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PRODUCTION OF BIOMASS AND LIPID YIELDS OF CHLORELLA SP. CULTIVATED IN AUTOTROPHIC AND MIXOTROPHIC MEDIA

PRODUCCIÓN DE BIOMASA Y RENDIMIENTOS LÍPIDOS DE CHLORELLA SP. CULTIVADO EN MEDIOS AUTÓTROFOS Y MIXOTRÓFICOS

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Production of Biomass and Lipid Yields of Chlorella sp. Cultivated in Autotrophic and Mixotrophic Media

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ABSTRACT

In this work, we analyze the biomass and lipid yields of *Chlorella sp.*, when it grows in synthetic wastewater with and without nitrogen limitation and in both cases, adding glucose to both medium, autotrophic and mixotrophic. Our results confirm that it is possible to expand their possibilities of use, which range from their use for the bioremediation of bodies of water to obtaining various biofuels due to their high content of lipids and carbohydrates. It was identified that both the biomass and lipids were higher in the media with mixotrophy with 535.71 mg L^{-1} and 244.60 mg L^{-1}, respectively. Similarly, the importance of nitrogen present in the growth medium was recognized as a determining variable for the accumulation of lipids in the species, while it is concluded that the use of *Chlorella* sp. eliminates a significant percentage of nitrogen and phosphorus present in wastewater, thereby reducing nutrient contamination. The nutrient stress to which the microalgae were subjected allowed a greater accumulation of lipids in the cells, which leads to the conclusion that in a large-scale study, *Chlorella* sp. It could be used as a raw material to obtain oils and their subsequent transformation into biodiesel.

Keywords: chlorella, biomass, lipid yields, bioremediation, mixotrophy medium

Producción de Biomasa y Rendimientos Lípidos de Chlorella sp. Cultivado en Medios Autótrofos y Mixotróficos

RESUMEN

En este trabajo, analizamos los rendimientos de biomasa y lípidos de Chlorella sp., cuando crece en aguas residuales sintéticas con y sin limitación de nitrógeno y en ambos casos, adicionando glucosa al medio, tanto autótrofo como mixotrófico. Nuestros resultados confirman que es posible ampliar sus posibilidades de uso, que van desde la biorremediación de cuerpos de agua hasta la obtención de diversos biocombustibles por su alto contenido en lípidos y carbohidratos. Se identificó que tanto la biomasa como los lípidos fueron mayores en los medios mixotroficos con 535,71 mg L^{-1} y 244,60 mg L^{-1}, respectivamente. De igual forma, se determinó la importancia del nitrógeno presente en el medio de crecimiento como variable determinante para la acumulación de lípidos en la especie, mientras que se concluye que el uso de Chlorella sp. elimina un importante porcentaje de nitrógeno y fósforo presentes en aguas residuals; reduciendo así la contaminación por nutrientes. El estrés nutritivo al que fueron sometidas las microalgas permitió una mayor acumulación de lípidos en las células, lo que lleva a concluir que en un estudio a gran escala, Chlorella sp. podría utilizarse como materia prima para la obtención de aceites y su posterior transformación en biodiesel.

Palabras clave: chlorella, biomasa, rendimiento de lípidos, bioremediación, medio mixotrofico

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INTRODUCTION

Microalgae represent a promising raw material not only in the food, pharmaceutical and cosmetic industries [1] but also due to their capacity to absorb CO_2 within photosynthetic their process, and their manipulable composition of lipids, proteins or carbohydrates, which can be used to obtain biofuels or bioproducts [2].

Its promising use as clean technology lies in the fact that, in any growing condition, it yields high population rates after just a few hours, compared to any terrestrial plant [3].

To produce microalgae, there are different forms of cultures; in any of them, it is necessary to use a device called photobioreactors, which allow the conversion of light and carbon mainly into biomass [4]. An adequate design of these photobioreactors involves considering the environmental conditions of the place where the microalgae are to be grown and characteristics of the type of microalgae that will be worked with [5].

However, the main challenge of microalgae technology lies in improving biomass production and being able to obtain high lipid accumulation for its subsequent transformation into value-added products, all this on a commercial scale and using only one type of culture [6]. These limitations come mainly from the low photosynthetic efficiency achieved by currently used devices and the cost of adding an organic or inorganic carbon source to the process [7, 8]. A viable option for increase the growth rate in species such as *Chlorella* sp. is the exploration of their metabolic pathway in different types of cultures [6].

Although autotrophic cultures would use CO_2 from the environment, it would be difficult to maintain a high cell density due to the variability in light penetration of the entire culture [7]. In contrast, while heterotrophic cultures would efficiently convert the organic carbon present, maintenance costs would be a negative factor [8].

Thus, in recent years, research has opted for the making of mixotrophic cultures, where there is an organic carbon source (such as glycerol, glucose, or acetate) and an inorganic one simultaneously while in the presence of light [9]. As a result, increased biomass productions are obtained, up to three times higher than autotrophic cultures [2].

In this type of culture, careful handling must be taken with the concentration of essential nutrients present in the medium, like carbon, nitrogen, and phosphorus; since their limitation or enrichment could trigger microalgal inhibition [10]. Research shows that nitrogen limitation stress favors the accumulation of lipids in cells; however, it decreases biomass production, which continues to represent a bottleneck production [11]. On the other hand, the addition of glucose represents a carbon source that allows increasing the growth rate of such microorganisms, some species of *Chlorella* have reported a higher biomassic and lipidic yield in mixotrophic cultures where the concentrations of carbon and nitrogen have been variable. [12].

It is possible to obtain a value-added product in the production of biofuels with microalgae; the culture medium can be wastewater from urban sources due to its high nitrogen and phosphorus content [13]. Which, once assimilated by the microorganisms, would represent a decrease in the eutrophication of waste water bodies [14].

For all the above, the present work evaluated *Chlorella* sp.´s growth and lipid content using synthetic wastewater with and without nutrient limitation, adding a source of organic carbon to the culture medium.

METHODOLOGY

Microalgae Culture

Chlorella sp. was cultured in synthetic medium BG11 [15] in 250 mL flasks, at 24 ± 2°C, for 15 days. With an artificial light source of 3000 lux of luminance at a 12:12 cycle photoperiod. Without the addition of external CO_2.

Growth

The Growth conditions are those described in Table 1. Autotrophic medium with nutrient (M_{a1}) were prepared with synthetic wastewater, composed of the following (per liter): NaCl, 7mg; $CaCl_2$, 4 mg; $MgSO_4 \cdot 7H_2O$, 2 mg; K_2HPO_4, 21.7 mg; KH_2PO_4, 8.5 mg; Na_2HPO_4, 33.4 mg and NH_4Cl, 3 mg [16]. Nitrogen-limited media did not contain NH_4Cl.

The mixotrophic growth of both cases, (M_c and M_{c1}) had the initial composition of M_a and M_{a1} respectively. In this case 10 g L^{-1} of dextrose monohydrate have been added to M_c and M_{c1} [11, 17].

Table 1 Growth mediums for *Chlorella* sp.

Media	Description of the medium
M_a	Nitrogen-limited synthetic wastewater.
M_{a1}	Synthetic wastewater with nitrogen.
M_c	Nitrogen-limited synthetic wastewater with glucose.
M_{c1}	Synthetic wastewater with nitrogen and glucose.

Growth was carried out for 21 days in glass bottles. Two replicates were established for each medium, all with a total volume of 100 mL, of which 10 mL corresponds to the portion of the inoculum mentioned above and a cell density of 1.2 x 10^6 ~ 1.9 x 10^6 cell mL^{-1}. With a light intensity variable from 1000 to 5000 lux at a 12:12 photoperiod of the light / dark cycle without the addition of external CO_2.

Cell count

Cell growth monitoring was carried out every 48 hours until reaching the stationary phase, collecting 1 mL aliquots for each treatment. Counting was performed using a Neubauer camera and an optical microscope with a 40 x objective. The cell concentration of each treatment was calculated with the equation [15]:

$$\frac{cell}{ml} = \bar{X}\ of\ cell\ in\ 1\ mm^2\ \cdot\ 10^4\ \cdot dilution \qquad ec.1$$

where $\bar{X}$, is the average number of cells that exist in 1 mm^2.

Quantification of Biomass

The recovery of the biomass was carried out by centrifugation for a period of 15 min at 4000 rpm. To know the growth in terms of dry weight, a volume of 10 mL (in duplicate) was filtered on GF / a filters, previously tared. Each filter was placed in the oven at a temperature of 105 °C for 4 hours, they were subsequently weighed until the constant weight was determined [10]. The total dry weight was obtained by the weight difference between the filters (empty and with sample), from the following equation [18]:

$$Biomass\ productivity\ (mg\ L^{-1}) = \frac{X_1 - X_2}{V_1} \qquad ec.2$$

where, X_1 represent weight of filter with sample (mg), X_2 weight of empty filter (mg) and V_1 is volume of filtered culture (L).

Extraction and Quantification of Lipids

The lipid extraction was carried out by the modified Bligh and Dyer method. Using a mixture of chloroform: methanol (1: 2) [15]. The quantification of the percentage was carried out using the following equation [19]:

$$\%\ lipids = \frac{weight\ of\ lipids}{weight\ of\ biomass}\ x\ 100 \qquad ec.3$$

Removal of Nutrients

To know the consumption of nutrients, present in the growth medium, the methodology proposed by Marin et al. [20] to measure the content of nitrogen present in the form of ammonium, and phosphate in the form of orthophosphates, both at the beginning and at the end of the crop.

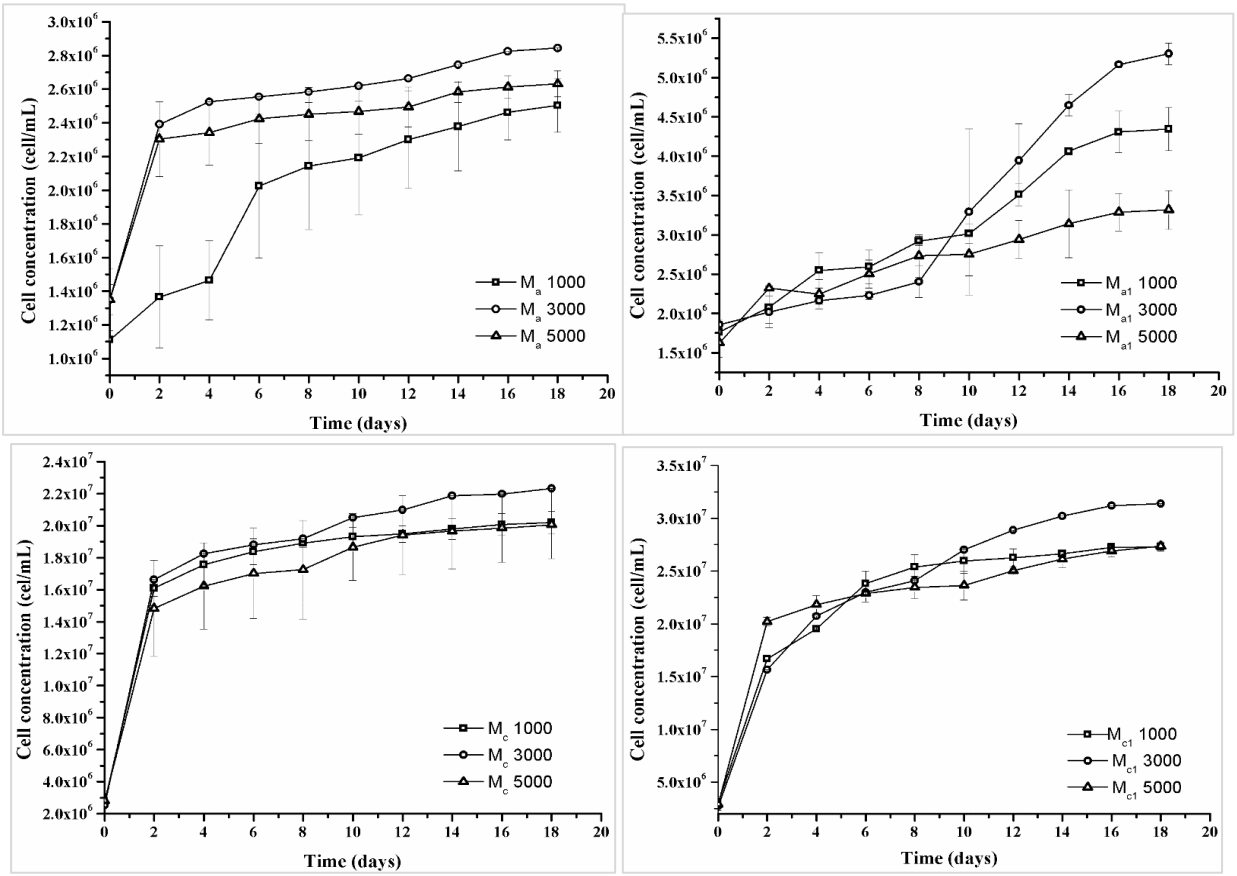
Statistical Analysis

The statistical analysis was carried out using Minitab version 19.2.0. The obtained data were analyzed statistically to determine the degree of significance at probability (P) < 0.05 using analysis of variance ANOVA.

Results and Discussion

The cell growth rate of *Chlorella* sp. is shown in Figure 1 for growth media M_a (Figure. 1.a), M_{a1} (Figure. 1.b), M_c, (Figure. 1.c) and M_{c1} (Figure. 1.d). It is possible to see that the growth rate was significantly higher for both media. When the species did not present nitrogen limitation (M_{a1} and M_{c1}), the cell concentration reached values of $5.30 \times 10^6 \pm 1.36 \times 10^5$ and $3.14 \times 10^7 \pm 1.95 \times 10^6$ cell m L^{-1}, respectively. Likewise, it is observed that the highest cell concentrations are found in all the mediums with a light flux of 3000 lux, having exponential growth from the eighth day.

Figure 1 Chlorella sp. growth kinetics in the media M_a (a), M_{a1} (b), M_c (c) and M_{c1} (d), subjected to light fluxes of 1000, 3000 and 5000 lux



The significant differences evaluated in this work about the specific growth rates, and the final cell density obtained in both media are agree with the results obtained by Rosales et al. [21]and Ortiz et al. [22]. Also, the main disadvantage of the physiological stress strategy by nutrients is associated with the reduced cell division and, therefore, the low generation of cells per day. These values can be seen in Table 2.

Table 2 Maximum kinetic growth parameters of the *Chlorella* sp., in each of the treatments

Growth medium	Initial cells concentration (cells mL^{-1})	Final cells concentration (cells mL^{-1})	Specific growth rate (generation day^{-1})	Generation time (day)	Divisions per day
M_a	$1.35 \times 10^6 \pm 1.82 \times 10^{5**\,a}$	$2.84 \times 10^6 \pm 1.85 \times 10^{5\,*}$	0.08^a	41.1	0.1
M_{a1}	$1.85 \times 10^6 \pm 5 \times 10^{4\,b}$	$5.30 \times 10^6 \pm 1.36 \times 10^5$	0.12^a	10.4	0.1
M_c	$1.41 \times 10^6 \pm 7.73 \times 10^{4\,d}$	$2.23 \times 10^7 \pm 1.13 \times 10^6$	0.24^b	33.8	0.3
M_{c1}	$1.34 \times 10^6 \pm 3.87 \times 10^{5\,e}$	$3.14 \times 10^7 \pm 1.95 \times 10^6$	0.28^b	22.1	0.4

* Average of two repetitions. ** The means (± standard error) within each column without common superscript differ significantly at P <0.05, performing an analysis of variance (ANOVA).

Figure 1 (d) shows that on the fourth day $M_{c\,1}$, an exponential growth begins , while the graphs refer to the medium M_c , which presents an average of 1.80 x $10^{\,7}$ ± 2.12 x $10^{\,6}$ cells m $L^{\,-\,1}$, in the same phase.

Until now, the study variables did not show significant changes in the treatments. By the middle of the experiment, the growth of the species in M_{c1} had increased by approximately 25%, with a cell average of 2.43 x 10^7 ± 9.89 x 10^5 cells m L^{-1}, compared to an increase in 18.5% of the medium M_c, with an average of 1.84 x10^7 ± 1.05 x 10^6 cells m L^{-1}. The significant difference points to what was previously indicated by other authors [11], even working in a mixotrophic medium, the stimulus that affects cell growth to a greater extent is nitrogen deficiency in the medium, since, the lack of physiological conditions prevented the increase in cell division, as does the present work, reaching day 7 of 16 in experimentation.

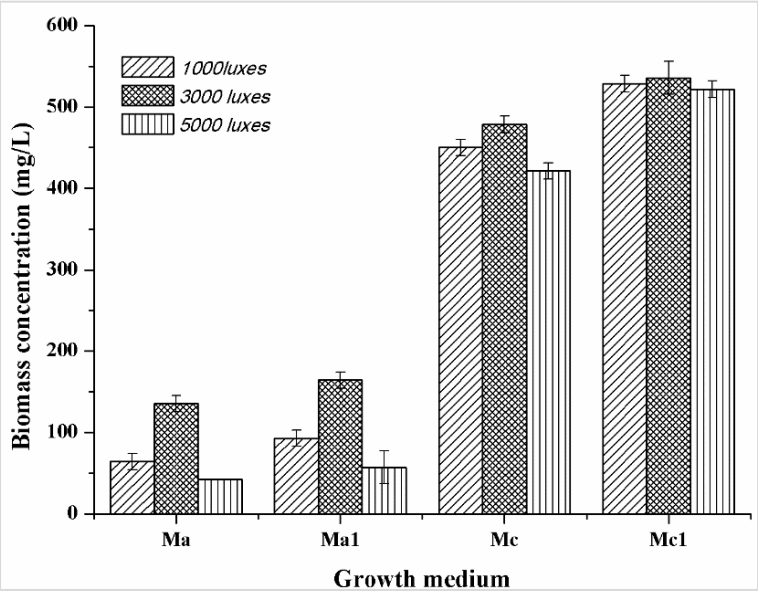
In the present work, the M_c and M_{c1} media (mixotrophic conditions), obtained higher concentrations when compared with M_a and M_{a1} (autotrophic conditions).

This can be attributed to the fact that glucose used as a source of organic carbon in this work, was easily metabolized by microalgae, a situation that concurred with that presented by Rodríguez et al[11]

Biomass Concentrations

Chlorella biomass yield has been reported in the ranges between 400 ~ 800 mg L^{-1}, as reported by Castillo et al. [23]. Figure 2 shows the results of the biomass concentration in media with nitrogen (M_a and M_c) and with limited nitrogen (M_{a1}y M_{c1}). The mixotrophic medium with nitrogen (M_{c1}) gave the highest yield, with an average of ~ 530 mg L^{-1} as indicated in Table 3. Some authors report that *Chlorella* presents better yields in medium with enrichment in organic carbon and the presence of nitrogen. (mixotrophic), compared to autotrophic medium, reporting values between 500 and 150 mg L^{-1}, respectively [11].

Figure 2 Biomass concentrations of *Chlorella* sp. in each of the treatments exposed to different light intensities.



Liang et al. [7] attribute the previous behavior to the willingness of species like *Chlorella* to modify their metabolic pathway with the presence of sugars. Additionally, Lang et al. [17] mention that the use of glucose for the cultivation of microalgae in mixotrophic conditions promotes an increase in biomass production, as presented in this research.

Table 3 Maximum biomass yield (mg L^{-1}) of *Chlorella* sp. species in the most favorable growth medium (3000 lux).

Growth medium	**Peak performance*** **(mg L^{-1})**
M_a	135.71 ± 10.10** [a]
M_{a1}	164.29 ± 10.10 [a]
M_c	478.56 ± 10. 10[b]
M_{c1}	535.71± 20.20[c]

* Average of two repetitions. ** The means (± standard error) within each column without common superscript differ significantly at P <0.05, performing an analysis of variance (ANOVA).

The averages of the maximum yield of the autotrophic medium M_a and M_{a1} did not present significant differences. However, there is a notable disparity between the autotrophic and mixotrophic media, as is the case of Ma and Mc, with a difference of ~350 ± 200 mgL^{-1} and in the M_{a1} and M_{c1} media with a difference of ~370 ± 272.74 mg L^{-1} which represents more than 100%. Li et al [24], report in their research that the biomass concentration doubled when there was nitrogen in the culture medium and a source of organic carbon, a scenario that is shared in the present work. This phenomenon is also explained by Freitas et al. When they mention that the formation of mixotrophic

cultures with the help of organic carbon compounds accelerates the metabolism of species such as *Chlorella* sp., as well as their cellular composition [25].

In the M_{c1} medium, the yields exceeded 500 mg L^{-1} , using only half the glucose than other reported works [11] . The 50% saving in the use of the organic carbon source, with respect to the reference, speaks of a cost reduction in the production of microalgae from this research.

It can be affirmed that the samples with higher light intensity favored a greater production of CO_2 and, with it, the obtaining of better cell concentrations and biomass [25]. As can be seen in Figure 2, the difference between cultures exposed to 1000 and 3000 lux ranges from 45 ~ 50 % in the case of autotrophic cultures and from 2 ~ 6 % for mixotrophic cultures. Some authors mention that the limitation of light prevents an efficient photosynthetic conversion and favors the appearance of shade gradients, a problem that affects cell density such a situation did not occur in this investigation [25, 26].

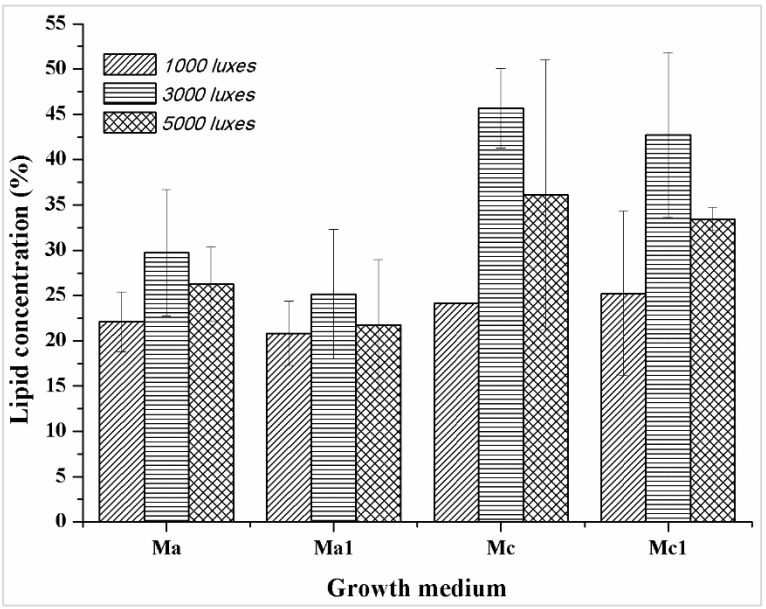
Overexposures then favored substantial increases in culture temperature, as well as excessive stress, which led to photooxidation and photoinhibition in the case of all experiments exposed to 5000 lux, thus proving that a variation in culture temperature for species like *Chlorella*, ranges from 20 ~ 30% [27, 28]

The difference in biomass increase between culture techniques can be attributed to what was described by Izadpanah et al., mentioning the biological compatibility of the species such as *Chlorella* sp. Between the isolation medium and the growth medium is noticeable. Since, mimicking environments similar to the one used in the isolation of the species favors and efficient the growth of the microalgae. Since, in this work, the species was found in an autotrophic medium of isolation [26].

Lipid Concentration

According to the analysis carried out, Figure 3 shows that the M_a and M_{a1} (autotrophic) medium has lower lipid yields than the M_c and M_{c1} (mixotrophic) medium, with percentages ranging between 20~30 % and 25~46%, respectively.

Figure 3 Lipid concentrations of *Chlorella* sp. in each of the treatments exposed to different light intensities



Also, Liang et al. [7] report that in an autotrophic medium with nitrogen limitation, *Chlorella* presented a yield close to 30 % against 36% in a mixotrophic medium using 1% v/v of organic carbon source. Such results, in the first instance, are similar to those of the present investigation since Ma achieved a lipid yield greater than 30% and secondly, the yield of M_c is 10% higher than that of that reference.

Table 4 Maximum lipid yield (mg L^{-1}) of the *Chlorella* sp., in the different growth media

Growth medium	Peak performance (mg L^{-1})
M_a	$^{*}40.36 \pm 6.96^{**a}$
M_{a1}	23.36 ± 7.12^{a}
M_c	244.60 ± 4.41^{b}
M_{c1}	204.40 ± 9.11^{b}

* Average of two repetitions. ** The means (± standard error) within each column without common superscript differ significantly at P <0.05, performing an analysis of variance (ANOVA).

The analysis of the information in Table 4, showed that the mediums M_a and M_{a1} (autotrophs) do not present significant differences between them, but there is a disparity within the mixotrophic mediums. We can attribute this to the composition of the mediums since M_c and M_{c1} are mediums with the addition of organic carbon in the form of simple sugar, while the remaining mediums are purely autotrophic. Rodríguez et al. [11], report that *Chlorella* yields in mediums with compositional

differences, such as those reported in the present investigation, range between 6 ~ 12% for purely autotrophic medium, and 16 ~ 38% for mixotrophic medium. In both cases, the mediums reported here surpass such results. It is important to point out that M_a and M_c show better results in lipid accumulation compared to M_{a1} and $M_{c;}$ Such effect was sought after from the beginning of the research, since according to Castillo et al. [23] subjecting species such as *Chlorella* to nutrient stress would directly impact fatty acid synthesis, thus increasing lipid production and achieving a maximum yield.

The analysis of the information in Table 4, showed that the M_a and M_{a1} (autotrophic) media do not present significant differences between them, but there is a disparity within the mixotrophic media. This can be attributed to the composition of the media, since M_c and M_{c1} are media with added organic carbon in the form of simple sugar, while the rest of the media are purely autotrophic. Some authors have reported that *Chlorella* yields in media with compositional differences, such as those reported in the present investigation, oscillate between 6 ~ 12% for a purely autotrophic medium and 16 ~ 38% for a mixotrophic medium [11]. It is important to note that M_a and M_c show better results in lipid accumulation results than to M_{a1} and M_c. This effect was sought from the beginning of the investigation, since according to Castillo et al. [23], subjecting species such as *Chlorella* to nutritional stress would directly impact fatty acid synthesis, thus increasing lipid production and achieving maximum yield.

Although the limitation of nitrogen, exclusively to decrease cell division (Ma and Mc), this restriction made it possible to redirect the synthesis of CO2 inside the cell, converting it mainly into neutral lipids. In 2020, Feng et al. found that crops with sufficient N stored a lower amount of lipids than those limited in this nutrient, a situation shared by both media in this research [29]. High light intensities are known to modify the growth rate and the composition of the resulting biomass. Some authors suggest that crops with an increase in simple sugars and medium intensities of white light (1500-2500 lux) potentiate the content of carbohydrates and lipids [25]. Such a case is presented for the Mc and Mc1 cultures, wich present an increase of 24 and 20% in lipid content, respectively, when going from 1000 to 3000 lux of light intensity, and only a difference of between 8 and 10%. For exposures from 3000 to 5000 lux, in the same cases.

It can be observed that after the cultures exposed to 3000 lux, those that 5000 present better lipid yields and this can be attributed to the fact that the overexposure of light allowed better of synthesizing the macro and micronutrients of each culture, in the initial stage of the experiment , declining for exactly the same reason once the species was acclimatized to each growth technique [28] [29].

Removal of Ammonium and Phosphate

According to the analysis, the mediums M_a and M_{a1} did not present significant differences in the initial content of both parameters; a similar case occurred in the mediums M_c and M_{c1}. After the culture time had elapsed, the removal values for each of the mediums were varied, see Table 5 The residual mediums did not show significant differences among themselves. For the cases of M_a and M_c, the initial and final values of nitrogen concentration in ammonium form were similar. The results are associated with the fact that both media were designed with nitrogen limitation, and it is presumed that the data reported here are due to the fixation of such compounds in the synthetic growth medium by the species [30]

Table 5 Initial characterization of the culture media for the growth of *Chlorella* sp. Ammonium (NH_4^+) and phosphate (PO_4^{3-})

Growth medium	NH_4^+ (PPM)	PO_4^{3-} (PPM)	NH_4^+ (PPM)	PO_4^{3-} (PPM)
	Initial	Initial	Final	Final
M_a	0.99 ± 0.04[a]	*1.50 ± 0.04**	0.85 ± 0.03	*1.38 ± 0.01**
M_{a1}	1.07 ± 0.03 [a]	1.64 ± 0.13	0.97 ± 0.01	0.98 ± 0.01
M_c [4]	1.75 ± 0.04[b]	1.42 ± 0.08	0.87 ± 0.04	1.34 ± 0.02
M_{c1} [5]	1.16 ± 0.01 [b]	1.42 ± 0.02	0.87 ± 0.01	0.99 ± 0.04

* Average of two repetitions. ** The means (± standard error) within each column without common superscript differ significantly at P <0.05, performing an analysis of variance (ANOVA).

Ramos et al. [31], reports in their work removal of ammonium NH_4^+, of 21.48%, with a synthetic residual medium, while in the present investigation similar removal values were reached in the media M_c and M_{c1} with 25.1 and 24.3 % respectively. In the case of the removal of total dissolved phosphate, the highest values were presented for M_a and M_c, with 40 and 30% respectively. García et al. [32], mention in their research that the good assimilation of this nutrient is directly related to the metabolic processes of microalgae that cause the biomass growth of the species.

CONCLUSIONS

Chlorella sp. was able to demonstrate its adaptability by growing in both autotrophic and mixotrophic media, the latter being the most viable option in terms of total energy yield, as it was able to store a percentage of lipids within the range already reported, while the amount of organic matter managed to harvest is not compromised. However, for future work it is important to mention that the choice of the culture medium will depend on the objectives that the work itself is planned. It should be noted that the biomass-lipid ratio required being symbiotic for this work due to its energetic implications.

For the specific case of this work, it is considered that a medium lacking in some nutrients, for example, nitrogen, presented better results in terms of cell concentrations, biomass and lipid yield, compared to an enriched medium, where both macro and micronutrients are available. In this sense, a parallel economic benefit is offered, since it is said that microalgae can be cultivated with a lower economic requirement than that already reported.

Finally, the potential of the Chlorella sp. species was not limited to energy fines; In addition, its great capacity to be used as an ecological agent in the treatment and bioremediation of wastewater will be confirmed. This opens the way to a biorefinery concept, an innovative sector with a great impact in the area of biotechnology recently. This methodology would be producing biomass with future uses in biofuels (biodiesel, bioethanol, biohydrogen), bioremediation of wastewater and simultaneously, products with high added value (biopolymers, biofertilizers, pigments, etc.).

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