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Obtaining polyhydroxyalkanoate and plastic film formation from the microalgae *Chlorella vulgaris* under light stress and nitrogen deficiency

Obtenção de polihidroxialcanoato e formação de filme plástico a partir da microalga *Chlorella vulgaris* sob estresse luminoso e deficiência de nitrogênio

Obtención de polihidroxialcanoato y formación de película plástica a partir de la microalga *Chlorella vulgaris* bajo estrés lumínico y deficiencia de nitrógeno

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Abstract

The microalgae *Chlorella vulgaris* is a potential source of biomass and several biopolymers. The aim of this study was to evaluate the production of polyhydroxyalkanoate and the formation of plastic film under constant light stress (24h) and photoperiod (12/12h – light/dark), in addition to the influence of inorganic nitrogen source deficiency ($NaNO_3$ – 50%), under cell concentration (X_m), productivity (P_X) and specific growth rate (μ). The polymer was also characterized by Fourier transform infrared spectroscopy (FTIR) and thermal analysis (TGA). Autotrophic conditions (CBBM24) under constant light stress (24h) showed better results, with X_m = 1163.45 ± 52 mg L^{-1}, P_X = 145.43 ± 7 mg L^{-1} day^{-1} and μ = 0.39 ± 0.00 day^{-1} during 8 days of cultivation. After extraction, the polymer obtained was characterized by FTIR, showing transmittance bands located at 1722 cm^{-1} that were attributed to the stretching vibration of the C=O

group (carbonyl ester) in the PHA polyester. Thermal analysis (TGA) showed that the polymer obtained from CBBM24 biomass showed T_{onset} (21%) at 91 °C and T_{decomp} (76%) at 295 °C. The plastic film was also produced using glycerol plasticizer, thus demonstrating that microalgae has strong potential in the production of biodegradable plastic.
Keywords: Light stress; Nitrogen deficiency; Bioplastic; Biopolymer; Polyhydroxyalkanoate.

Resumo

A microalga *Chlorella vulgaris* é uma fonte potencial de biomassa e diversos biopolímeros. O objetivo deste estudo foi avaliar a produção de polihidroxialcanoato e a formação de filme plástico sob constante estresse luminoso (24h) e fotoperíodo (12/12h – claro/escuro), além da influência da deficiência de fonte inorgânica de nitrogênio ($NaNO_3$ – 50%), sob a concentração celular (X_m), produtividade (P_X) e taxa de crescimento específico (μ). O polímero também foi caracterizado por espectroscopia no infravermelho com transformada de Fourier (FTIR) e análise térmica (TGA). Condições autotróficas (CBBM24) sob estresse luminoso constante (24h) apresentaram melhores resultados, com X_m = 1163,45 ± 52 mg L^{-1}, P_X = 145,43 ± 7 mg L^{-1} dia-1 e μ = 0,39 ± 0,00 dia_{-1} durante 8 dias de cultivo. Após a extração, o polímero obtido foi caracterizado por FTIR, apresentando bandas de transmitância localizadas em 1722 cm^{-1} que foram atribuídas à vibração de estiramento do grupo C=O (éster carbonílico) no poliéster PHA. A análise térmica (TGA) mostrou que o polímero obtido da biomassa CBBM24 apresentou T_{onset} (21%) a 91 °C e T_{decomp} (76%) a 295 °C. O filme plástico também foi produzido utilizando plastificante glicerol, demonstrando assim que as microalgas têm forte potencial na produção de um plástico biodegradável.
Palavras-chave: Estresse luminoso; Deficiência de nitrogênio; Bioplástico; Biopolímero; Polihidroxialcanoato.

Resumen

La microalga *Chlorella vulgaris* es una fuente potencial de biomasa y varios biopolímeros. El objetivo de este estudio fue evaluar la producción de polihidroxialcanoato y la formación de película plástica bajo estrés lumínico (24h) y fotoperiodo (12/12h – luz/oscuridad) constante, además de la influencia de la deficiencia de una fuente inorgánica de nitrógeno ($NaNO_3$ – 50%), bajo concentración celular (X_m), productividad (P_X) y tasa de crecimiento específica (μ). El polímero también se caracterizó mediante espectroscopia infrarroja por transformada de Fourier (FTIR) y análisis térmico (TGA). Las condiciones autótrofas (CBBM24) bajo estrés lumínico constante (24h) mostraron mejores resultados, con X_m = 1163.45 ± 52 mg L^{-1}, P_X = 145.43 ± 7 mg L^{-1} día-1 y μ = 0.39 ± 0.00 $día^{-1}$ durante 8 días de cultivo. Luego de la extracción, el polímero obtenido se caracterizó por FTIR, mostrando bandas de transmitancia ubicadas a 1722 cm^{-1} que se atribuyeron a la vibración de estiramiento del grupo C=O (éster carbonílico) en el poliéster de PHA. El análisis térmico (TGA) mostró que el polímero obtenido de la biomasa CBBM24 presentó T_{onset} (21%) a 91 °C y T_{decomp} (76%) a 295 °C. La película plástica también se produjo utilizando un plastificante de glicerol, lo que demuestra que las microalgas tienen un gran potencial en la producción de plástico biodegradable.
Palabras clave: Estrés lumínico; Deficiencia de nitrógeno; Bioplástico; Biopolímero; Polihidroxialcanoato.

1. Introduction

The high rate of generation and disposal of plastic waste in the environment has been causing serious damage to modern life. Due to their petrochemical composition, these plastics have a great resistance to deterioration and several methods for disposing of these plastics have been used, including landfilling, incineration and even deposit in the environment (Koller, 2019). The deposits of this plastic waste in the marine environment, for example, cause various damages to human health, due to the ingestion of microparticles and nanoparticles, which enter the food cycle (Koller, 2019; Campanale et al., 2020). Therefore, alternative means to replace and mitigate the damage caused by these plastic wastes have been severely researched, and an alternative is polyhydroxyalkanoates (Silva & Houllou, 2022). Polyhydroxyalkanoates (PHAs) are biopolymers produced by a variety of microorganisms, in recent decades, microalgae. Because these polymers have properties similar to those of synthetic polymers, they are great substitutes for conventional plastics, originating from petroleum, due to their biodegradable capacity (Costa et al., 2019). Microalgae have been presented as a potential alternative for the production of polyhydroxyalkanoates and cultivation models are the main tools for production efficiency (Afreen et al., 2021). Silva and Houllou (2022) verified the potential for PHA production from the microalgae *C. vulgaris* and *Tetradesmus obliquus* using agroindustrial residue corn steep liquor as a source of carbon/nitrogen, García et al. (2020) evaluated the production of PHA from the microalgae *Scenedesmus* sp. under conditions of nitrogen deficiency, Roja et al. (2019) evaluating the growth profile of four species of photosynthetic microorganisms for the production of polyhydroxyalkanoate used only the conventional ASN III medium, but the luminosity factor is not very well described in the literature. Therefore, photosynthetic microorganisms,

being photoautotrophic, demonstrate an advantage over bacteria, since they can survive in adverse conditions, and require minimal metabolic requirements, such as CO_2, N, P, and light. Thus, strategies for optimizing culture conditions and increasing PHA production, such as reducing nitrogen and phosphorus, exposure to light, CO_2 dynamics and carbon saturation, have been frequently evaluated by researchers (Afreen et al., 2021). The reduction of nitrogen in the cultivation medium causes microalgae to reduce the production of primary compounds, modifying their metabolic route to produce secondary compounds such as PHAs (Costa et al., 2018a; Mendhulkar & Shetye, 2017). In this study, the parameters of cell production, productivity and specific growth rate of the microalgae *C. vulgaris* were evaluated, as well as the potential for production and formation of polyhydroxyalkanoate plastic film. The biopolymer was also characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Thermal Analysis of Thermogravimetry (TGA). This study aimed to evaluate the influence of light stress (24h), photoperiod (12/12h light/dark) and nitrogen deficiency (NaNO3 - 50%) on the growth parameters (X_m, P_X and μ) of the microalgae *C. vulgaris*, the characterization of the functional groups and the chemical composition of the biopolymer (FTIR), its thermal characterization (TGA) and the formation of plastic biofilm.

2. Methodology

Experimental, laboratory research of a quantitative nature was carried out (Pereira et al., 2018) and supported by statistical analysis (Shitsuka et al., 2014; Vieira, 2021).

2.1 Microorganism and culture conditions

Chlorella vulgaris UTEX 1803 was obtained from UTEX (University of Texas, Austin) and cultivated under conditions of constant light stress (24h), photoperiod 12/12h (light/dark) and $NaNO_3$ reduction. The culture used Bold's Basal Medium (Bischoff & Bold, 1963) as standard medium. The microalgae were inoculated with an initial biomass concentration of 50 mg L^{-1}, temperature of 27 ± 1 °C, light intensity of 4500 lux, under constant aeration.

2.2 Biomass concentration

Cell concentration was determined by measuring the optical density (OD) at 685 nm (Xu et al., 2008) by a UV–Visible spectrophotometer (Thermo Scientific™ GENESYS™ 180 UV-Visible) and expressed in milligrams of dried biomass per liter of medium (mg L^{-1}) through a calibration curve relating OD to dry biomass weight.

2.3 Determination of biomass productivity

Biomass productivity (P_X, mg L^{-1} dia^{-1}) during the culture period was calculated from the Eq. (1), where Xt was the biomass concentration (mg L^{-1}) at the end of the exponential growth phase (t_x) and X_0 the initial biomass concentration (mg L^{-1}) at t_0 (day):

Eq. (1)

$$P_X = \frac{X_t - X_0}{t_X - t_0}$$

2.4 Specific growth rate

The specific growth rate (μ, day^{-1}) was calculated from the Eq. (2), where N1 and N2 were the concentration of cells at the beginning (t1) and at the end (t2) of the exponential growth phase, respectively.

Eq. (2)

$$\mu = \frac{ln\ N2 - ln\ ln\ N1}{t_2 - t_1}$$

2.5 Biopolymer Extraction

The extraction was performed according to Silva and Houllou (2022) with slight modifications. Lyophilized biomass of *C. vulgaris* were subjected to motor agitation with a solution of hypochlorite (3%) + chloroform (v/v), then the samples were centrifuged (5000 rpm, 10 minutes, temperature environment) and the organic phase was collected, evaporated and subjected to spectroscopic analysis.

2.6 Plastic film formation

The polymer obtained was solubilized in chloroform at 80°C and left under stirring for 10 minutes, then 30% (w/w) of glycerol was added and the solution was stirred for 2 minutes and then poured into a Petri plate. The solvent was evaporated at room temperature and the plastic film was obtained.

2.7 Fourier Trasnform Infrared Spectroscopy (FTIR)

Samples of PHAs were qualitatively analyzed with Fourier transform infrared spectroscopy (FTIR, Shimadzu, model IRTracer 100) between 4000 and 600 cm^{-1} using ATR accessory with a zinc selenide crystal.

2.8 Thermal analysis of PHA samples

The thermal properties of polyhydroxyalkanoates synthesized from *Chlorella vulgaris* were obtained from Thermo Gravimetric Analysis (TGA). TGA was performed on the NETZSCH STA 449F3 Simultaneous Thermal Analyzer from 30 °C to 500 °C with a heating rate of 10 °C min-1 in a nitrogen atmosphere.

2.9 Statistical analysis

Data represent the mean ± SD of different assays. Statistical significance was determined by one-way analysis of variance followed by the Turkey method, at 5% of significance. The STATISTICA software (Version 5.5, 1999 Edition; Statsoft Inc., Tulsa, OK, USA) was used for all statistical analyses.

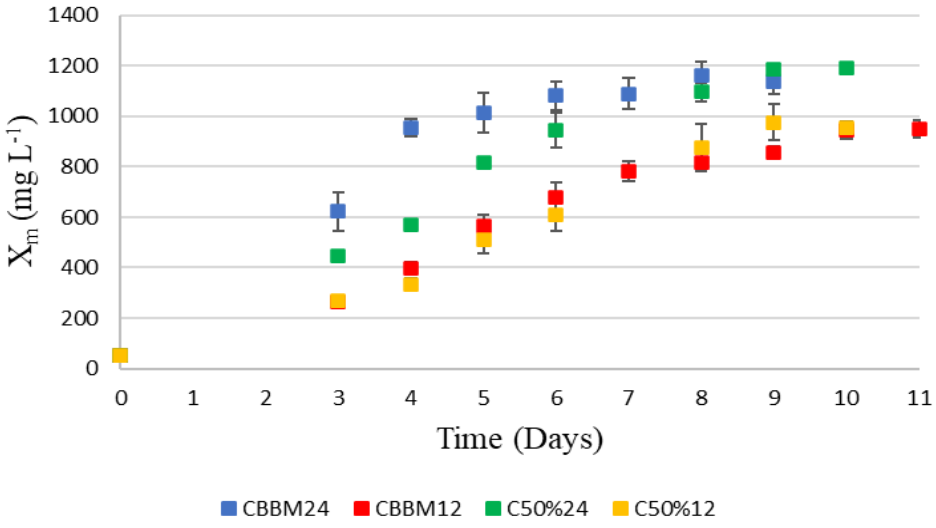
3. Results and Discussion

3.1 Cell concentration, productivity and specific growth rate of cultures under constant light stress (24h), photoperiod 12/12h (light/dark) and $NaNO_3$ reduction

The growth profiles of the microalgae *C. vulgaris* under conditions of constant light stress (24 h), photoperiod 12/12h (dark light) and $NaNO_3$ reduction can be seen in Figure 1 and Table 1. Cultivation under light stress (24h) and complete standard medium (CBBM24) showed the end of the exponential phase after 8 days of cultivation, obtaining a maximum cell concentration (X_m) of 1163.45 ± 52 mg L^{-1} and productivity (P_x) of 145.43 ± 16 mg L^{-1} day^{-1} and specific growth rate (μ) at the end of the exponential phase of 0.39 ± 0.00. Costa et al. (2018b) cultivating the microalgae *C. minutissima* in standard medium obtained the same productivity and specific growth rates as in the present study (CBBM4). However, cultivation under photoperiod (12/12h) and complete standard medium (CBBM12) showed the end of the exponential phase at 10 days of cultivation, obtaining a maximum cell concentration (X_m) of 945.45 ± 34 mg L^{-1} and productivity (P_x) of 94.54 ± 3 mg L^{-1} day^{-1} and specific growth rate (μ) at the end of the exponential phase of 0.29 ± 0.00. CBBM24 cultivation proved to be better than CBBM12, achieving approximately 18% increase in maximum cell concentration and 35% increase in productivity, in addition to the 2-day reduction to reach the end of the exponential phase of cultivation. Bazdar et al. (2018) when cultivating the microalgae *Chlorella vulgaris* under continuous light also obtained a higher concentration of biomass, compared to the photoperiod regime (12/12h, light/dark). Silva et al. (2015, 2017, 2018) also obtained similar results in the production of the

microalgae *C. vulgaris* under constant light. Cultures subjected to NaNO3 deficiency (50%) and 24h light (C50%24) and photoperiod (C50%12) were also evaluated. C50%24 showed the end of the exponential phase at 10 days of cultivation, obtaining a maximum cell concentration (X_m) of 1191.82 ± 10 mg L^{-1} and productivity (P_x) of 119.18 ± 1 mg L^{-1} day^{-1} and specific growth rate (μ) at the end of the exponential phase of 0.31 ± 0.00. The C50%12 culture showed the end of the exponential phase at 9 days of cultivation, obtaining a maximum cell concentration (X_m) of 977.41 ± 20 mg L^{-1} and productivity (P_x) of 108.60 ± 8 mg L^{-1} day^{-1} and specific growth rate (μ) at the end of the exponential phase of 0.33 ± 0.00. When compared, C50%24 obtained approximately 18% increase in maximum cell concentration and 10% increase in productivity, and an increase of 1 day to reach the end of the exponential growth phase. Cultures CBBM and C50%24 did not show significant differences in terms of maximum cell concentration, as well as CBBM12 and C50%12 did not show any difference between them. As for productivity and specific growth rate, all cultivation conditions showed significant differences. Kumari et al. (2022) point out that it is a well-known fact that the survival, growth and productivity of any organism, including microalgae, are strongly affected not only by its physiological and biochemical processes, but also by biotic and abiotic factors in the environment, and that these factors can directly affect the production of bioactive compounds and other economically important products, such as polyhydroxyalkanoates (PHAs).

Figure 1 – Growth profile of microalgae *Chlorella vulgaris* cultivation. (■) CBBM24; (■) CBBM12; (■) C50%24; and (■) C50%12. The figure shows the maximum cellular concentration (X_m – mg L^{-1}) as a function of time (days).



Fonte: Autores (2024).

Table 1 – Growth parameters of the microalgae *Chlorella vulgaris* cultivated under constant light stress (24h, CBBM24) and photoperiod 12/12h (CBBM12) and constant light stress with $NaNO_3$ deficiency (50%) (24h, C50%24) and photoperiod with $NaNO_3$ deficiency (50%) (12/12h, C50%12).

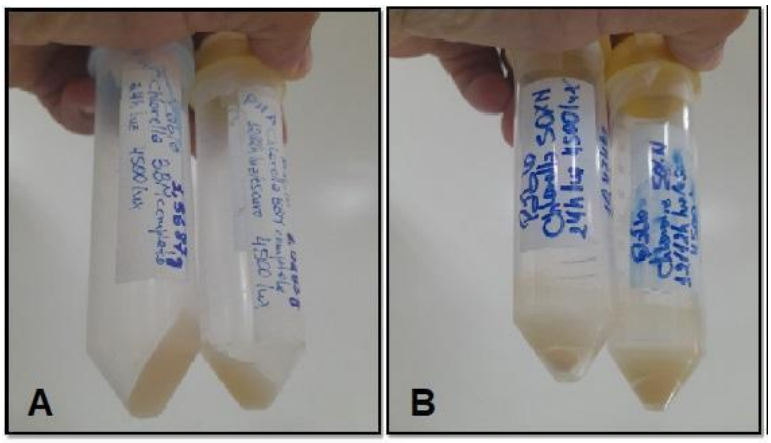
Cultivation conditions	Time (days)	X_m* (mg L^{-1})	P_X** (mg L^{-1} dia^{-1})	μ*** (dia^{-1})
CBBM24	8	1163,45 ± 52[a]	145,43 ± 7[a]	0,39 ± 0,00[a]
CBBM12	10	945,45 ± 34[b]	94,54 ± 3[b]	0,29 ± 0,00[b]
C50%24	10	1191,82 ± 10[a]	119,18 ± 1[c]	0,31 ± 0,00[c]
C50%12	9	977,41 ± 20[b]	108,60 ± 8[d]	0,33 ± 0,00[d]

Data are expressed as mean ± standard error.
Means in the same column followed by different letters represent significant differences ($p < 0.05$).
*Xm = Biomass concentration at the end of cultivation.
**PX = Biomass productivity at the end of cultivation.
***μ = Specific growth rate during the end of the exponential phase
Fonte: Autores.

3.2 Fourier Transform Infrared Spectroscopy (FTIR)

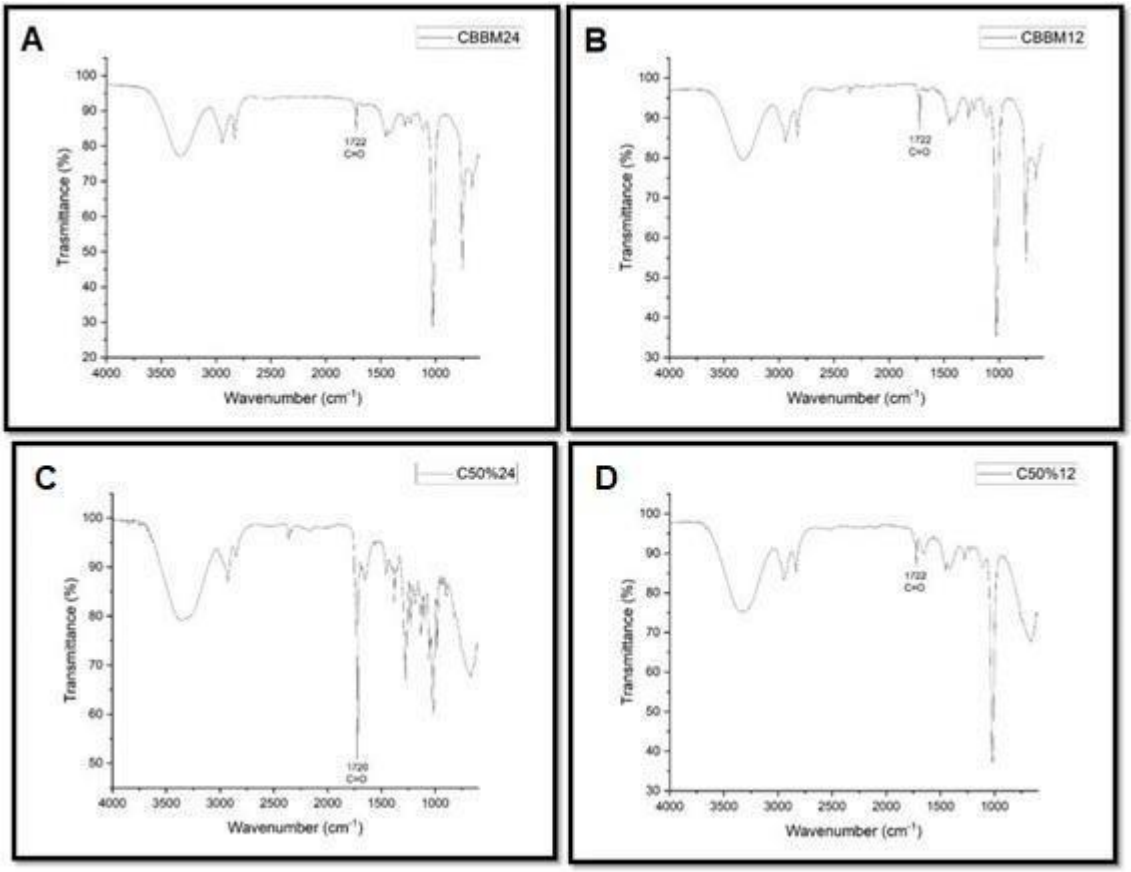
Figure 2 shows the polymers obtained after extraction, and the figures (Figure 3) below show the FTIR spectra of the PHA samples obtained, and show a similarity between the samples in general. The transmittance bands located at 1720-1722 cm^{-1} were attributed to the stretching vibration of the C=O group (carbonyl ester) in the PHA polyester. Characteristic bands of the C-O-C groups appear in the spectral region from 1285 to 1310 cm^{-1}. The transmittance region from 2800 to 3100 cm^{-1} corresponds to the stretching vibration of the C-H bonds of the methyl (CH3) and methylene (CH2) groups. Shrivastav et al. (2010) obtained FTIR spectra of the biopolymer from samples obtained from the biomass of *Spirulina subsalsa* and reported bands at 1724 cm^{-1}, corresponding to the carbonyl ester group. Kavitha et al. (2016) analyzed the FTIR spectra of polyhydroxybutyrate extracted from the biomass of the microalgae *Botryococcus braunii* and identified absorption bands at 2933 cm^{-1}, corresponding to the -CH3 group; at 1728 cm^{-1}, corresponding to the extension of the C=O group, characteristic of PHB; and at 1232 cm^{-1}, which is characteristic of the asymmetric and symmetric stretching vibrations of the C-O-C groups; these data indicate that the biopolymer was obtained. Figure 4 shows the PHA film obtained from the biomass of CBBM24 cultivation (This study).

Figure 2 – Tubes with polymers obtained from the extraction of biomass from the microalgae *C. vulgaris*. (A) CBBM24 and CBBM12, respectively; (B) C50%24 and C50%12, respectively.



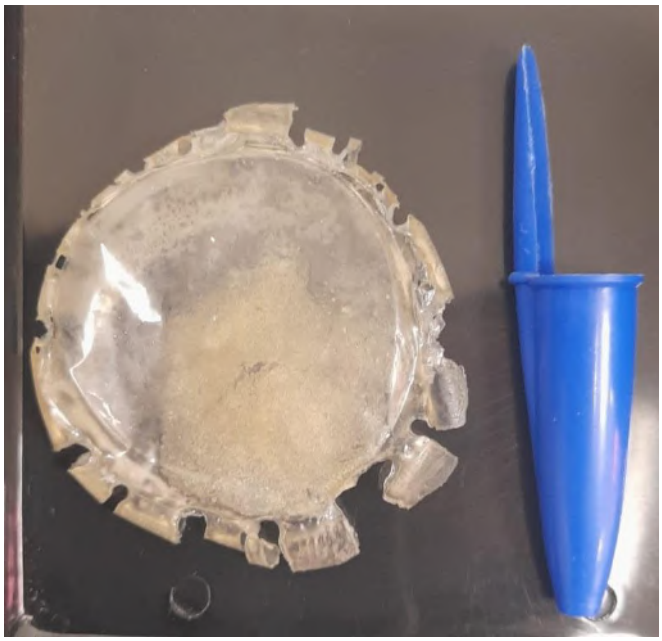
Fonte: Autores.

Figure 3 – FTIR spectra of PHAs extracted from *C. vulgaris*. (A) CBBM24; (B) CBBM12; (C) C50%24; and (D) C50%12.



Fonte: Autores (2024).

Figure 4 - Film obtained from CBBM24 biomass by casting technique with glycerol plasticizer. *In blue the cap of a pen to reference the size of the film (40 mg of polymer).

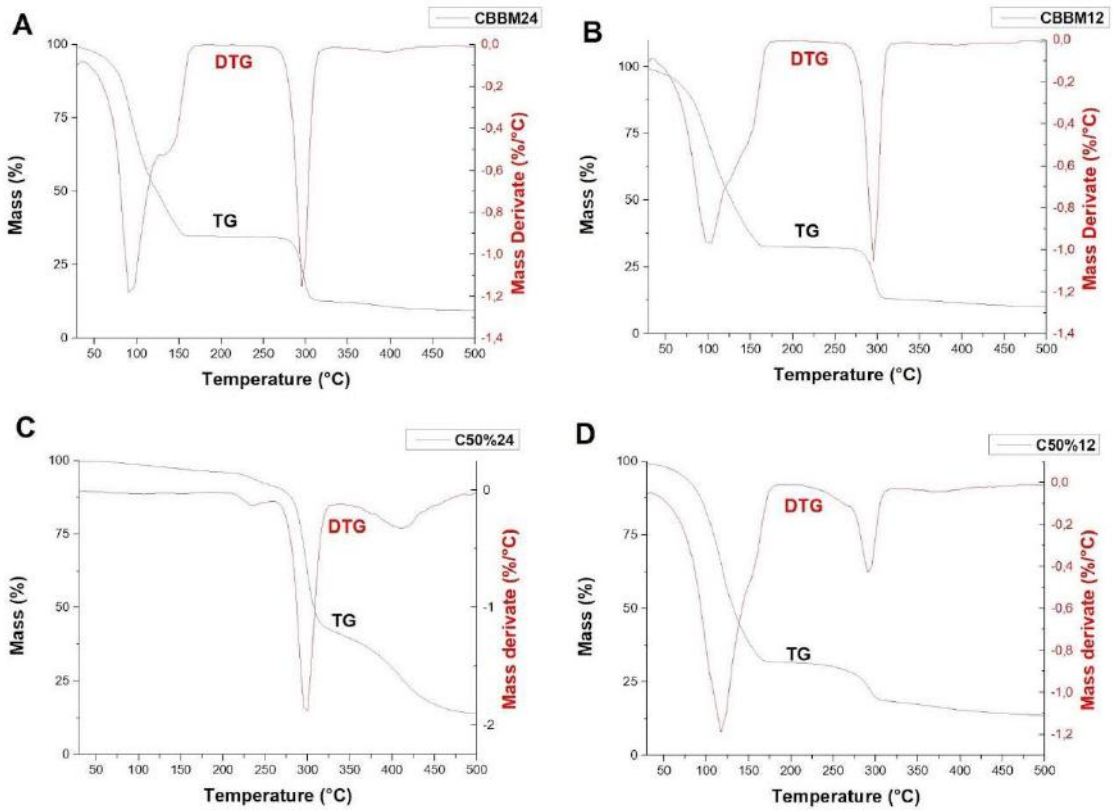


Fonte: Autores (2024).

3.3 Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC)

The thermal stabilities of the obtained PHAs were evaluated by TGA and presented in Figure 5. The results showed that the PHA extracted from *C. vulgaris* in BBM medium under light stress 24h light (CBBM24) presented T_{onset} (21%) at 91 °C and T_{decomp} (76%) at 295 °C, and under photoperiod 12/12h light /dark (CBBM12) T_{onset} (32%) at 104 °C and T_{decomp} (78%) at 295 °C. On the other hand, *C. vulgaris* samples with 50% inorganic nitrogen deficiency (NaNO3) and under constant light stress of 24h light (C50%24) showed T_{onset} (39%) at 300 °C and T_{decomp} (74%) at 410 ° C, and with 50% inorganic nitrogen deficiency (NaNO3) and 12/12h light/dark (C50%12) showed T_{onset} (34%) at 117 °C and T_{decomp} (76%) at 290 °C. The thermal analysis of PHAs obtained by Roja et al. (2019) from 4 different microalgae started between 217-261°C, with an initial weight loss at 150°C, due to the presence of water and impurities in the sample, corroborating the present study. Ansari and Fatma (2016), evaluating the thermal properties of PHB obtained from the microalgae *Nostoc muscorum* NCCU-442, found that the polymer presented T_{onset} = 256 °C and T_{decomp} = 284 °C. The synthesis of PHA with thermal characteristics similar to conventional plastics from microalgae makes these microorganisms promising machines for producing this biopolymer, at a low cost rate and contributing to the environment (Ranganadhareddy, 2022), Abdo and Ali (2019) also emphasize that algae bioplastics have a great plasticization capacity.

Figure 5 – TGA thermograms and their respective DTGs of PHAs extracted from the microalgae *C. vulgaris*. (A) CBBM24; (B) CBBM12; (C) C50%24; and (D) C50%12.



Fonte: Autores (2024).

4. Conclusion

The results show that the microalgae *C. vulgaris* has great potential in the production of polyhydroxyalkanoates, a biopolymer of great industrial application. It also shows that luminosity can directly influence the production of maximum cell concentration, productivity and specific growth rate, even with the reduction of inorganic nitrogen ($NaNO_3$). Therefore, microalgae have great industrial potential and can be a very attractive source in the production of plastic films and in reducing the use of conventional plastics (petrochemicals).

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Conflicts of Interest

The authors declare to have no conflict of interest.

Data availability

Data sharing and data citation is encouraged.

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