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Total Lipid Production of Microalgae Chlorella sp. and Chlorella Vulgaris under Mixotrophic Culture Conditions

Producción de lípidos totales de la Microalga Chlorella sp. y Chlorella Vulgaris en condiciones mixotróficas de cultivo

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Resumen La microalga de agua dulce *Chlorella sp.* y *Chlorella Vulgaris* se cultivaron en un fotobiorreactor a escala de laboratorio y se evaluó su cinética de crecimiento y producción de lípidos totales. Estas especies se cultivaron en medio Conway modificado, con fotoperíodo 8:16 y ventilación mecánica. El tiempo de evaluación fue de veinte días, la densidad celular se determinó diariamente mediante cámara de Neubauer y mediante densidad óptica mediante espectrofotometría a 685 nm. Se determinó la tasa de crecimiento y el porcentaje de lípidos totales producidos, las biomasas obtenidas se analizaron por espectroscopia infrarroja transformada de Fourier (FTIR). La comparación de las cinéticas de crecimiento mostró una diferencia significativa entre el crecimiento de las especies, alcanzando valores máximos de crecimiento de 7.022.000 cel·mL-1 para *Chlorella sp*. y 8.750.000 células · mL-1 para *Chlorella vulgaris.* La especie *Chlorella sp* mostró una tasa de crecimiento de 0,298 días-1, ligeramente superior a la de *Chlorella vulgaris*, que fue de 0,279 días-1. Los resultados también muestran que la especie con mayor producción total de lípidos fue *Chlorella sp.* (27,3%), frente a *Chlorella vulgaris* (21,5%).

Palabras claves Microalgas; *Chlorella sp.; Chlorella vulgaris;* lípidos; cinética.

Abstract The freshwater microalgae Chlorella sp. and Chlorella Vulgaris were cultured in a laboratory-scale photobioreactor and their growth kinetics and total lipid production were evaluated. These species were grown in a modified Conway medium, with an 8:16 photoperiod and mechanical air ventilation. The evaluation time was twenty days, the cell density was determined daily using the Neubauer chamber and by optical density using spectrophotometry at 685 nm. The growth rate and the percentage of total lipids produced were determined, the biomasses obtained were analyzed by Fourier transform infrared (FTIR) spectroscopy. The comparison of the growth kinetics showed a significant difference between the growth of the species, reaching maximum growth values of 7,022,000 cells · mL-1 for Chlorella sp. and 8,750,000 cells · mL-1 for Chlorella vulgaris. The Chlorella species showed a growth rate of 0.298 days-1, which was slightly higher than Chlorella Vulgaris, which was 0.279 days-1. The results also show that the highest total lipid production species was Chlorella sp. (27.3%), compared to Chlorella vulgaris (21.5%)

Keywords Microalgae; *Chlorella sp.; Chlorella vulgaris;* lipids; kinetics.

1 Introduction

As oil reserves are on the way to depletion, microalgae-based biotechnology has become an area of considerable interest as it is one of the most promising alternative sources of renewable energy due to its high productivity (Khoo et al, 2020) (Banerjee et al, 2016).

In addition, microalgae may produce various substances of commercial interest such as nutrients, pigments, drugs, minerals, etc. These microorganisms may be used in bioremediation processes of water contaminated with different pollutants and may become an efficient alternative for wastewater treatment (Nagappan et al, 2021) (Matamoros et al, 2016).

Microalgae are prokaryotic or eukaryotic unicellular photosynthetic organisms with a simple structure that allows their rapid cell growth, due to this they may produce a large amount of biomass in short periods under controlled conditions (dos Santos et al, 2015). Microalgae require varied factors to grow, such as light and a carbon source, which are their main energy sources. Other limiting factors for the growth of microalgae are temperature, salinity, pH, photoperiod, and the addition of nutrients (Tagliaferro et al, 2019). The microalgae *Chlorella sp*. and *Chlorella vulgaris* are species with biotechnological potential, they belong to Chlorophyceae (green algae), a color obtained from chloroplasts, which are the structures in charge of photosynthesis.

The microalgal culturing may be autotrophic or heterotrophic and a combination of both, i.e., mixotrophic. In such conditions, the organism simultaneously assimilates CO_2 from the culture medium to carry out photosynthesis and uses organic carbon sources from the medium to also produce metabolites for survival and reproduction (Morais et al, 2021). Currently, oleaginous microalgae are considered a source of second-generation biofuels because they can accumulate substantial amounts of triglycerides when subjected to environmental stress conditions (Barahoei et al, 2020). The lipid components of microalgae serve as the basis for different bioenergetic uses such as biodiesel, bioethanol, biogas, and biohydrogen. Neutral lipids accumulated during photosynthesis by microalgal cells are stored as TAG (triacylglycerols). These neutral fats are made up of a triple chain of esters, attached to a glycerol molecule. TAGs are subsequently converted into efficient biodiesel by transesterification (Ananthi et al, 2021).

Total lipids, which comprise polar membrane lipids (phospholipids and glycolipids) and intracellular neutral lipids, generally constitute 15-35% of algal cell mass on a dry basis and may be isolated from cell mass with the use of various methods that use solvents, such as chloroform, methanol, hexane, and polar / non-polar solvent mixtures, such as methanol/chloroform (2: 1 v / v) or isopropanol/hexane ((Manisali et al, 2019) (Yang et al., 2015).

To establish a microalgae production system, a deep knowledge of the isolation, purification and maintenance techniques of strains is necessary, as well as knowledge of their morphology, life cycle, and biochemistry. This will determine the feasibility of the final biotech use. This research aims to compare the growth kinetics and the production of total lipids of the microalgae *Chlorella sp*. and *Chlorella vulgaris* under mixotrophic culture conditions.

2 Materials and methods

2.1 Microalgae cultures

The microalgae *Chlorella sp.* and *Chlorella vulgaris*, were obtained from the collection of microalgae cultures in the Biotechnology laboratory of SENA - CINAFLUP, located in the city of Cartagena de Indias, Colombia. These species were selected for their oleaginous characteristic and for their adaptive capacity to the culture conditions established in the laboratory.

The procedure began with the inoculum. Each culture was prepared or bioaugmented from 10% inoculum and 90% working medium. The time for each bioaugmentation phase was 6 days. The inoculum for each test was obtained as follows: 10 mL of culture (1

mL of the *Chlorella sp*. Strain with 9 mL of medium) was placed in a test tube for 6 days, then this culture was passed to an Erlenmeyer flask of 250 mL and it was brought to a volume of 100 mL, then 500 mL and finally, an arrangement with two bioreactors, both with a capacity of 4 L. The growth of the microalgae was fully monitored by reading the optical density and cell counting daily until death phase. In the culture, the working conditions were temperature of 27 ± 2 ° C, pH of 7 and it was controlled by a buffer solution (Beuckels et al., 2015) (Carpogno et al. 2015). A mechanical blower without CO_2 injection was used for the air supply. A light intensity of 450 Lm was used (Ouyang et al., 2015) with photoperiods of 8h: 16h (day: night). The cultures and bioassays were done in triplicate.

2.2 Culture medium

Modified CONWAY® medium was used for culturing the strains (Tompkins et al., 1995). The culture medium was prepared in the biotechnology laboratory of SENA – CINAFLUP with the composition listed in Table 1.

Table 1. Composition of the Conway® medium.

Component	
$FeCl_3 \cdot 6H_2O$	26 g
$MnCl_2 \cdot 4H_2O$	0,72 g
H_3BO_3	67,2 g
EDTA	90 g
$Na_2HPO_4 \cdot 12H_2O$	40 g
$NaNO_3$	200 g
Na_2SiO_3	40 g
H_2O	2 L
Solution of metallic traces.	2 mL
Vitamin solution.	100 mL

Table 2. Solution of metallic traces.

Component	
$ZnCl_2$	2,1 g
$CoCl_2 \cdot 6H_2O$	2 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0,9 g
$CuSO_4 \cdot 5H_2O$	2 g
Distilled water	100 mL

Table 3. Vitamin solution.

Component	
Decamil	210 mg
Distilled water	100 mL

2.3. Cultivation System

To carry out the cultures, an arrangement was made with two bioreactors, both with a capacity of 4 L. The culturing system consisted of a rectangular structure 44 cm wide by 55 cm long, in which an arrangement of 6 fluorescent bulbs (5.5 W) was arranged as a source of artificial lighting with irradiation of 450 Lm and a photoperiod of 8:16. Aeration was mechanical using blowers (Power LifeTM, P-500) without CO_2 injection.

Figure 1. Microalgae culture system

2.4. Cell density

Cell density was determined by counting in a Neubauer chamber (Boeco® Germany), applying the respective cell counting techniques (Alvear, Castillo and Henao, 2011) under an optical microscope (Leica® dm-500). Counting was done in duplicate for each replica. The number of cells / ml was obtained according to (Shen et al., 2010).

$$\text{Cell/mL} = \frac{\text{Total Cell}}{\text{No.of counted squares}} \times 10{,}000 \qquad (1)$$

Furthermore, the growth of the microalgae was monitored by measuring the absorbance of chlorophyll in a UV-VIS spectrophotometer (Thomson, Gold

Spectrumlab 54) at a wavelength (λ) of 685 nm (Baldiris et al, 2017).

2.5. Microalgal growth rate

The growth rate was calculated from daily microscope counts, using the following formula (Sorokina et al., 2020):

$$\mu = \frac{\ln(N_2/N_1)}{t_2 - t_1} \quad (2)$$

Where: N_2 = number of cells or optimal density at the end time; N_1 = number of cells or optimal density at initial time; t_2 = final time; t_1 = initial time.

The doubling time (Santos-Ballardo et al., 2015), was determined using the expression:

$$t_d = \frac{\ln(2)}{\mu} \quad (3)$$

Where: μ = specific growth rate for each organism and culture medium.

2.6. Biomass production

To obtain the residual biomass of microalgae, the light source was removed and the aeration was suspended in each of the bioreactors after 20 days of culture, thus guaranteeing the death of the microalgae. After two days, the biomass was separated by centrifugation at 4000 rpm for 5 min (Indulab®, D-04) and washed with sterile distilled water. Finally, the moisture was removed in a drying and heating oven (Thermo Scientific ™, OMH 180 / Coated) at 60 ° C for 8 h.

2.7. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was performed on the biomass obtained from the mixotrophic cultures to show the presence of the functional groups associated with the accumulation of lipids in the microalgae. Infrared absorption spectra were recorded using the Jasco® brand FT / IR-4000 spectrophotometer in a wavelength range of 4000 - 500 cm^{-1}.

2.8. Lipid extraction

Total amount of lipids in the microalgae biomass was extracted by a modification of the protocol described by (Gutiérrez et al., 2015). 20 mL of a chloroform-methanol mixture (Panreac®) in a 2: 1 v / v ratio was added to 100 mg of dry microalgae and sonicated (Daihan Scientific ™, WUC-A06H) for 1 h. The extract obtained was homogenized for 30 s after the addition of a NaCl solution (Panreac®) at 0.9% w / v. Finally, the mixture was centrifuged for 8 min at 3000 rpm (Indulab®, D-04) and the organic phase was filtered (1882-047, Whatman). Finally, the organic solvent of the lipids was completely evaporated in a heating oven. This method is frequently used for the extraction of total lipids from oleaginous microalgae due to its simplicity and effectiveness. The amount of total lipids extracted was calculated with the following equation:

$$\%\ \text{of lípid} = \left(\frac{P_L}{P_M}\right) \times 100\% \quad (4)$$

Where: P_L = Dry weight of total lipids; P_M = Dry weight of microalgae.

2.9. Statistical analysis

The average and standard deviation were determined to know if there are significant statistical differences in cell density, growth rate, dry microalgae biomass and total lipid production. For statistical calculations, OriginPro 8 and R-Studio software were used.

3. Results y Discussion

The growth curve (Figure 3) shows that the microalgae Chlorella sp. and Chlorella vulgaris were able to grow under the proposed conditions. Taking into account the phases of a typical growth curve, no latency or adaptation is observed in the growth curve,

this is because the microalgae were in an exponential growth phase and were placed in a similar culture medium, in the presence of nutrients contained in the modified CONWAY® medium (Tompkins et al., 1995); This behavior was similar to the study carried out by (Domínguez et al., 2013). The microalgal density showed a significant difference, although not very marked, between the growth of the species, reaching maximum growth values of 7,022 x 10^6 cel · mL^{-1} for Chlorella. sp. and 8.75 x 10^6 cells · mL^{-1} for Chlorella vulgaris.

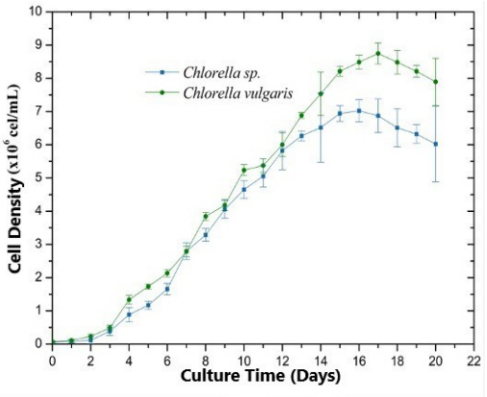

Figure 3. Cell density for *Chlorella sp* y *Chlorella vulgaris*

In Figure 3 it is observed that the exponential growth phase began from day 2 for all cultures and lasted until day 16 and 17, for *Chlorella sp*. and *Chlorella vulgaris*, respectively. The cell death phase extends until the last day of culture (day 20) and where the mortality rate is higher than the growth rate, which generated a decrease in cell density (Kiran et al., 2014). Applying the previously established equations, the kinetic data recorded in Table 4 were calculated.

Table 4. kinetic parameters for Chlorella sp y Chlorella vulgaris.

	μ (div/day)	t_d (days)
Chlorella sp.	0,298	2,326
Chlorella vulgaris	0,279	2,480

The population kinetic parameters obtained show that the higher specific growth speed presented by the *Chlorella sp*. It was 0.298 day^{-1} with a doubling time corresponding to 2.326 days, these conditions being more favorable for growth. However, the highest specific growth rate for the *Chlorella vulgaris* culture was 0.279 day^{-1} with a doubling time corresponding to 2.48 days. Other investigations report values of 0.17 day^{-1} using air and NPK fertilizer (Ortíz et al., 2012), reaching 0.32 day-1 using a mechanical blower and culture medium f / 2 Guillard (Infante et al., 2011).

Other investigations report values of 0.14 day^{-1} (Converti et al., 2009); 0.25 day^{-1} using air and 0.61 day^{-1} with 2% CO_2 (Chiu et al., 2008); 1.17 day^{-1} using 10% CO_2 (Sasi et al., 2011) and 1.32 day^{-1} using $NaHCO_3$ as a carbon source (Yeh et al., 2010), according to the above, the value that was obtained in this investigation is among the range of these investigations.

Regarding the influence of the variables that were studied for the harvest of both crops, it was evidenced that growth was less favorable for *Chlorella sp*., Despite the fact that the same amount of nutrients and light intensity were used with respect to the microalgae. Chlorella vulgaris. The results obtained by other authors regarding the harvesting of microalgae show that the initial density of a culture that will later be bio augmented is quite important; the supplied strain of *Chlorella vulgaris* used as inoculum to start the growth stage had a higher cell density than the *Chlorella sp*. strain, which represented an advantage in the growth of *Chlorella vulgaris*. In addition, the exponential phase of each culture lasted until days 16 and 17, for *Chlorella sp*. and *Chlorella vulgaris*, respectively, that is, the latter had an additional day to complete this phase and increase its cell density. In other investigations, high cell densities of approximately 3 x 10^7 cells mL-1 were obtained for *Chlorella sp.* (Huo et al, 2018) and 80 x 10^7 cells mL-1 (Kim et al, 2019), cell densities of 8 x 10^6 cells / mL (Soleimanikhorramdashti et al, 2021) and 70 x 10^7 (Zhang et al, 2019).

The FTIR analyzes of the biomasses obtained from the mixotrophic cultures of the species are presented in figure 5. From 13 to 10 signals were detected in the range of 500 to 4000 cm-1 for the spices. The two samples show a total of 8 similar bands (3268, 2357, 1640, 1017, 580, 561, 549, and 537) this is attributed to the nature of the material since *Chlorella vulgaris* is

composed of 75% *Chlorella so*. and indeed, its spectrum is similar to that of pure microalgae (Chiu et al., 2008).

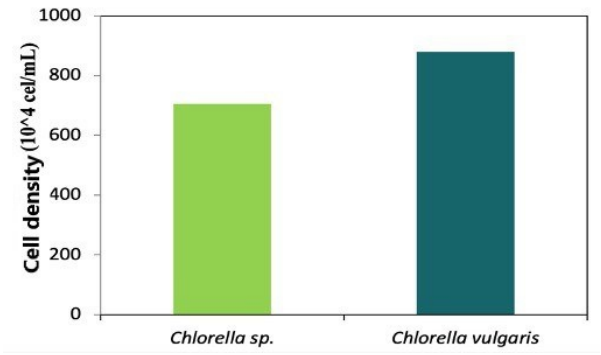

Figure 4. Cell growth of the spices

The first signal of 3485 wavenumber (cm-1), corresponds to stretching vibrations of -NH, that is, a primary amine (Csutak et al., 2010). The second in the range of 3100 to 3200 wavenumber (cm-1), correspond to the -OH stretching of simple hydrogen bonds (Habibzadeh et al., 2018). Signals close to 2350 wavenumber (cm-1) are attributed to accumulated double and triple carbon bond voltages (C = C and C≡C) (Eslami et al., 2018). The signals at the value of 1640 wavenumber (cm-1) correspond to the identification of amines in the sample (San Miguel et al., 2009). Signals close to 1015 wavenumber (cm-1) are identified as bonds to a sulfoxide group S = O in the sample. The signals detected below 600 (cm-1), are attributed to bonds with halogens, such as Cl, Br, and I. (Miglio, 2013). The accumulation of lipids was monitored employing FTIR analysis in the region (3050–2800cm − 1) typical of hydrocarbons and for triglycerides (TAGs) in the typical peak of 1745. TAGs are neutral primary lipids composed of saturated fatty acids (SFA) and monounsaturated (MUFA) and are the preferred compounds to produce biofuels (Grace et al, 2020). Also, in the biomass of microalgae the region of 1200–950 cm-1 is associated with stretch bonds of polysaccharides (C – O – C) (Dean et al, 2010). These results are also comparable with those reported in the identification of phospholipids at 2960 cm-1 (Miglio, 2013) in microalgae.

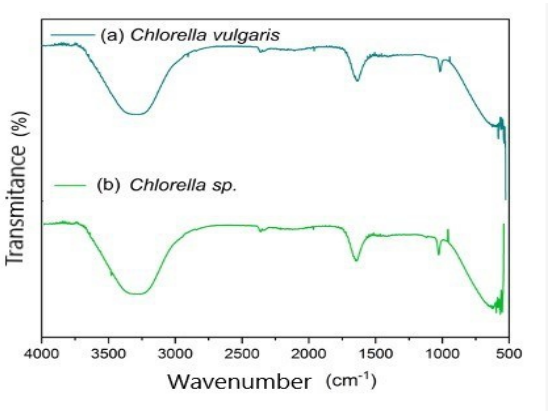

Figure 5. FTIR of the obtained biomass

The values of the lipid percentages after performing the sonification and extraction procedures by the Soxhlet method are summarized in Figure 6. A lipid recovery percentage of 27.3% was obtained for the *Chlorella sp* microalgae and the *Chlorella vulgaris* of the 21.5%.

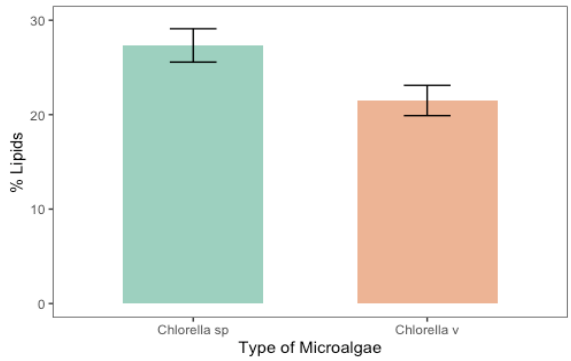

Figure 6. Lipid Production from microalgae.

For the microalgae Chlorella sp. of freshwater, other authors report values of 19% (Zheng et al., 2021), 30% (Kuo et al., 2015), and 45% (Zhu et al., 2017) for the percentage of total lipids produced by the microalgae. After carrying out the same procedure for the microalgae *Chlorella Vulgaris*, a percentage of lipids corresponding to 21.5% was obtained. Other investigations report values of 15% (Converti et al., 2009), 24.5% (dos Santos, 2015), and 36% (Thirugnanasambantham et al., 2020) for the percentage of total lipids generated by freshwater *Chlorella vulgaris.* These results show that the native

strains with which we work have good possibilities of being used as feedstock in the production of biofuels.

4. Conclusions

In this research, the growth and lipid production of two species of the *Chlorella* microalgae were compared. The data showed maximum cell densities of $7.06x10^6$ and $8.80x10^6$ cells / mL, for *Chlorella sp.* and *Chlorella vulgaris*, respectively. The microalgae presented the typical peaks for the storage of triglycerides and polysaccharides in the FTIR analysis. To determine the percentage of lipids, the method with organic solvents chloroform: methanol (2: 1) was used followed by a sonification procedure, which is the method that has shown the highest efficiency for the extraction of lipids, the tests showed that the microalgae *Chlorella sp*. had a better performance in the production of total lipids. These results show that the strains of microalgae used can generate good amounts of lipids which can be a raw material for obtaining biofuels in the future.

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