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BIOTECHNOLOGICAL POTENTIAL OF MICROALGAE IN HIGH-VALUE COMPOUNDS EXTRACTION AND BIOFUELS PRODUCTION

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Abstract. *Microalgae potential for biofuel production, such as biodiesel, bioethanol, or biohydrogen, is well-known in the academic community. However, to reach the technical and financial feasibility of those processes, it is necessary to utilize microalgal biomass in other operations, reinforcing concepts related to biorefineries. One possibility is the use of biomass and its compounds in agriculture, with the possibility of partial substitution of synthetic fertilizers. The goal of the present work was to determine biostimulating activity of the algae *Tetradismus obliquus* cultured in photobioreactors over cucumber cotyledons. Biomass was subjected to two protocols of water extraction, which were used to determine cytokinin-like activity in recently-germinated cotyledons. The results point out that the extracts have biostimulating activity related to the presence of cytokinins, which implies a higher rate of cellular division and mass gain of the cotyledons. Although preliminary, the results are promising, since microalgal biomass use as a source of molecules with biostimulating action does not interfere with the later utilization of biomass for biofuels production. Therefore, it is expected to contribute to the increase in the technical and financial feasibility of the production of biofuels and other biotechnological products from microalgae biomass.*

Keywords: *Microalgae, Biomass, Biorefinery, Biostimulant.*

1. INTRODUCTION

1.1 Microalgae

Microalgae are photosynthetic unicellular organisms, namely, they transform inorganic carbon into organic carbon (Coutteau, 1996). Hence, they are organisms of major trophic importance in water ecosystems, being responsible for the greatest part of photosynthesis on our planet. These microorganisms can be used as raw material in biotechnological processes for the production of several bioproducts, besides having the capacity to decrease the concentrations of pollutants in the atmosphere through CO₂ fixation (Martinez, 2018). Microalgae can be found in the most diverse environments and are considered a potential indicator of environmental pollution, once their growth can happen in rivers and lakes with high quantities of inorganic compounds.

There are countless species of microalgae all over the world with diverse taxonomic, morphological, ecological, and evolutive characteristics. The freshwater algae *Tetradismus obliquus* (Chlorophyceae) has been used as an agent in nitrogen and phosphorus removal from wastewater (Martinez et al., 2000) and has already been subject to studies in bioremediation of effluents, biofuel production such as biodiesel, green diesel and biohydrogen, fish and dog food at the Núcleo de Pesquisa e Desenvolvimento de Energia Autossustentável (NPDEAS), at the Universidade Federal do Paraná (Taher, 2013; Balen et al., 2015; Corrêa, et al., 2017; Miyawaki et al., 2021; Costa et al., 2022).

1.2 Culture mediums

Several biotic and abiotic factors influence the growth of microalgae, from conditions of pH and temperature to ecological interactions of competition and predation. Among the abiotic factors, the main ones are the concentration of nutrients such as phosphorus, nitrogen, and carbon dioxide, besides incident light energy (Blanken et al., 2013). Microalgae culture can take place in synthetic mediums, with their composition defined by inorganic salts, as CHU medium (Chu, 1942), or in alternative mediums, with undefined composition and elevated organic load, as agroindustrial diverse wastewaters. The source of luminous energy used in the culture can be natural or artificial. As a natural source, solar energy is commonly used, because it is free and abundant. However, this source carries some disadvantages such as luminous cycles (photoperiod), dependence on climatic conditions, and changing seasons. As an alternative, it is possible to use light energy derived from artificial sources, such as light bulbs, which will increase the productivity of the culture in exchange for a higher added price to the production (Blanken et al., 2013).

1.3 Culture systems

Microalgae can be cultured in open or closed systems. Open systems, such as pools, water tanks, and lakes are more widely used due to their low cost of construction and maintenance. However, this model of culture requires large areas, besides being more susceptible to contamination, excessive loss by evaporation, and low diffusion of solar light in the system. Closed systems of microalgae culture, on the other hand, are entitled to photobioreactors (Santos, 2016). Photobioreactors have a better performance compared to open systems. Among its advantages, we can mention higher control of culture conditions, lower rate of contamination and higher productivity per square meter of the area occupied, and the adaptable construction related to the installation environment (Taher, 2013).

1.4 Commercial uses

Microalgae can be utilized in several areas as raw material in the production of bioproducts, just as biofuels, chemicals, and cosmetic materials, as well as it can be used in animal and human feeding and aquaculture. Besides, they can be a source of high-added-value molecules, like oily acids used in human food supplementation and pigment production (Spolaore, et al., 2006). Still, microalgae can be used in the treatment and production of biofertilizers, products with biostimulating and elicitor activity (Zanette, et al., 2019).

One of the biggest potential markets for microalgae is biodiesel production. Climate change and the great dependence on fossil fuels as energy sources boost more and more investment in innovative and renewable technologies as energetic alternatives, like biodiesel, bioethanol, and biohydrogen. Though, around 70% to 80% of the production cost of biofuels comes from raw material acquisition (Santos, 2016). For this reason, microalgae have a great potential to lower the cost of production due to their high productivity. In face of this context, Brazil is one of the countries that have the favorable edaphoclimatic conditions for microalgae to be one of the main sources of biofuels in the future (Ho et al., 2014).

1.5 Presence of high-added-value molecules

According to Spolaore et al. (2006), microalgae present some molecules in its composition that possess high added value, such as oily acids, used in food supplementation and pigment production, or carbohydrates, which are found in the forms of starch and other polysaccharides, besides glucose, and can be used as raw material in the industry.

One of the most abundant molecules in microalgae is chlorophyll, the main responsible for capturing photons in synthesizing organisms and thereby realizing photosynthesis. Chlorophyll can be used for the production of chlorophyllin, which is a derivative used as a dye by the food, textile, and paper industries (Santos et al., 2021). Along with it, carotenoids are also pigments found in microalgae cells. Astaxanthin, xanthophylls, zeaxanthin, canthaxanthin, and echinenone are some examples that are exploited in nature, coloring animals like birds, fish, reptiles, and amphibians. These molecules act as well in the social dynamic of species, indicating the social level and attracting sexual mates. And, as well, one of the more important purposes: to serve as camouflage and toxicity alert.

In the industry, carotenoids are employed due to their antioxidant capacity, since that, in nature, they have the purpose of absorbing excessive luminous energy and, in that way, avoid damage to the cell and even to chlorophyll (photo-oxidation). Astaxanthin is a good example of industrial use, being applied mainly by the pharmaceutical industry due to its antioxidant properties, photo-protection, increase in immune response, and treatment of degenerative diseases. And besides carotenoids, there are other compounds of high added value, such as docosahexaenoic acid (DHA) and eicosapentaenoic (EPA), which can be commercialized for therapeutical or pharmaceutical applications, some being studied as antimicrobial compounds. Carbohydrates are also present in microalgae cells, and they have great value for presenting compounds such as B 1,3-glucan - which shows immunostimulating, antioxidant, and blood cholesterol-reducing activities - and polysaccharides that are applied in anti-adherent therapies and bacterial infections, alginate, and cellulose, which are used as emulsifiers and stabilizers in the food field (Santos et al., 2022).

1.6 Microalgae in agriculture

Microalgae are major allies in agriculture, once they are present in great agricultural activities, in extensive crops, and even in the farmers that move local economies. Microalgae culture applied to agriculture points to a great economical and sustainable potential, to the extent that they have compounds with biostimulating activities that can replace synthetic fertilizers. Organic agriculture is expressive over the global economy, and using natural fertilizers paves the way to the creation of more healthy crops, fit to adverse factors, such as biotic and abiotic stresses, plant resistance, quality, and productivity (Corrêa et al., 2021). On microalgae, biostimulating activity is associated with the presence of various substances such as proteins, amino acids, peptides, vitamins, and minerals, and mainly phytohormones (classes of hormones present in vegetables). According to Navarro et al. (2021), extracts obtained from microalgae can be used as biostimulants, providing a plain development in plants that are threatened with extinction, as an example, *Cattleya warneri*, an orchid with great ornamental potential and a delicate reproductive cycle, having seeds that do not nourish completely the embryo, depending on the substrate in which it is to grow normally. Microalgae can yet be used in cultures for food production, as in organic tomatoes and other vegetable cultivation (Mazepa et al., 2021).

1.7 Microalgae biorefineries

Understanding that microalgae have much to offer, it is fundamental to develop processes that allow the obtainment of different bioproducts stem from the same microalgal raw material, establishing a concept of a biorefinery. From the cultivation of microalgae, a variety of co-products can be obtained, with emphasis on pigments such as carotenoids and chlorophyll, which are applied to dietary, pharmaceutical, and cosmetic fields. Chlorophyll can be used in textile and food industries (in vitaminic supplements, for example) and utilization of carotenoids as astaxanthin, xanthophylls, and zeaxanthin - pigments that serve as antioxidants (Santos et al., 2021).

Microalgal biomass can be used in the extraction of oil for biodiesel production, or even extraction and fractioning of green diesel (Costa et al., 2022). Biodiesel derived from microalgae fits in the third generation of biofuels, which is different from the first and second generations by the reduced impact on the environment, not needing great lots of land and, consequently, not decreasing local biodiversity, therefore making it possible for its production in places such as ponds and oceans. That decreases its impact on the hydric and carbon footprint along the production chain (Siddiki, et al., 2021). In working with biodiesel production through biomass, it is also possible to extract different co-products from it, which can be applied in other activities, for example, ethanol, biogas, and hydrogen that are produced by biochemical processes; pigments, vitamins, and antioxidants that are used in pharma and cosmetic industries; biomass itself that can be used in dietary supplementation; bio-electricity that come from microalgal fuel cells; and even being used biomass extracts, which act as plant fertilizers (Chandrasekhar, et al., 2021).

2. MATERIALS AND METHODS

The microalgae *Tetradesmus obliquus* was cultivated in a synthetic medium for microalgal production, used for extraction of compounds and determination of biostimulating activity, according to the following described methodologies.

2.1 Cultivation execution facilities

Experiments were carried out at the Núcleo de Pesquisa e Desenvolvimento de Energia Autossustentável (NPDEAS) from the Universidade Federal do Paraná, UFPR. Cultures for biomass production were performed in photobioreactors of the Airlift kind, with a total volume of 12 L (FIG. 1), constituted of transparent PVC tubes, set up in the external area of NPDEAS, turned to sunlight, and exposed to climatic environmental variations of the city of Curitiba - PR (Brazil).



Figure 1. FBR Airlift for microalgae cultivation.

2.2 Information about cultivation

The algae utilized in the experiments was the chlorophyte *Tetradesmus obliquus*, a native microalga from Curitiba and the main microorganism used in the research developed at NPDEAS. The medium utilized for microalgae growth was CHU medium (Chu, 1942), composed of inorganic salts of macro and micronutrients: NaNO_3 (250 mg L⁻¹), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (25 mg L⁻¹), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (75 mg L⁻¹), K_2HPO_4 (75 mg L⁻¹), KH_2PO_4 (175 mg L⁻¹), NaCl (25 mg L⁻¹), EDTA (50 mg L⁻¹), KOH (31 mg L⁻¹), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (5 mg L⁻¹), H_3BO_3 (11.4 mg L⁻¹), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (8.8 10⁻³ mg mg L⁻¹), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.4 10⁻³ mg L⁻¹), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (1.2 10⁻³ mg L⁻¹), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.6 10⁻³ mg L⁻¹), $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.49 10⁻³ mg L⁻¹). Each culture carried in photobioreactor contained 10% inoculum previously grown in 2L Erlenmeyer flasks in temperature and light conditions controlled. The culture scheme adopted for the production of biomass in the Airlift reactors was in batches, namely, without nutrient influx between system inoculation and biomass collection.

2.3 Culture growth evaluation

For the tracking of the culture, samples were taken periodically from each of the photobioreactors. The measured parameters were the cell number growth, pH from the cultures, and dry biomass. Determination of cell concentration in the sampling was carried out with the aid of a Neubauer chamber and an optical microscope from the brand Bioval, using ampliation of 400x. Countings were made in triplicates and a growth curve was produced from the average of the obtained data from the photobioreactors.

Dry biomass was determined through a process of vacuum filtration of a known volume of the culture, carried out in duplicates. To make sure of the dryness of the biomass, after the end of filtration, filters were kept in a glasshouse at

60°C for a period of 24 hours. The mass of microalgae was determined using an analytical scale from the brand Shimadzu, model AUW220D, with the precision of 0,0001g. A profile of cell concentration was drawn from the average of the data obtained from the filtration of samples.

2.4 Water extractions

For the production of microalgae extract and determination of biostimulating activity, dry microalgal biomass was macerated and resuspended in deionized water in the proportion of 10% (m/v). Two extracts were produced, one of them at room temperature and another one at the temperature of 80°C. In both conditions of extraction, the mixture was kept under mechanical agitation for two hours, followed by centrifugation under 2600 x g for 10 min for solid-phase separation (biomass) from liquid phase/supernatant (water extract). The extracts were then separated and stored in a freezer until utilization in bioassays of biostimulating activity.

2.5 Biostimulating activity assays

The assays for the determination of biostimulating activity were carried out using cucumber seeds (*Cucumis sativus*) acquired in the local market of the city of Curitiba. The seeds germinated in Petri dishes with agar 0.7% (m/v) and were kept in the dark in B.O.D incubators for 3 days at 25 ± 1 °C. After this period, the cotyledons were excised and distributed in Petri dishes lined with filter paper soaked in 3 mL of the different water extracts (room temperature and 80°C). Each treatment consisted of 3 repetitions with 10 cotyledons in each dish. For obtained answer correlation by the fractions with a possible cytokinin-like activity, were carried out bio-assays with the plant regulator Benzylaminopurine (BAP) as a reference cytokinin, in different concentrations (0; 0.1; 1. 5; 50; and 100 $\mu\text{g mL}^{-1}$) The dishes remained in the B.O.D incubator with a temperature of 25 ± 1 °C for 3 days and after that period the cotyledons were scaled in analytical scale for mass gain evaluation.

3. RESULTS AND DISCUSSION

Biomass used to obtain the water extracts was produced from the cultivation of *Tetrademus obliquus* cells in photobioreactors for a period of 8 days. Figure 2 presents the growth profile and cell density of the cultures conducted in the Airlif photobioreactor.

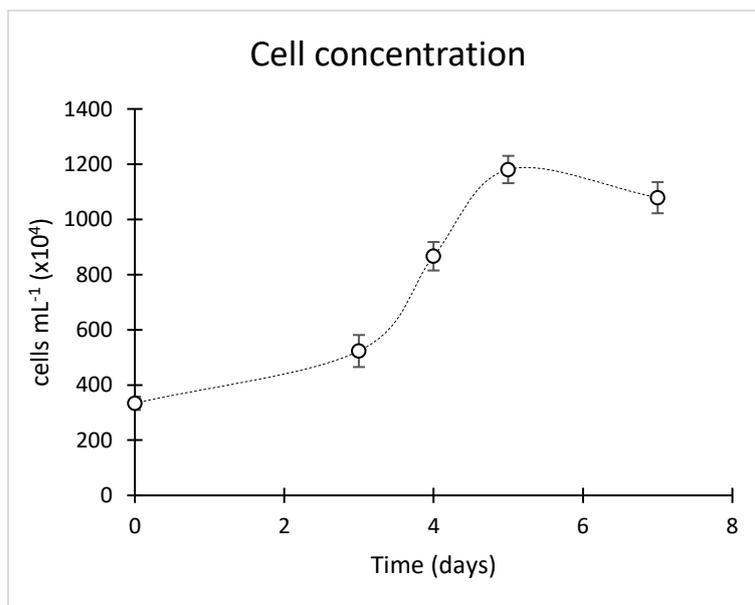


Figure 2. Cell density of *T. obliquus* cultured in FBR airlift.

According to the graphic, cell concentration follows a phase of adaptation between days 1 and 4, followed by a stage of fast growth between days 4 and 6, until the moment in which cell concentration reaches its optimal point. After this period, a reduction in cell concentration was observed, indicating the beginning of the death phase of the cultures. For this reason, culture was taken in its most productive point for biomass obtainment. In a standard growth curve for the cell concentration of microalgae (and for other microorganisms such as bacteria), it is possible to note the different

phases of growth as well as it is visible in this experiment. The first phase is called lag, where there is cell activity from the microalgae, which utilize the medium they are to obtain molecules and synthesize proteins that are necessary for its reproduction. The following phase is called log (or exponential) and highlights asexual reproduction among microalgae that reproduce exponentially, having high metabolic activity at this moment and using up medium compounds to reproduce. The stationary phase comes next. Due to the increase of individuals in the medium, the available nutrients and free volume to keep reproduction decreases, showing them to be inversely proportional. During this period there is a break in the exponential reproduction, keeping the cell concentration stable for a certain time. Thus, comes the last phase, which is death. For not having nutrients and needed space to keep the plain reproduction of the organisms, residues are generated and many dead cells show up, decreasing, therefore, the number of live cells and making the graphic plunge, emphasizing, then, the end of the growth curve.

For a cytokinin-like activity, the results were obtained through differences in averages and standard deviations made by duplicates of each extract, BAP treatment, and negative control, after germination of cucumber seeds, cotyledon excision, and maintenance of bioassays in the glasshouse. Figure 3 presents the data of the increase of cucumber cotyledons mass in the cytokinin-like activity assay.

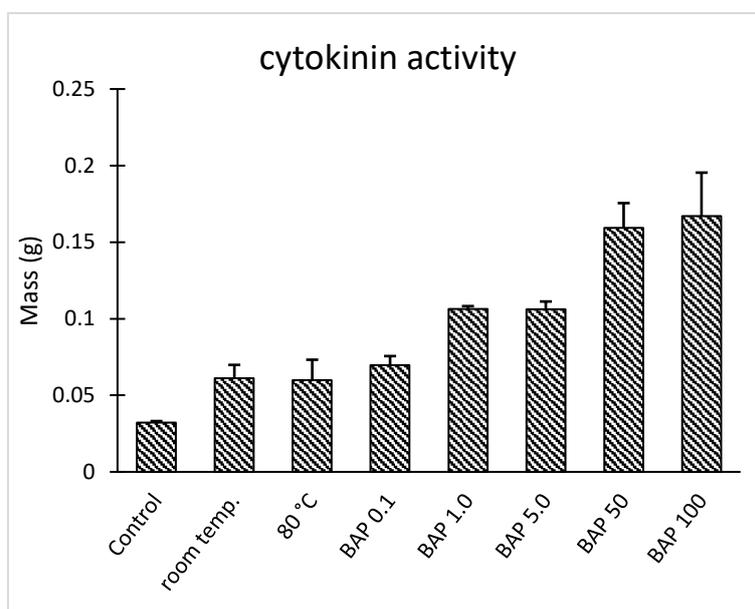


Figure 3. Increase in cotyledon mass in the cytokinin activity bioassay.

The results point out that microalgae extracts have cytokinin-like activity over cell multiplication, growth, and mass gain of the cucumber cotyledons. Compared to control, the extracts resulted in bigger mass cotyledons, equaling its results with the activity of the synthetic plant regulator BAP in the concentration of 0,1 $\mu\text{g mL}^{-1}$. Higher concentrations of BAP, equivalent to 50 e 100 $\mu\text{g mL}^{-1}$ presented elevated cytokinin-like activity, although they present higher standard deviations. Cytokinins such as BAP, are one of the groups of plant hormones that act over the plants inducing their growth through cell division. The morphological response of growth observed for the microalgae extracts is indicative of the presence of similar compounds to this phytohormone in microalgal biomass.

The water extract obtained in this work is a compound that can contain besides plant hormones as cytokinins, auxins, gibberellins, and abscisic acid, proteins, amino acids, mono, oligo, and polysaccharides that can result in the cytokinin-like activity observed (Mazepa, *et al.*, 2021; Corbelini, *et al.*, 2020; Navarro, *et al.*, 2021). Besides cytokinin-like activity, microalgae biomass and its compounds can be used in agriculture in several ways, promoting the enrichment of soil for CO_2 e N_2 fixation and the decrease in the quantity of macro and micronutrients needed to develop organisms, besides protection against bacteria, fungi or nematodes (Siddiki, *et al.*, 2021).

According to the results obtained, it is possible to infer that the microalgae extract from the species *Tetradismus obliquus* has biostimulating activity similar to the cytokinin phytohormone benzylaminopurine, once it induced a similar response to the synthetic plant regulator BAP. According to Corrêa *et al.* (2019), equivalent activities were observed using the extracts of the microalgae from the species *Desmodesmus subspicatus* applied in the germination of seeds of *C. warneri*, indicating that they have biotechnological potential that can be applied in different proposals. The obtained results show the potential to expand the studies of microalgae extracts and how they are correlated to the plant phytohormones, possessing a notable biotechnological application for modern agriculture.

The results also exemplify ways of extraction of varied compounds from the same raw material. Starting its activities in the dietary field, microalgae are passing through several studies for their applicability, going through areas of aquaculture, cosmetics, biochemistry (through the handling of oily acids), pharmaceutical, and renewable energy. And,

therefore, even being recent, it is possible to make the same raw material, such as the microalgae biomass, provide different compounds for commercial applications (Lourenço, 2007).

Companies that boost these areas can use biomass to take out their products, such as oil for biodiesel production, for example, at the same time that they can work with others that are not completely interconnected, like biostimulating extracts/biofertilizers (the percentage of the compounds extracted from biomass is not equivalent to its total mass, what allows the industries to work over the same raw material to obtain their compounds). Biomass having this diversity in its composition does not compromise or invalidate the primary processes that a company has and provides different ways to work with different already existing compounds that can be refined and developed in the future.

Microalgae potential is extensive and still is in its initial phases of study due to limitations over biomass. Therefore, to gain progress in its applicabilities industries need to understand that biomass must be studied and its production and acquisition methods have to be improved, implying not only the increase in production of primary compounds of interest but also the use of secondary compounds that can serve to reach different ways of production and research.

4. CONCLUSIONS

The use of extracts of *Tetrademus obliquus* to determine biostimulating activity indicated that microalgal biomass has potential for the development of products with direct application in agriculture, especially in practices related to organic crops. It is important to highlight that the viability of the acquisition of this product, or others such as pigments, proteins, and biofuels can only be reached with the development of methodologies and processes that allow the exploration of biomass for the obtainment of co-products from this same raw material, establishing the concept of microalgae biorefineries.

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