washed 4x with distilled water at 5°C. The extracted starch was dried at 40°C until constant weight and the starch content was expressed as percentage (g/100g). To verify the starch ultrastructure of *Chlorella* sp. under different treatments, it was used scanning electron microscopy (SEM). The dried starch was powdered using a ball mill (Vieira, SP, Brazil) and the ground tissue was fixed in stubs, metalized with an alloy of gold/palladium and observed under a scanning electron microscope with energy dispersive X-ray analysis (SEDX).

2.6. Fatty acids composition

The fatty acid methyl esters (FAMEs) profile of *Chlorella* sp. was determined by transesterification. The algae powder was saponified with a solution containing $NaOH\text{-}CH_3OH$ at 75°C in a thermostated water bath for 30 min. A 1:2 (v/v) boron trifluoridemethanol solution was added to the saponified samples that were shaken for 30 min. The esterified oil samples were then mixed with hexane and the upper-layer FAMEs profile was characterized by a Varian 3400CX gas chromatograph (Varian, USA) fitted with a DB-FFAP Megabore column (0.545 mm inner diameter x 30 cm length) and a flame ionization detector. 1 µL of FAMEs solubilized in chloroform was injected at chromatograph by splitless mode using nitrogen at 2 mL/min as carrier gas. The injector and detector temperatures were 250 and 270°C, respectively. The initial oven temperature was set at 120°C for 1 min, followed by 3 sets of temperature: (*i*) increasing to 180°C at a rate of 5°C/min, and then held at this temperature for 2 min; (*ii*) increasing to 200°C at a rate of 2°C/min, and then held at this temperature for 2 min; (*iii*) increasing to 220°C at a rate of 5°C/min, and then held at this temperature for 25 min. The identification of FAMEs was performed using Varian Star software v. 4.51, comparing retention times and peak areas of the samples with known standards.

III. RESULTS AND DISCUSSION

3.1 Effects of medium composition on biomass, lipid contents and total sugars of Chlorella sp.

Microalgae cultivation is a challenging process that is pivotal for biomass productivity and lipid quality and quantity [15]. The nutrient requirements, light intensity and carbon sources are important factors determining microalgae growth. Thus, this study attempted at enhancing biomass and lipid accumulation in a new strain of *Chorella*, isolated from a farm located in the semiarid region of Paraíba, Brazil, aiming to explore new possibilities in developing affordable and scalable algal lipids for biofuels. *Chlorella* sp. was grown in open-system bioreactors known as raceway ponds, taking advantage of the prolonged sunlight periods of this specific Brazilian region. First, the microalgae were cultured in a medium previously established for *Chlorella ellipsoidea* (CT treatment) by), [8] where N and K are provided as nitrate and phosphate (Table 1). Using MC as culture medium throughout 16 days, it was observed a maximum biomass accumulation of 4.74 g/L on day 10 (Fig. 1A). Changing the medium composition, by decreasing the levels of N, P, K, and supplementing it with $NaHCO_3$ as an additional carbon source (Table 1), had different effects on biomass accumulation. In general, decreased levels of NPK negatively affect biomass accumulation in *Chlorella* sp. When the culture was performed with medium T3, T4 or T5, which presented the lowest levels of NPK (Table 1), biomass accumulation decreased by 12.8%, 13.9% and 18.5% on day 10, respectively, when compared to MC medium (Fig. 1A). These results were not surprising since N, P and K are essential nutrients for algae growth, especially N, the main constituent for protein and chlorophyll synthesis. However, the nutrients are one of the most cost-involving factors for growth, and decreasing the amounts of nutrient requirements can provide affordable industrial processes using algae as producers of value-added chemicals. The lack of nutrients in the culture medium can be perceived by algae as a stress factor. The lipid content is known to increase under stress conditions such as high light and temperature, low nitrate and phosphate levels, carbon imbalance and salinity [16]. Therefore, this strategy was used here to enhance lipid accumulation in *Chlorella* sp.

As observed in Fig. 1B, lower amounts of N, P, K and Fe and an additional carbon source ($NaHCO_3$), obtained when the algae was grown in T4 and T5 enhanced lipid accumulation, despite the decreased amount of biomass (Fig. 1A). Compared with algae grown in MC, which presented 4.3% of lipid accumulation of total biomass, T4 and T5 provided 18.7% and 17.8% of lipid accumulation on day 10 (Fig. 1B), where maximum growth was achieved for MC (Fig. 1A). The maximum levels of lipids were observed on day 10, with longer periods of culture decreasing algae lipid contents in our system. These results demonstrated that lipid accumulation in *Chlorella* sp. may be enhanced up to 4-fold when culture conditions are modified. Lipid biosynthesis is a complex process that involves several biochemical reactions and depend mainly on the physiological status of the algae. The pathway leading to lipid biosynthesis initiates with the formation of acetyl CoA by the acetyl-CoA carboxylase gene (*ACCase*), which carboxylates the acetyl CoA that can be directed to fatty acids biosynthesis, depending on the algal physiological needs. This is considered a key step towards carbon assignment for lipid synthesis [17]. Microalgae are able to store lipids in different cell types in the form of oil globules [16], and it has been shown that neutral lipids are produced in these cells through the conversion of either starch or carbon to lipids [18]. The types and levels of lipid accumulation greatly vary among different algal strains, and the microalgal lipids that can be used for biofuel production usually have from 14-20 carbon chains [19]. Our results demonstrated that N, P, K and Fe deprivacy is able to enhance lipid accumulation in *Chlorella* sp. These results are in accordance to other studies, which demonstrated that reduced levels of these nutrients was able to increase lipid accumulation up to 70% of dry cell weight [20], [21], [22], [23]. However, most of the studies where lipid levels enhanced drastically were performed in culture flasks, which allow controlled conditions. Our study demonstrated enhanced lipid accumulation under industrial scale, since the microalgae was cultured in open-system bioreactors. A possible explanation for increased lipid levels in *Chlorella* under nutrient depletion is discussed by [24], which demonstrated that an increase of reactive oxygen species and decrease of photosynthesis in *Chlorella pyrenoidosa* were accompanied by dynamic gene expression changes directly related to lipid accumulation, suggesting that this phenomenon was achieved through physiological stress.

The total sugar content analysis revealed that the treatments T3, T4 and T5 that presented higher levels of $NaHCO_3$ were responsible for increased sugar content in *Chlorella* sp. (Fig. 1C). In these treatments, it was observed sugar levels representing 12%, 17% and 20% of dry biomass for treatments T3, T4 and T5, respectively. Total sugar contents of the treatments that presented lower levels (T1, T2) or absence (TC) of $NaHCO_3$ were, on average, around 8%. These results suggest that the additional carbon source used to supplement the culture medium was effective, since it was observed a concentration-dependent increase of sugars as $NaHCO_3$ (used as additional C source) increased. However, we cannot ruled out the effect of low levels of N on enhanced sugar content in our strain since other studies demonstrated that decreased levels of N increased the production of sugars. For instance [25] verified that decreasing the levels of N from Bold Green Medium (BG11) led to an approximately 30% increase of total sugar contents in *C. vulgaris*. It is possible that N deprivacy deviates the metabolic pathway from amino acid synthesis to sugar production, but this phenomenon cannot be confirmed at this point.

3.2. Physicochemical and biochemical analysis of Chlorella sp. grown in different medium compositions

The physicochemical data were analyzed in the dry biomass after 16 days of *Chlorella* grown in different culture media (CT, T1, T2, T3, T4 and T5) and are shown in Table 2. The moisture content of the samples presented similar levels, around 6.7% on average, with treatment T3 showing a statistically significant increase of moisture content (6.89%) compared to control CT (6.74%). Similar results were obtained for water activity (a_w), which is the measurement of the free form of freezable water. The a_w is a very important parameter since water strongly affects the microbiological and chemical stability, physical properties and technological processes of algal biomass [26]. Our results demonstrated that, in general, a_w variability between the different treatments was not statistically significant in the dry biomass of the algae. The ashes content decreased in the biomass as the levels of NPK were lower and the additional C source was higher, in a dose-dependent manner, varying from 26.3% to 21.67%. It is well known that the ash content of several algae differs according to the media composition, environmental factors and climate conditions [27], [28], but the reason why this phenomenon occurs is not understood. Ash is an important component that negatively affects industrial downstream processes, and decreasing the ash content in microalgae for biofuel production is desirable [29].

The total protein levels of the new *Chlorella* strain is considerably high, representing 42% of the dry biomass when the algae were grown in the nitrogen-rich medium (CT). As expected, when N content was lowered, the total amount of proteins also decreased in a nitrogen concentration-dependent manner, as verified for the treatments T1 to T5. Interestingly, increases in the carbon source in the medium with less N led to a more pronounced decrease of total proteins, as verified from treatments T3 to T5 (Table 1), indicating an imbalance

between C and N partitioning when algae were grown under these conditions. The amino acid profile of Chlorella grown under different treatments is shown in a supplemental table provided in supplementary data available in online version of the manuscript. Most of the analyzed amino acids decreased by 50% for T5 treatment (lowest level of N and higher C) compared to control treatment, with exception of aspartate and glutamate, which reduced 83% and 72%, respectively, for T5 compared to TC. The pH of the dry biomass for all treatments was alkaline, ranging from 7.94 to 8.21, in general not statistically different from each other, indicating that the changes observed in cell composition did not alter the pH drastically. The photosynthetic pigments chlorophyll and pheophytin also decreased in *Chlorella* sp. grown in low levels of N, especially for the treatments T3 to T5, where N concentration was very low. The decrease in the photosynthetic pigments might be responsible for the lower accumulation of algal biomass verified for these treatments (Fig. 1A), since the photosynthesis may be impaired under these conditions. We also performed an analysis of the mineral profile in the dry biomass of *Chlorella* sp. for the different treatments (Table 3). Under control conditions (CT medium), the most accumulated minerals were iron, manganese and copper, representing 42%, 18.9% and 18.7%, respectively, in addition to low levels of zinc (0.7%) and sodium (1.03%). Most of the mineral levels diminished when the algae were grown in low levels of NPK, with exception to calcium for the treatments T2 to T5, which presented increases up to 144% compared to control treatment. The comprehensive analysis of mineral composition in *Chlorella* is important especially because the microalgae have been increasingly being considered as a source of these components for animal and human nutrition [30].

Altogether, the physicochemical data revealed that changes in the growth conditions can alter important parameters of algal physiology such as protein and photosynthetic pigments levels, but the physical parameters responsible for successful downstream industrial processes were not drastically affected. The next steps are the metabolic engineering of this new *Chlorella* strain and the improvement of the media modification in order to enhance lipid contents with no penalties on biomass accumulation.

3.3. Starch analysis of Chlorella sp. grown in different medium compositions

A key feature of microalgae-based production of biofuels is their ability to rapidly switch their energy storage forms from polysaccharides, such as starch, to lipids [24]. It is known that nitrogen limitation is one factor that can be used to increase starch production [31], [32]. Therefore, we aimed to study the starch accumulation in our *Chlorella* strain grown in different levels of N in the medium. As observed in Fig. 2A, under control conditions, the algal production of starch was 3.53% of the dry biomass. The decreased levels of N in the treatments T1 to T5 led to enhancement of starch levels after 16 days of culture in open photobioreactors, with levels ranging from 4.47% to 13.85%. It can be noticed that the starch content greatly increased when the addition of the carbon source was enhanced, revealing a 4-fold increase for the treatment T5 (13.85%) when compared to control. In addition, we performed scanning electron microscopy (SEM) to verify the ultrastructure of the starch granules produced by our strain. When algae were grown under control conditions where starch production has normal levels (CT), the granules seem to be agglomerated and could be observed fragments of the cell wall attached to them (Fig. 2B - CT). As we decreased the levels of NPK and increased the carbon source in the medium, the starch granules turned more clearly visible, as observed in Fig. 2B (T1 to T5). The micrographs showed that the starch granules from *Chlorella* sp. were irregular and apparently brittle, possibly because of the mechanical extraction method that was used. Other methods can be used to obtain spherical and defined starch granules from algal cells, such as chemical treatments where the pigments are extracted first and the starch is hydrolyzed, as described in [33]. However, the structure of the granules obtained by mechanical extraction, which is an economically viable procedure, does not interfere with downstream industrial processes.

The metabolic feature that allows some algae to switch from starch-rich heterotrophy to lipid-rich photoautotrophy has a great impact in commercialization of large-scale microalgal production systems as the switch between food and fuels allows flexibility for commercial operations [24]. The comprehension of molecular mechanisms behind starch-to-lipid metabolism is emerging. A recent study using transcriptome analysis of *Chlorella pyrenoidosa* demonstrated that under heterotrophy, the genes most up-regulated were from the tricarboxylic acid cycle, respiratory chain, oxidative phosphorylation, gluconeogenesis, glyoxylate cycle and amino acid metabolism, while the less expressed were from fatty acid and oxidative pentose phosphate metabolism. It was verified that the switch from heterotrophy to autotrophy was marked with a redirection of metabolism, where carbon skeletons from photosynthesis and starch degradation may be directly channeled into fatty acid and protein biosynthesis [24]. Our results also suggest that in *Chlorella sp.* grown under different medium compositions, a redirection of metabolism was achieved, as the decrease of NPK and supplementation with additional carbon source changed the accumulation of biomass (Fig. 1A), levels of lipids (Fig. 1B), total sugars (Fig. 1B), protein content (Table 2) and fatty acid levels (Table 2) and profile (Table 3; see below).

3.4. Effects of medium composition on FAMEs profile of Chlorella sp.

Fatty acid methyl esters (FAMEs) are esters of fatty acids produced by transesterification of lipids. The physical characteristics of FAMEs are closer to those of fossil diesel fuels, therefore FAMEs can be used as a renewable energy source, commonly known as biodiesel. The advantage of biodiesel relies on its non-toxicity and biodegradability. Here, we used a strong base as catalyst to perform the transesterification of *Chlorella* sp. lipids produced under different culture conditions and analyzed the profile of the FAMEs that were generated. As observed in Table 2, in control conditions (MC medium) the monounsaturated fatty acids (MUFAs) represented the majority of produced FAMEs, achieving ~30% of the dry weight, followed by 22.6% of saturated fatty acids (SFAs) and 15.6% of polyunsaturated fatty acids (PUFAs). Omega-6 and omega-3 fatty acids represented 12.37% and 3.2% of the total dry weight of lipids produced from MC medium culture, respectively. To improve the yield of biofuel production and provide superior oxidative stability, a high percentage of SFAs and MUFAs is preferred [15]. Thus, our results demonstrated that lipids from *Chlorella* sp. grown in MC medium might be a good source for biodiesel generation, due to the high levels of these fatty acids. However, SFAs- and MUFAs-rich lipids are prone to solidify in a low temperature environment, a negative factor for industrial processes. In this case, PUFAs have a better performance on the cold-flow property of biofuels, but the high level of unsaturated bonds in fatty acids favors oxidation, which is also not desirable in industrial biofuel production. In this context, *Chlorella* sp. grown in MC4 medium, that presented low levels of NPK, demonstrated interesting results for biodiesel production, mainly because of the balance between SFAs, MUFAs and PUFAs, with levels of 22.69%, 26.24% and 24.21%, respectively (Table 2). Our study also evidence that changes in the culture medium composition can have major effects on FAMEs levels. For instance, the levels of omega-3 (ω -3) drastically increased in medium containing low contents of NPK compared to MC medium that presented only 3.2% of ω -3. For MC1, MC2, MC4 and MC5, the average level of ω -3 was ~15%, while in MC3 these levels reached 8.2% (Table 2), evidencing that even small changes in the culture medium could lead to great changes in FAMEs contents. In this case, the only difference between MC3 and MCs 1, 2, 4 and 5 is the lower amount of the additional carbon source, $NaHCO_3$ (Table 1).

The changes in the culture medium not only modified the fatty acids levels in *Chlorella* sp., but also modified FAMEs profile. As observed in Table 3, when the algae were grown in control MC, the fatty acids produced had from C12 to C18, with high proportions of C16-C18 fatty acids, a composition usually found in *Chlorella* and that favors filter plugging point for fuels produced by algal biomass [33]. However, when the algae were grown in the culture mediums with low amounts of NPK the lipids produced C20 to C24 fatty acids, and the levels of these additional fatty acids also varied between the different culture conditions (Table 3). At this point, we are not able to rationalize the phenomenon behind the additional FAMEs produced by *Chlorella* sp. grown in low levels of NPK.

3.2. Effects of the culture conditions on pH, temperature and light intensity

Algae are phototrophic organisms that require for optimal growth sunlight in a proper light intensity, CO_2, water, inorganic salts such as N, P and K and an optimal temperature ranging from 20-30°C [34]. As described above, the main goal of this study was to establish optimal conditions for industrial scale lipid production by a new strain of *Chlorella* isolated from a farm located in Paraiba, a semiarid region of Brazil. Since the algal growth was performed in raceway ponds, which are open bioreactors, we decided to verify if some important growth conditions would change throughout the daylight period. Thus, we measured pH, temperature and light intensity parameters from 7 am to 4 pm in our different culture conditions (Fig. 3). As observed in Fig. 3A, light intensity had an increase from ~70000 lux at 7 am to ~90000 lux at noon, which is expected for this daytime as the sunlight reaches its maximum. The pH was, on average, kept at 8.5, with small but not statistically significant changes between different time periods and culture conditions (Fig. 3B). The temperature increased from 26°C at 7 am to 30°C at 12 pm, and finally decreased to 27°C by the end of the day. Even the small increase in temperature at noon, which is expected because of the high irradiance, did not reach levels above the optimal algal growth temperature range, usually from 20-30°C, as mentioned above. With respect to the compositions of the culture medium (MC-MC5), none of them was able to modify drastically the parameters examined. These results suggest that our growth conditions of *Chlorella* sp. in raceway ponds are suitable for large-scale production of algal biomass for enhanced lipid accumulation and further biodiesel production. We are currently optimizing such conditions and engineering this new *Chlorella* strain in order to adjust an improvement of biomass with increased levels of lipid accumulation.

IV. CONCLUSION

This study reinforced that lipid accumulation in microalgae can be enhanced by decreasing the levels of NPK in the culture medium. The levels and the profile of fatty acids methyl esters that can be used for biodiesel

production were also modified when the algae were grown in low nutrient levels and an additional carbon source. The new strain of *Chlorella* grown in raceway ponds located in a semiarid region of Paraiba, Brazil, present new opportunities for the development of suitable, affordable and scalable microalgae-based biofuels.
E-supplementary data of this work can be found in online version of the paper.

ACKNOWLEDGMENTS

The authors would like to thank the Tamanduá farm team and to CAPES (Coordination for the Improvement of Higher Education Personnel) for granting the scholarship during the whole period of JFL's PhD.

Funding

This work was supported by CAPES (Coordination for the Improvement of Higher Education Personnel).

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Figure captions

Figure 1. Total biomass (A), lipid contents (B) and total sugar levels (C) of *Chlorella* sp. grown in different medium composition. The algae were grown for 16 days in open photobiorecators and samples were collected every 2 days for the measurements. The results are from three independent experiments, performed in triplicate for each treatment and are expressed as percentage of dry biomass. Statistics were performed applying the Tukey test at 5% probability. CT: control treatment.

Figure 2. Time-course analysis of pH (A), luminosity (B) and temperature (C) of the culture media for *Chlorella* sp. growth. The samples were collected from photobioreactors at 7 am, 12 pm and 4 pm for the different treatments. Results are expressed as the average of three independent experiments, using three biological replicates for each experiment. Statistics were performed applying the Tukey test at 5% probability. CT: control treatment.

Figure 3. Starch analysis of *Chlorella* sp. grown in different medium composition. (A) Starch levels (% of dry biomass) of *Chlorella* sp. grown during 16 days in open photobioreactors. **(B)** Scanning electron microscopy of starch granules processed from the dry biomass for each treatment. The micrographs are representative from three independent experiments. CT: control treatment.

Figures and Tables

Table 1. Physicochemical and biochemical analyzes of the dry biomass of *Chlorella* sp. grown for 16 days for different treatments

Parameter	Treatment					
	CT	T1	T2	T3	T4	T5
Moisture content (g/100g)	6.74bc	6.81b	6.64de	6.89a	6.59e	6.71cd
Water activity (a_w)	0.496ab	0.486bc	0.503a	0.490abc	0.463d	0.473cd
Ash content (g/100g)	26.28a	24.15b	22.39c	22.26c	21.88d	21.67e
Proteins (g/100g)	41.72a	36.57b	32.44b	28.82c	21.89d	21.70d
pH	8.21a	8.15ab	8.08ab	7.94b	8.02ab	8.14ab
Chlorophyll (mg/g)	7.67a	6.54b	6.30b	5.50c	5.50c	5.20c
Pheophytin (mg/g)	4.43a	4.18a	3.70b	3.49b	3.43bc	3.12c

Note: averages followed by the same lowercase letter in the lines do not differ statistically by the Tukey test at 5% probability.

The International Journal of Engineering and Science (IJES)
|| Volume || 7 || Issue || 6 Ver. I || Pages || PP 88-99 || 2018 ||
ISSN (e): 2319 – 1813 ISSN (p): 23-19 – 1805

Table 2. Media composition for growth of *Chlorella* sp. in open photobioreactors

	Macronutrients			Micronutrients					C source
Medium	KNO_3	$KH_2PO_4.7H_2O$	$FeSO_4.7H_2O$	H_3BO_3	$MnCl_2.4H_2O$	$ZnCl_2$	$CuSO_4.5H_2O$	3. $(NH_4)_2O.7MoO_3.4H_2O$	$NaHCO_3$
CT	1.25	1.25	0.02	2.9	1.81	0.11	0.08	0.018	NA
T1	0.312	0.625	0.01	2.9	1.81	0.11	0.08	0.018	0.4
T2	0.156	0.312	0.005	2.9	1.81	0.11	0.08	0.018	0.4
T3	0.156	0.156	0.0025	2.9	1.81	0.11	0.08	0.018	0.8
T4	**0.156**	**0.156**	**0.0025**	**2.9**	**1.81**	**0.11**	**0.08**	**0.018**	**1.0**
T1	0.156	0.156	0.0025	2.9	1.81	0.11	0.08	0.018	1.2

The nutrients are expressed as g/L. CT refers to control treatment, which was the medium composition where the algae presented the higher biomass accumulation. T1 to T5 are the different medium compositions. In bold is represented the treatment where the lipid accumulation in *Chlorella* sp. was higher when compared to CT.

Table 3. Mineral profile of *Chlorella* sp. dry biomass for the different treatments

Parameter	Treatment					
	CT	T1	T2	T3	T4	T5
Calcium (g/100g)	7.15e	5.60f	17.50a	16.48b	9.60d	15.65c
Cobalt (mg/100g)	5.62a	4.90b	3.67c	2.87e	3.22d	3.55c
Nitrogen (g/100g)	5.36a	4.61b	4.27c	4.03cd	3.84d	3.55e
Copper (mg/100g)	18.70a	14.20b	12.40c	8.70f	11.20d	9.77e
Sulfur (mg/100g)	4.60e	4.30f	5.31d	7.61a	6.38b	5.75c
Iron (mg/100g)	42.10a	35.50b	24.12e	26.76c	23.00f	24.64d
Phosphor (g/100g)	4.18b	1.45c	1.27d	1.05f	5.12a	1.15e
Magnesium (g/100g)	1.66e	2.25b	2.19c	2.35a	1.36f	2.03d
Manganese (mg/100g)	18.90a	13.10b	6.91d	5.74f	7.44c	6.39e
Nickel (mg/100g)	8.90a	7.69b	5.28d	4.58e	6.10c	5.24d
Potassium (g/100g)	2.02a	1.00b	0.53d	0.46e	0.42e	0.91c
Sodium (g/100g)	1.03a	0.49d	0.21e	0.55c	0.62b	0.47d
Zinc (mg/100g)	0.70e	1.2b	1.60a	0.89cd	0.93c	0.87d

Table 4. Fatty acid levels (g/100g) of *Chlorella* sp. grown for 16 days in open photobioreactor under different treatments

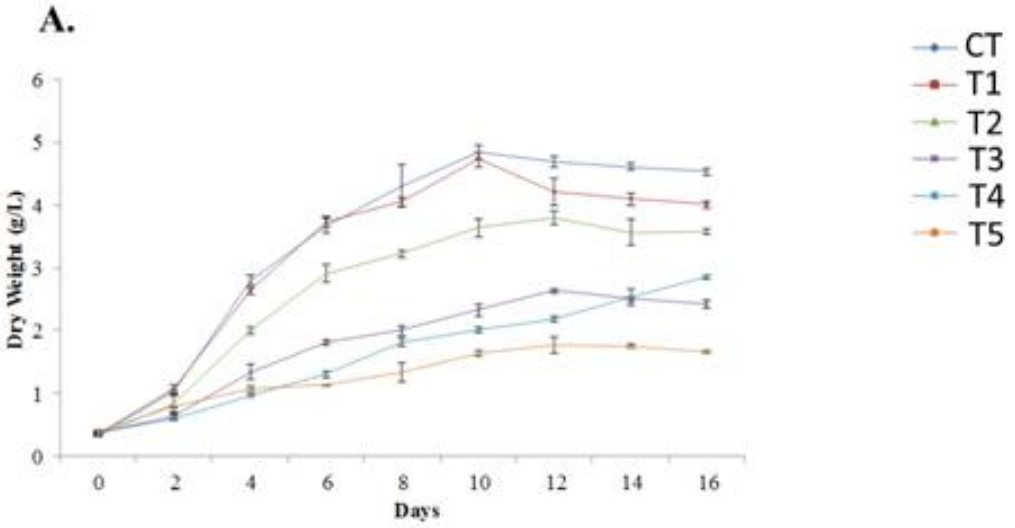
Fatty acids	Treatment					
	CT	T1	T2	T3	T4	T5
Saturated	22.67	21.16	18.91	24.39	22.69	19.31
Monounsaturated	30.47	26.45	26.53	18.48	26.24	28.50
Polyunsaturated	15.57	26.21	28.09	15.93	24.21	27.36
Omega-3	3.20	13.67	15.54	8.18	13.06	14.79
Omega-6	12.37	12.54	12.55	7.75	11.15	12.57
Total trans isomers	0.41	3.02	3.80	2.91	3.04	3.16
NI	26.47	18.76	18.28	33.89	19.43	17.27

NI – Not identified

Table 5. Fatty acids profile of *Chlorella* sp. grown for 16 days in open photobioreactor under different treatments

Fatty acids (g/100g)		Treatment					
		TC	T1	T2	T3	T4	T5
C12:0	Lauric	1.49	0.65	0.61	1.22	0.85	0.76
C13:0	Trichloroic				0.11		
C14:0	Myristic	0.58	0.63	0.63	2.18	0.70	0.72
C15:0	Pentadecanoic	2.54	1.21	1.04	1.52	1.75	1.28
C16:0	Palmitic	10.67	13.24	12.73	13.42	13.17	12.30
C16:1	ω 7 Palmitoleic	-	0.50	0.54	1.01	0.35	0.54
C17:0	Margaric	3.62	1.05	0.71	0.86	1.35	0.94
C17:1	Cis-10-heptadecanoic	9.83	6.60	6.52	0.18	7.69	8.11
C18:0	Estearic	3.77	3.82	2.61	3.76	4.03	2.95
C18:1	ω 9 trans Elaidic	-	0.19	0.35	0,61	-	0,28
C18:1	ω 9 Oleic	20.64	19.36	19.25	17.08	18.20	19.85
C18:2	ω 6 trans-linoleic	0.41	2.83	3.44	1.96	3.04	2.89
C18:2	ω 6 Linoleic	12.37	12.54	12.55	7.75	11.15	12.57
C 20:0	Araquidic				0.31		
C20:1	ω 11 cis-11- eicosenoic		-	0.21	0.20	-	-
C 18:3	ω 3 trans-linolenic				0.33		
C18:3	ω 3 alpha-linolenic	3.20	13.67	15.54	8.18	13.06	14.79
C22:0	Behenic	-	0.33	0.35	0.21	0.58	0.35
C24:0	Lignoceric	-	0.21	0.22	0.43	0.25	-

Figure 1.



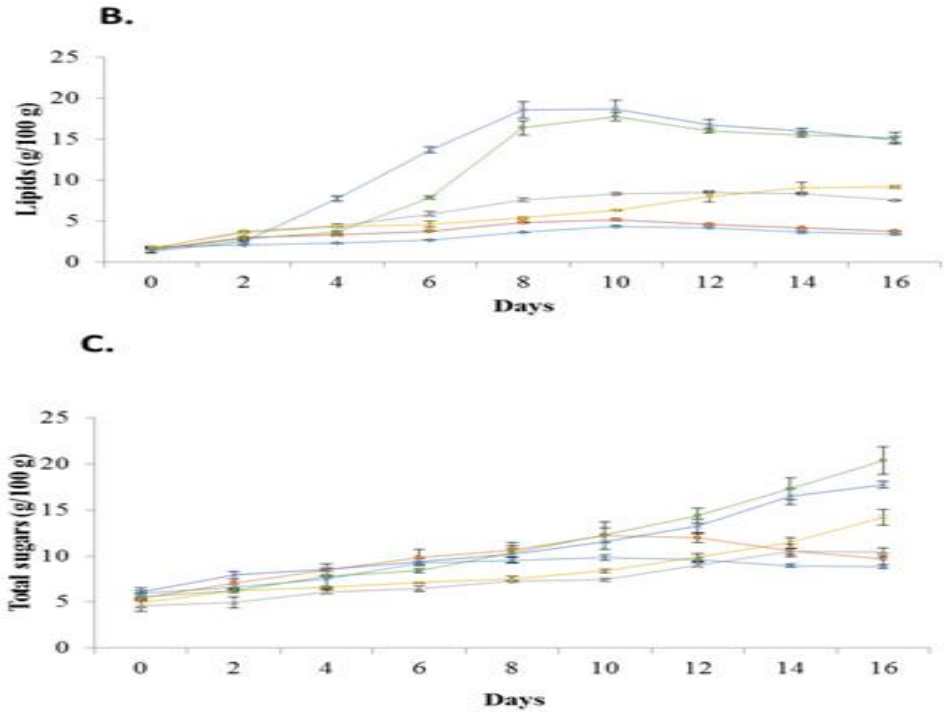
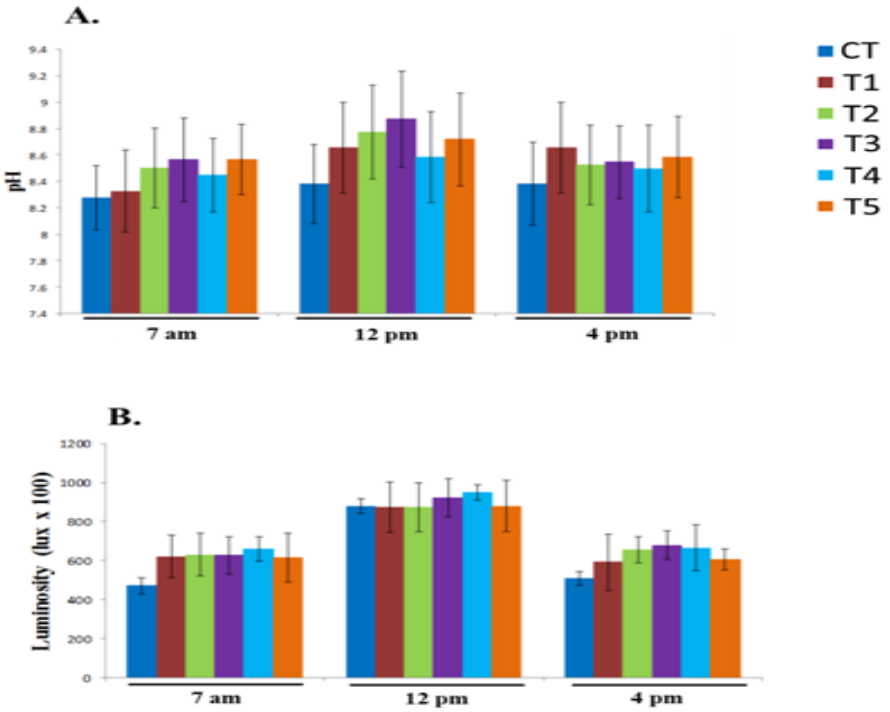

B.
Lipids (g/100 g)
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Days
C.
Total sugars (g/100 g)
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Days

Figure 2.


A.
pH
7 am
12 pm
4 pm
CT
T1
T2
T3
T4
T5
B.
Luminosity (lux x 100)
1200
1000
800
600
400
200
0
7 am
12 pm
4 pm

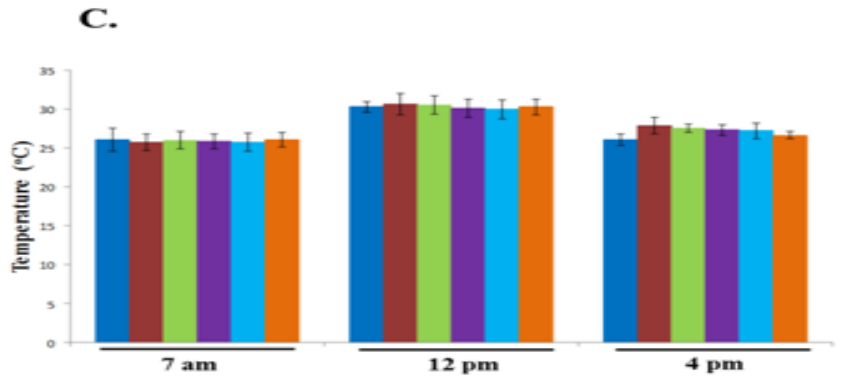
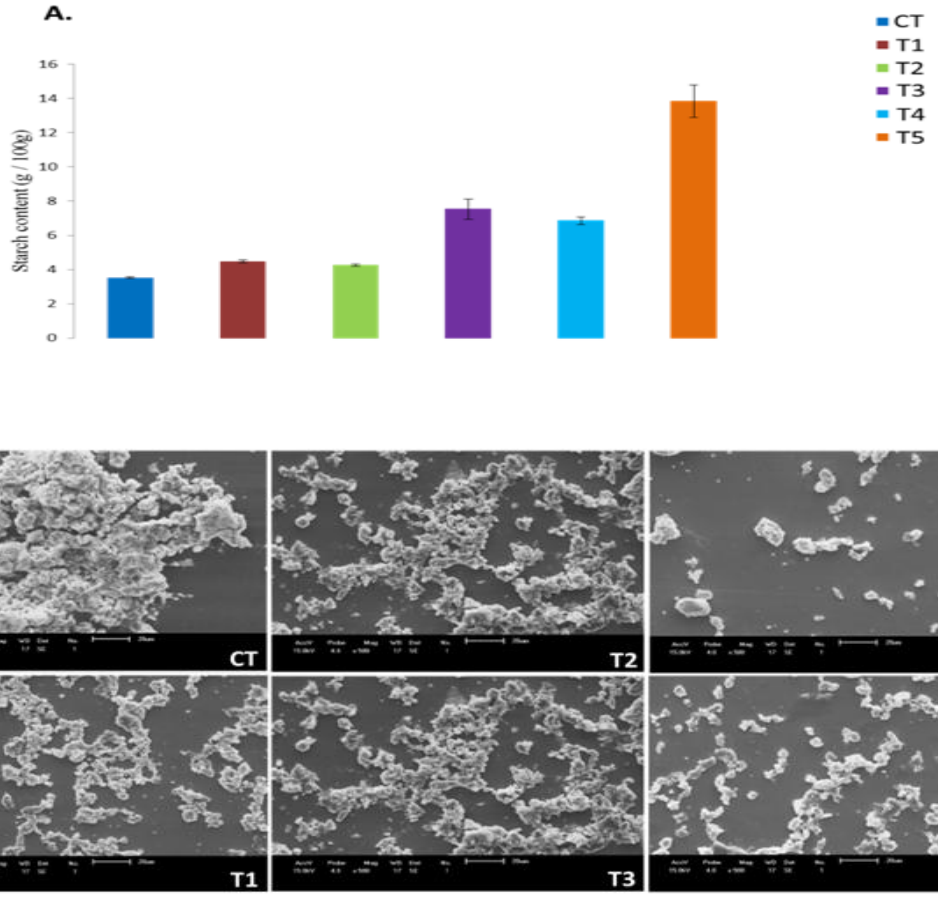


Figure 3.



José Franciraldo De Lima.” Enhancement of Lipid Accumulation in Chlorella Sp. Farming Culture Held in Open Photobioreactors." The International Journal of Engineering and Science (IJES) 7.6 (2018): 88-99