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BIOSTIMULANT POTENTIAL OF THE MICROALGAE *TETRADESMUS OBLIQUUS* CULTIVATED IN AIRLIFT PHOTOBIOREACTORS AS A COPRODUCT OF BIOFUELS GENERATION

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Abstract. *Microalgae have great potential in biofuels production, such as biohydrogen, bioethanol, and biodiesel. Technical-scientific knowledge about these microorganisms increase every year, but it is also necessary to make biomass and bioproducts production processes viable, per developing the concept of biorefinery. Regarding microalgae, biomass utilization enables several applications such as in agriculture, with potential for partial substitution of synthetic fertilizers. Therefore, the aim of the present work was to evaluate the biostimulant activity of the microalgae Tetradesmus obliquus cultivated in airlift photobioreactors over lettuce seeds. The biomass was subjected to extraction generating the extract that was used in soil applications germinated with lettuce seedlings (Lactuca sativa). fresh and dry mass of the lettuce plants were measured to determine biostimulating activity and lipid levels were analyzed to correlate the concept of biorefinery from microalgal biomass. Regardless of being preliminary, the results shows that the microalgal biomass used for obtaining biostimulant extracts have biostimulating activity related to the overall growth of the plants and does not interfere with the later utilization of biomass for biofuels production. For this reason, it is expected to contribute to technical and financial viability increase, aiming the production of biofuels and other products from microalgal biomass.*

Keywords: *microalgae, biostimulant, biofuels, biomass, photobioreactor*

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 Tetradesmus 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. Some disadvantages that natural light brings with it are the existence of light cycles (photoperiod), dependence of climatic conditions and the change of seasons (Blanken et al., 2013). Alternatives that are used to circumvent these problems are the use together of lamps that provide artificial light and increase the production of microalgae, but this makes the procedure with a higher cost (Blanken et al., 2013).

1.3 Culture systems

There are two systems that microalgae can be cultivated, the open and the close 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 sollar light in the system. Close 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 applied in several areas, being used as a raw material in the production of bioproducts such as biofuels, chemicals, materials, and cosmetics, 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 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 to produce 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.

Carotenoids are employed in various industries due to their antioxidant capacity. In nature, they have the purpose of absorbing excessive luminous energy and, in that way, preventing damage to cells and chlorophyll through photo-oxidation. Astaxanthin is a notable 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. Furthermore, apart from 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. Microalgae cells also contain carbohydrates, and they have great value for presenting compounds such as Β 1,3-glucan - which shows immunostimulating, antioxidant, and blood cholesterolreducing 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), $CaCl_2.2H_2O$ (25 mg L-1), $MgSO_4.7H_2O$ (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), $FeSO_4.7H_2O$ (5 mg L-1), H_3BO_3 (11.4 mg L-1), $ZnSO_4.7H_2O$ (8.8 10 -3 mg mg L-1), $MnCl_2.4H_2O$ (1.4 10-3 mg L-1), $Na_2MoO_4.2H_2O$ (1.2 10-3 mg L-1), $CuSO_4.5H_2O$ (1.6 10-3 mg L-1), $Co(NO_3)_2.6H_2O$ (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 to produce biomass in the Airlift reactors was in baches, 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 Extraction of the cultive

The assays to produce the microalgae extract in natura cultivation was used in 12L photobioreactors. Through it, three extracts were produced with different concentrations, namely 0.5, 1, and 2 g/L. For each concentration, a dilution process with deionized water was performed since the cultivation was at a concentration of 2.57 g/L. At the end of the dilution, 200 ml of each concentration were stored for application in the phase two of the experiment.

2.5 Assay of biostimulant Activity

In the first phase of the experiment, seed germination was tested using different concentrations of microalgae extracts to determine the most effective concentration. Lettuce seeds (*Lactuca sativa*) are the most suitable for laboratory studies involving germination due to their manageable size and good response to external stimuli. A total of 180 lettuce seeds were used and placed in germination boxes (germabox) containing filter papers, which served as a medium for their fixation. For comparison purposes, triplicates of each extract with different concentrations were prepared, along with a negative control containing only water ionized.

In the first part of the phase 1, 15 lettuce seeds were added to each germination box, and then 15 ml of each extract was applied to the filter papers, ensuring a thorough mixing of the medium and seed fixation. The boxes were kept in a B.O.D. (Biological Oxygen Demand) incubator, wrapped in aluminum foil, at a temperature of 25°C for approximately 5 days. At the end of the fifth day, the boxes were removed, and seedling growth was evaluated, focusing on the radicle growth. A software program was used to identify which radicles exhibited the most growth compared to others, facilitating the decision on which extract to use.

After determining the optimal concentration to proceed with the assays, lettuce seedlings were used and placed in germination trays with fertilized soil. Approximately 12 seedlings per treatment were used. They were placed back in the B.O.D. incubator at a temperature of 25°C. Applications of the chosen extract and the negative control (water deionized only) were applied in the soil every seven days, in addition to regular daily watering. The experiment lasted for approximately 45 days, which was sufficient to obtain relevant data regarding seedling growth.

3. RESULTS AND DISCUSSION

The cultivation of *Tetradesmus obliquus* on a laboratory scale enable the production of extracts on different concentrations. Figure 2 presents the generic growth profile of the microalgae in CHU synthetic medium. This profile determines the peak of major productivity of cells, with implies in a large amount of biomass that can be used to produce the extracts.

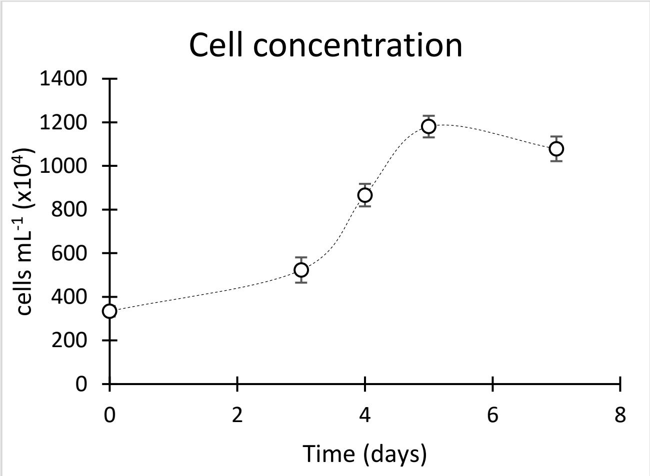

Figure 2. Cell density of *T. obliquus* cultured in CHU medium.

According to the graphic, the microalgae follow an exponential growth, a type very usual in the growth of microorganisms. It has three phases: lag, log, and stationary. Between days 1 and 4, it is in the lag phase, which determines the adaptation of the microalgae to the environment and the provided nutrients. During this phase, it produces molecules and synthesizes proteins necessary for its reproduction. After that, comes the log phase which occurs between days 4 and 6. This phase is also know as exponential phase, because the microalgae are already adapted to the environment and utilizes the nutrients to multiply rapidly. At the end, there is the stationary phase (death phase), where the microalgae no longer multiply with the same intensity as the log phase due to nutrient depletion. The multiplication rate decreases, and the remaining cells seeks it surviving until its eventual death, resulting in the accumulation of waste and dead cells.

The extracts were obtained around the fifth day of the cellular growth, which is when it reaches and optimum point of productivity before entering the stationary phase. The biostimulating activity in the first phase of the experiment showed different results according to the different concentrations of *T. obliquus* cultivation. The results were obtained through triplicates for each parameter used, including concentrations of 0.5 g/L, 1 g/L, 2 g/L, plus a negative control. Figure 3 presentes data on the average root growth of lettuce seeds in response to the different concentrations.

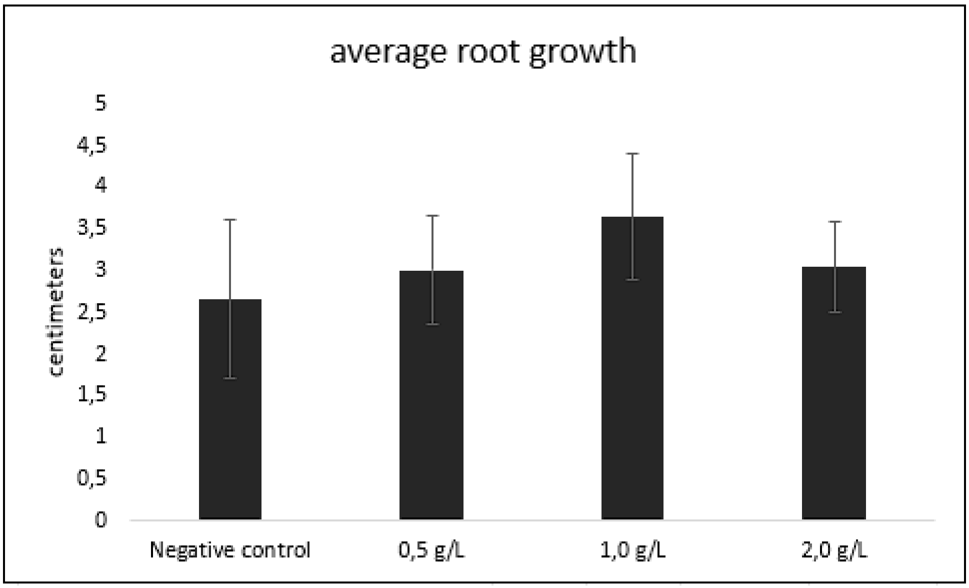

Figure 3. Biostimulating activity of *Tetradesmus obliquus*.

4. CONCLUSIONS

The preliminar result indicates that the use of extracts of *Tetradesmus obliquus* have biostimulating activity on lettuce seeds. The microalgal biomass has potential for development of products with direct application in agriculture, especially in practices related to organic crops. Besides, it is important to highlight that the viability of the acquisition of this product, such as biofuels, pigments, proteins, can only be reached with the development of technologies and methodologies that allow the exploration of biomass, a raw material that enables the obtention of various co-products, optimizing the concept of microalgae biorefineries.

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