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# OUTDOOR CULTIVATION OF *Scenedesmus obliquus* BR003 IN STIRRED TANKS BY AIRLIFT

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**ABSTRACT**: The use of microalgae as a source of raw material for biofuel production has been highly targeted. Compared to other biological options for the capture and use of $CO_2$, microalgae crops have the following advantages: high productivity, lack of competition with feed and food-based products, use of unproductive and non-arable land, and allowance of the use of wastewater for its cultivation. The use of wastewater from industrial, agricultural, and domestic sewage has been indicated as an alternative means to reduce the cost of crops, since such waste is generally released into the environment without previous treatment. This work addresses the use of petrochemical wastewater (ARP) from effluent produced at the Gabriel Passos Refinery (Petrobrás refinery unit) for the cultivation of a strain of *Scenedesmus obliquus* BR003. Twelve experimental units of 20 L each were constructed in the experimental area of the Biofuel Laboratory of the Federal University of Viçosa, Minas Gerais, Brazil in an uncontrolled environment, and the cultures were evaluated by quantification of total carbohydrates, total lipids, dry biomass, and growth evaluated by absorbance. The results showed that the wastewater from petrochemical effluent is an efficient growth medium for the growth of *Scenedesmus obliquus* BR003 microalgae for large-scale cultivation on land that is not suitable for other agricultural crops.

**KEY WORDS**: wastewater treatment, microalgae, biofuel.

## INTRODUCTION

With growing attention focused on clean technologies, sustainable development, and environmental concerns, microalgae appear as an ideal solution. According to the research conducted by Teixeira & Morales (2006) of the National Institute of Technology, microalgae have characteristics that guarantee the production of an efficient and clean biofuel with high growth rates, assimilation of carbon dioxide, and expressive contents of lipids and carbohydrates without worry over the risks of its use. Considering the advantages of microalgae, the production of biofuels, in certain aspects, is attractive regarding the reuse of waste from other production processes. The use of agro-industrial wastewaters for the production of microalgae appears as a solution to reduce production costs in the composition of the culture medium and to minimize the effects caused by waste discharges (Cereda, 2002).

Microalgae can be grown in various production systems, with their volume varying from as little as a few liters to billions. The systems commonly used are unsophisticated because they occur in the open, under natural lighting conditions, and with low or no control over these environmental variables (Molina-Grima, 2003). Finding microalgae species that are apt for growth is possible; however, producing unialgal crops for biodiesel is not a trivial activity, since they have similar characteristics and require special care, such as avoiding contamination by other species of microalgae. The main problem in the cultivation of microalgae in open systems, usually on a large scale, is that species with higher oil content are not necessarily the fastest to reproduce. Because these systems are open, it is important to note that these systems are more susceptible to contamination by other species of microalgae and bacteria (Volkmann et al., 2008).

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The number of species that have been successfully cultivated in open systems is relatively small. In addition, there is less control over water temperature, $CO_2$ concentration, and lighting and mixing conditions in these systems. As a consequence of these factors, the production of algal biomass is lower when compared to cultures in closed systems, also called photobioreactors (Santos et al., 2013). However, the low operational and maintenance costs of open systems, the possibility of taking advantage of agroindustrial residues, and the ease of operation of the crop units have encouraged several authors to examine feasible outdoor crops (Park et al., 2011; Chiaramonti et al., 2013; Waller et al., 2012, Moazami et al., 2012). Considering the above, this work had the objective of evaluating and comparing cultures in outdoor open tanks of *Scenedesmus obliquus* BR003 in ARP, aiming at the production of biomass, carbohydrates and lipids, which comprise the raw materials needed to produce ethanol and biodiesel.

## MATERIAL AND METHODS

### Microalgae strain and culture conditions

The strain *Scenedesmus obliquus* BR003 was obtained from the Collection of Cyanobacteria and Microalgae of the Petrobrás Project from the Laboratório de Crescimento de Plantas of the Department of Plant Biology of the Universidade Federal de Viçosa (UFV). The experiment consisted of twelve polystyrene tanks (26 cm in diameter and 40 cm in height) using 20 L of useful volume for the cultures.

In this work, the culture media B4, based on agricultural fertilizers, and BG11 were used. BG11 medium (Andersen, 2005) was prepared with analytical purity reagents and used as a control treatment, and B4 medium was formulated using ammonium monophosphate (0.688 g $L^{-1}$), potassium chloride (0.174 g $L^{-1}$), and calcium nitrate (0.0517 g $L^{-1}$) (Soares, 2012). Different from the B4 culture medium, the BG11 medium presents low potassium content and uses $NaNO_3$ as a source of nitrogen. Twelve experimental units were used, in triplicate, consisting of cultures in B4 and BG11 ARP medium and cultures in the same medium using water from the public treatement system (control). The nutrients were added in two steps, 50% at the beginning and 50% after the ninth day of cultivation, which corresponds to a fertilizer concentration of 0.457 g $L^{-1}$ at each dose. The composition of the ARP is shown in Table 1.

TABLE 1. Characterization of pretreated petrochemical wastewater (ARP).

| Parameter | Value | Unit |
|---|---|---|
| pH | 8.14 | - |
| TDS | 12,660 | mg $L^{-1}$ |
| Conductivity | 17,728 | µS $cm^{-1}$ |
| Calcium | 724 | mg $L^{-1}$ |
| Magnesium | 273 | mg $L^{-1}$ |
| Sodium | 2,869 | mg $L^{-1}$ |
| Potassium | 642 | mg $L^{-1}$ |
| Strontium | 21.7 | mg $L^{-1}$ |
| Barium | 1.06 | mg $L^{-1}$ |
| Ammonia | 3.75 | mg $L^{-1}$ |
| Bicarbonate | 1,482 | mg $L^{-1}$ |
| Sulfate | 1,265 | mg $L^{-1}$ |
| Chloride | 5,291 | mg $L^{-1}$ |
| Flouride | 4.81 | mg $L^{-1}$ |
| Nitrate | 51.1 | mg $L^{-1}$ |
| Phosphate | 3.16 | mg $L^{-1}$ |
| Silica | 29 | mg $L^{-1}$ |

Source: Refinery Gabriel Passos, Betim, Minas Gerais (Machado, 2011).

For sanitization of the culture tanks and culture media, ozone gas was used at a concentration of 120 ppm. Each experimental unit was inoculated with 25% inoculum (v $v^{-1}$), previously acclimated to the outdoor culture conditions in the respective culture media. The cultivation experiments were performed during 24 days under photoautotrophic conditions in a greenhouse with an average radiation of 613.6 μmol photon $m^{-2}$ $s^{-1}$, measured daily with a radiometer (LI-COR, model LI-185B), at a temperature of 25 ± 0.1 ºC and pH of 7.6 ± 0.1. The solar radiation attenuation by the greenhouse cover was 58%, as measured by the mean external radiation (1058 μmol photon $m^{-2}$ $s^{-1}$) and internal radiation (613.6 μmol photon $m^{-2}$ $s^{-1}$). The agitation of the cultures was carried out by means of compressed air at a total flow of 0.28 L $s^{-1}$ using a 2-hp compressor (Motomil, model MAM-8.7/24) and measured by means of a rotating blade anemometer (Minipa, model MPA-20). The pH was adjusted daily by the addition of aliquots of HCl or NaOH stock solutions with a concentration of 1 M (mol $L^{-1}$), and the volume of the cultures was kept constant by the addition of water previously treated with ozone gas at 120 ppm (Ozone and Life, model O & L 50.0 RM).

The cultures were supplemented with industrial purity carbon dioxide ($CO_2$, class 3, White Martins, Brazil) injected through a cylinder at a flow rate of 28 L $h^{-1}$, which was measured by means of a rotameter (Myka, model SM37) installed at the start of the distribution line. The $CO_2$ injection line was installed separately from the aeration line to increase the residence time of the gas in the crops.

Due to the strong absorption of infrared radiation from sunlight, the temperature of the cultures was controlled by means of an aluminum heat exchanger and arranged inside each tank. The thermal exchange fluid used was water circulating from a cooling and heating unit (Tamson Mark, model TLC40-14, Netherlands), maintained at 25 ± 0.1 °C.

**Growth curves and the composition of the algal biomass**

Culture growth was monitored by optical density, also known as absorbance. This methodology is often used to predict the concentration of biomass in cultures of bacteria and other unicellular microorganisms (Kumar et al., 2010). Absorbance measurements were performed at wavelengths of 680 nm and 720 nm in a spectrophotometer spectrum of microplates (Thermo Scientific, Multiskan GO, Finland). Three repetitions and 8 replicates were performed daily for each experimental unit.

The dry biomass concentration, expressed as g $L^{-1}$, was determined every 5 days using aliquots of 15 mL from the culture with membrane filters of 0.22 μm pore diameter previously oven dried at 60 °C. This membrane can be used because it has a pore diameter smaller than the mean diameter of the cells of the genus *Scenedesmus* (10 μm, Santos et al., 2011). The filtration membrane was conditioned in an oven at 60 °C until reaching a constant mass, being later removed and immediately placed in desiccator for cooling. After this period, the membranes were weighed, and the dry mass was determined, discounting the mass value of the membrane.

Ash content was measured by calcination of membranes containing dry biomass. Before the test, the crucibles were calcined in muffle at 575 °C for 1 hour. After calcination, the crucibles were placed in a desiccator for cooling and mass determination. The ash-free biomass value was obtained by the difference between the crucible mass and the mass value of the crucible with biomass after calcination.

The concentration of lipids in the cultures, expressed as mg $L^{-1}$, was determined by gravimetry. For the extraction of lipids, an adaptation of the Schmid-Bondzynski-Ratzlaff method (IDF, 1986) was used. A total of 0.1 g of previously lyophilized microalgae biomass, obtained by centrifugation of 300 mL samples of cultures, were weighed in test tubes previously oven dried at 102 °C and cooled in a desiccator. A total of 1 mL of 8 M HCl was added to these test tubes, which were incubated in thermostated bath at 70 °C for 30 minutes. After the procedure, 2 mL of ethyl alcohol, 2.5 mL of ethyl ether, and 2.5 mL of petroleum ether were added. The material was put in an ultrasonic bath for 5 minutes. After homogenization, the material was poured into 15-mL tubes

and centrifuged at 5000 g for 2 minutes. The supernatant was removed and transferred to glass tubes that were previously oven dried, identified, and weighed. The extraction procedure for ethyl ether and petroleum ether was repeated twice to ensure removal of the lipids from the sample. After evaporation of the solvents, the tubes were placed in an oven at 102 °C until complete drying of the material. After cooling in a desiccator, the tubes were again weighed. The calculation of the total lipids was performed by mass difference. With this mass difference, one can calculate the content of lipids (mg lipids per g of sample). Multiplying this content by the concentration of free ashes biomass, the concentration of lipids in the cultures was determined.

The total neutral carbohydrate contents were measured by the phenol-sulfuric acid method adapted for microplates, as recommended by Masuko et al. (2005), with spectrophotometer readings (Thermo Scientific, Multiskan GO, Finland) at 490 nm. In parallel, a standard curve was prepared using glucose concentrations ranging from 0 to 1.4 mg $L^{-1}$.

**Nitrogen and phosphorus concentration**

As carbon is supplied continuously to microalgae cultures, the deficiency of the macronutrients nitrogen and phosphorus may limit growth. Thus, throughout the cultures, the levels of ammonium, nitrate, and phosphate were quantified daily, expressed in mg $L^{-1}$ of culture.

The determination of ammonium was carried out only in the cultures that used the medium B4, since the BG11 medium has an insignificant amount of ammonium in its formulation (approximately 0.54 mg $L^{-1}$). The protocol proposed by Riley (1953) was adapted for ammonium quantification in 2-mL microtubes. A 1.5-mL culture sample was centrifuged at 5000 g for 10 minutes, and 1 mL of the supernatant was collected in 2-mL microtubes. Then, 266 μL of the sodium phenate solution, 133 μL of the sodium hypochlorite solution, 66 μL of the sulfate solution, and 200 μL of deionized water were added. The sample was incubated in an ultrathermostatic bath at 70 °C for 45 min. After 10 minutes, the spectrophotometer (Thermo Scientific, Multiskan GO, Finland) was read at 625 nm.

The determination of nitrate was carried out only in the cultures that used the BG11 medium, since the B4 medium does not have nitrate. For nitrate determination, the protocol described by Oliveira (2007) was used without modifications.

Phosphate determination was performed on cultures with B4 and BG11 medium. The protocol of Murphy & Riley (1958) was adapted for quantification of phosphorus in the form of phosphate in microtubes. A 1.5-mL culture sample was centrifuged at 5000 g for 10 minutes, and 1 mL of the supernatant was collected in 2-mL microtubes. Then, 200 μL of a reagent containing the solutions of 5 M sulfuric acid, 4% ammonium molybdidate, and 0.1 M ascorbic acid were added. Subsequently, 50 μL of deionized water was added. After homogenization, the sample was conditioned in an ultrathermostatic bath at 60 °C for 30 minutes. After ten minutes, a spectrophotometer reading (Thermo Scientific, Multiskan GO, Finland) was performed at 827 nm.

**Experimental design**

The experiments were assembled in a completely randomized design. All the results were submitted to analysis of variance (ANOVA), and the means were compared by the Tukey test at 5% probability.

The culture flasks were arranged arbitrarily on a bench in the greenhouse for drawing lots that give all units the same chance of receiving any treatment. In this way, the experimental units have equal chances of being given an advantage or disadvantage by some manifestation of characteristics of the environment external to the culture.

## RESULTS AND DISCUSSION

A significant increase in absorbance values for the two wavelengths can be observed from the 18th day of culture (Figure 1). This fact is explained by the decrease in water depth due to the collection of material for lipid analysis. On days 18, 20, 22, and 24, a liter of culture was withdrawn

from each unit for analysis. Removal of this volume was sufficient to alter the culture conditions and promote cell growth. To confirm these growth rate variations, the slopes of the curves before and after the 18th day of cultivation (Table 2) were compared, showing a significant difference for all treatments after that period. It was also possible to observe that for the set of 3 replicates of each treatment, the standard error associated in all the curves was not expressive (0.035 to 0.14 for 680 nm and 0.018 to 0.051 for 750 nm), which demonstrates good repeatability and fidelity of the growth results.

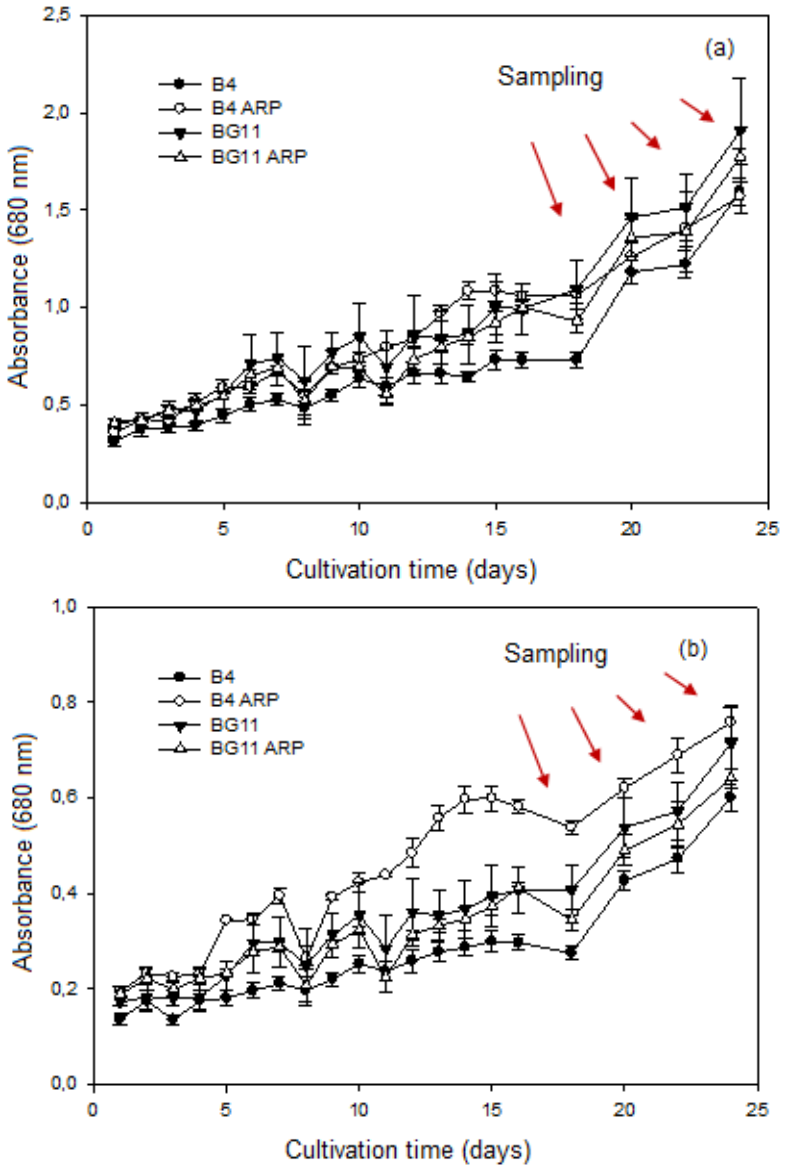



FIGURE 1. Absorbance growth curves at (a) 680 nm and (b) 750 nm.

TABLE 2. Mean and standard errors (in parentheses) for the growth rates of microalgae cultures before and after the 18th day of cultivation.

| Culture Medium | Wavelength | | | |
|---|---|---|---|---|
| | 680 nm | | 750 nm | |
| | Before day 18 | After day 18 | Before day 18 | After day 18 |
| B4 | 0.026 (0.0015) aA | 0.132 (0.0041) bA | 0.010 (0.0008) aA | 0.051 (0.0011) bA |
| B4 ARP | 0.047 (0.0030) aB | 0.083 (0.0005) bB | 0.026 (0.0024) aB | 0.036 (0.0002) bB |
| BG11 | 0.039 (0.0030) aC | 0.125 (0.0035) bA | 0.015 (0.0013) aC | 0.048 (0.0011) bC |
| BG11 ARP | 0.033 (0.0035) aC | 0.128 (0.0041) bA | 0.011 (0.0017) aA | 0.047 (0.0009) bC |

Means on the same row followed by the same lowercase letter and in the same column followed by the same capital letter do not differ statistically by Tukey's test in the same block (absorbance at 680 nm and 750 nm) at 5% probability.

In general, the behavior of the growth curves measured at 680 and 750 nm were similar, except for the culture in B4 ARP. At the 680 and 750 nm wavelengths, the absorbance values ranged between 0.3–2.0 and 0.1–0.8, respectively. The distinct behavior between growth curves for B4 ARP crops suggests higher non-algal suspended solid contents. The absorbance readings at the wavelength of 680 nm correspond to a peak absorption of the chlorophyll pigments, while the absorbance at 750 nm corresponds to a low absorption region (Griffiths et al., 2011b) in microalgae samples, being indicative of turbidity. Interestingly, the disagreement between the two growth curves corresponds to the higher content of opaque particles that diffract the light, being usually suspended mineral solids.

The production of dry biomass of *Scenedesmus obliquus* BR003 in medium B4 with ARP was superior to the other treatments (Figure 2). One hypothesis investigated was the precipitation of salts in this medium. The occurrence of precipitates was so significant for this treatment that it was possible to observe the formation of a white layer deposited on the bottom of the tanks. The ash-free dry biomass concentration analyses for this treatment were performed on the 15$^{th}$, 18$^{th}$, 20$^{th}$, 22$^{nd}$, and 24$^{th}$ days of cultivation, and the results are presented in Table 3. The amount of ash present in the other treatments was not significant for the method used and is therefore considered negligible.

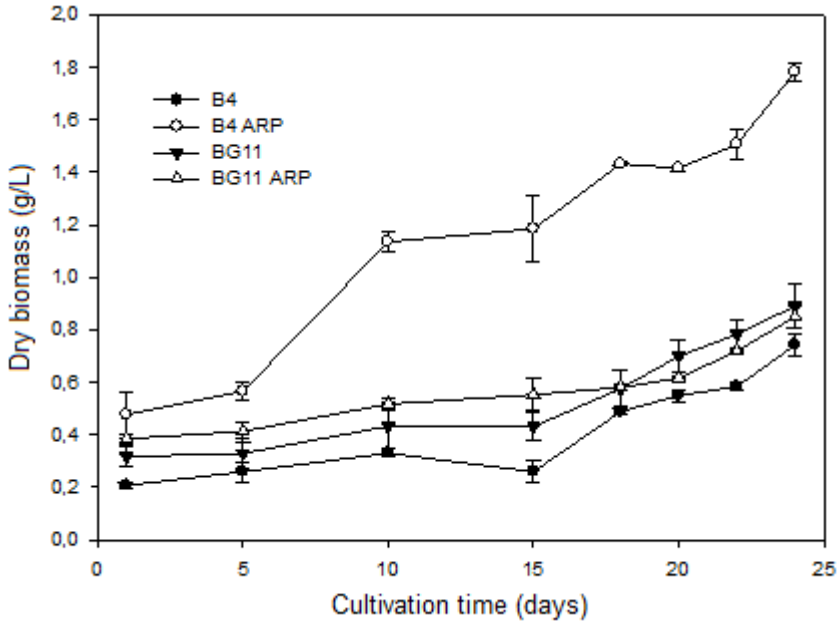



FIGURE 2. Dry mass of *Scenedesmus obliquus* BR003 during cultivations.

TABLE 3. Dry mass, ash content, and ash-free dry mass for cultures on B4 medium in ARP.

| Day of culture | Dry mass (g $L^{-1}$) | Ash content (%) | Ash-free dry mass (g $L^{-1}$) |
|---|---|---|---|
| 15 | 1.18 | 44 | 0.66 |
| 18 | 1.43 | 34 | 0.94 |
| 20 | 1.42 | 30 | 0.99 |
| 22 | 1.50 | 32 | 1.02 |
| 24 | 1.78 | 23 | 1.37 |

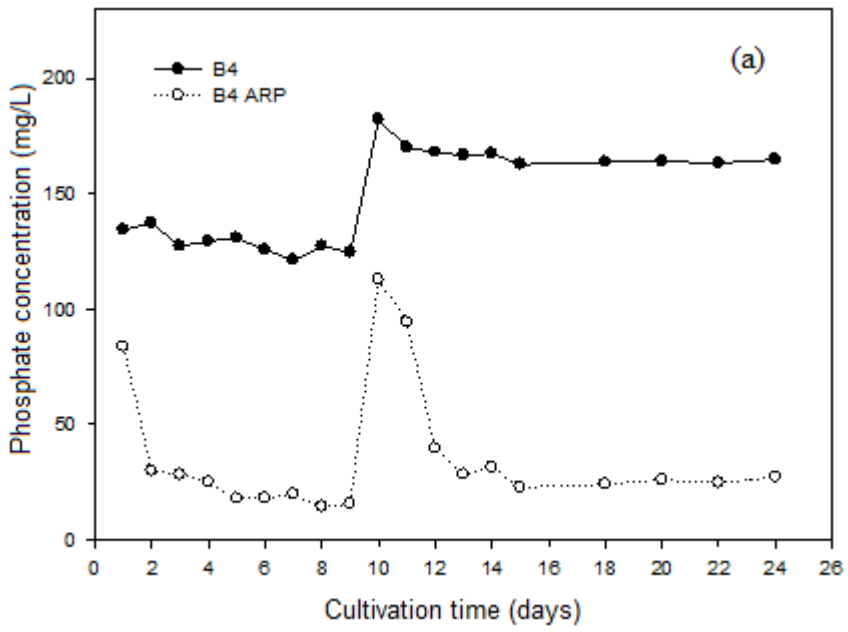
The ash-free biomass is an important parameter for the analysis of the amount of organic matter present in the culture. The ash content in microalgae varies with the species, the type and amount of nutrient, and the water used for cultivation. This assay also identifies nutrient precipitation by chemical reactions and pH variations, such as calcium and magnesium phosphates and carbonates.

The high ash content is particularly undesirable in the context of biorefinery and biomass conversion to energy, as it affects operating and maintenance costs. Phukan et al. (2011) examined

the physical and chemical characteristics of a strain of *Chlorella* sp. and found an ash content equivalent to 5.93%. In a thermogravimetric investigation of the same species, Chen et al. (2012) found an ash content of 10.3%. Biller et al. (2012) analyzed the chemical characteristics of *Scenedesmus* sp. and *Chlorella* sp. and obtained an ash content of 11.8% and 7.0%, respectively.

The production of microalgae biomass in open cultures is lower when compared to closed systems (photobioreactors). This can be attributed to many determining factors, including evaporative losses, crop temperature fluctuations, $CO_2$ deficiencies, inefficient agitation systems, and especially high light shading (Wang et al., 2013). By means of growth curves (Figures 1 and 2) and ash-free dry biomass data (Table 3), one can verify the effect of self-shading on biomass production. When the samples were collected from day 15, there was a reduction of the volumes of the cultures, and with that, the depth was reduced. The reduction of self-shading increases the photosynthetic efficiency of the culture and therefore increases the generation of algal biomass.

In the cultivation of *Scenedesmus obliquus* BR003 in B4 with ARP, a decrease in the phosphate concentration was observed after the second day, followed by a more gradual decay on the other days (Figure 3a). This phenomenon occurred again after day 10 when more fertilizers were applied to the culture (0.457 g $L^{-1}$). The strong phosphate decay in ARP treatment can be explained by the precipitation of the nutrients with calcium, which is rich in B4 medium with ARP (approximately 395 mg $L^{-1}$ of Ca). According to Song et al. (2002), depending on the pH and the molar ratio between $Ca^{2+}$ and $PO_4^{3-}$, compounds such as phosphate dicalcium dihydrate ($CaHPO_4.2H_2O$), calcium phosphate ($CaHPO_4$), octa-calcium phosphate ($Ca_4H(PO_4)_3.2{,}5H_2O$), amorphous calcium phosphate ($Ca_3(PO_4)_2$), hydroxylapatite ($Ca_5(PO_4)_3OH^-$) and calcium phosphate ($Ca_3(PO_4)_2$) can be generated. Precipitate formation was not evident for the BG11 cultures with ARP due to the low phosphate concentration in the medium. According to Carlsson et al. (1997), at pH close to neutrality, at least 50 mg $L^{-1}$ of P and 100 mg $L^{-1}$ of Ca are required for precipitation to occur. Because the phosphorus concentration in BG11 medium was below 10 mg $L^{-1}$, precipitate formation was not favored. In addition to calcium, other bivalent cations, such as magnesium, may cause variation in phosphate concentrations in the medium, which can be verified in BG11 cultures with and without ARP. This hypothesis could be confirmed by the higher variation in phosphate concentration in BG11 medium with ARP (Figure 3b), where higher concentrations of these metals were observed (Table 1).



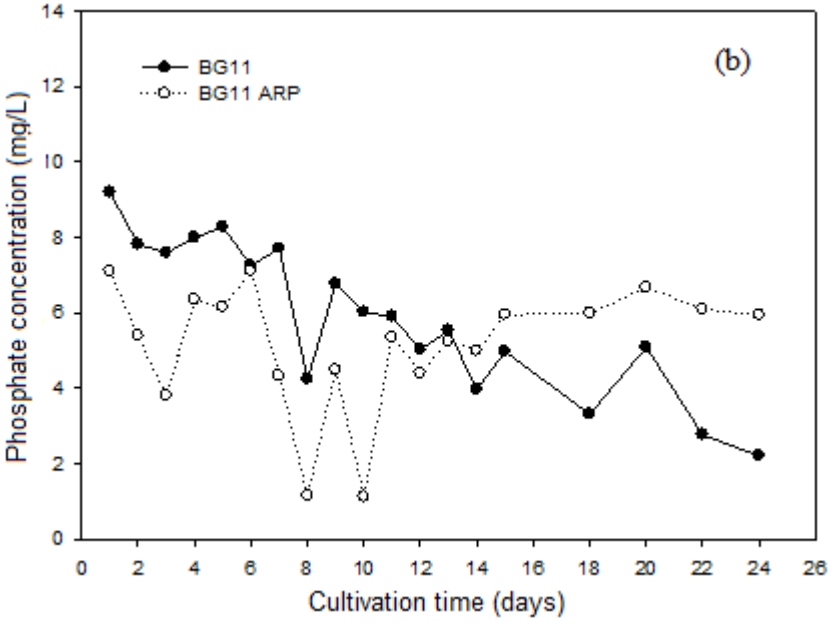



FIGURE 3. Concentration of phosphate during cultivation of *Scenedesmus obliquus* BR003 in culture media (a) B4 and (b) BG11.

The variation in the phosphate concentration observed in Figure 3b can be explained by the accumulation of polyphosphate inside the microalgae, called luxury consumption. Powell et al. (2008) studied the consumption of luxury phosphorus by microalgae and observed that these organisms can regulate both the release and the assimilation of phosphorus, depending on the temperature and luminous intensity of the cultures. In the BG11 and ARP cultures, the temperature was controlled, but the light intensity was dependent on the incidence of solar radiation on the tanks.

Figure 4 shows the variation in ammonium concentration throughout the culture in medium B4, with and without ARP.

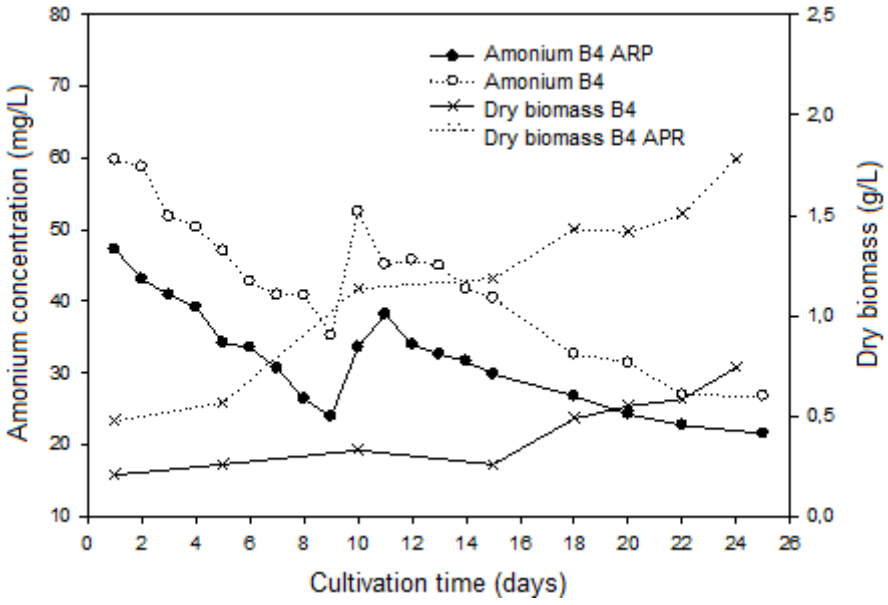



FIGURE 4. Ammonium concentration during cultivation of *Scenedesmus obliquus* BR003 in medium B4.

It can be observed that the concentration of ammonium continued to decrease until the last day in both treatments. This fact can be explained by the consumption of the microalga and not by the volatilization of the ammonium in the form of ammonia, since the pH is maintained at 7.6 ± 0.1.

According to Park et al. (2010), the elevation of pH favors the loss of nitrogen due to the displacement of the balance between ammonium and ammonia. Abeliovich & Azov (1976) tested various ammonium and pH levels on *Scenedesmus obliquus* cultures and found that photosynthesis and growth inhibition at concentrations and pH values above 36 mg $L^{-1}$ and 8.0, respectively. According to the authors, the presence of ammonium predominates at pH below 8.0, but, with increasing pH, ammonia levels increase, which acts as an inhibitor of photosynthesis and microalga growth. According to Nuñez et al. (2001), between 25-33% of the initial ammonium was used by the *Scenedesmus obliquus* microalgae, assuming that the majority was lost as gas to the atmosphere and resulting from the combination of high pH values and aeration of the culture. Benavides & Torzillo (2012) cultivated *Chlorella vulgaris* with a pH close to 7 and obtained an ammonium use by the microalga of 85%, reducing losses by volatilization (15%). Notably, growth inhibition was not observed, even with ammonium levels higher than those reported by Abeliovich & Azov (1976) for the same species, which can be explained by the adaptation of the strain used in this study to cultures in ammonium salts. Additionally, it is recorded that the native microalga *Scenedesmus obliquus* BR003 was collected in fish ponds of the Federal University of Viçosa, which is an aquatic environment where ammonium concentrations are usually high. This fact suggests the importance of the prospection of local lineages to produce biomass and other metabolites of interest.

The ammonium decay rate was 1.85 mg $L^{-1}$ $d^{-1}$ and 2.67 mg $L^{-1}$ $d^{-1}$ for cultures in B4 medium with ARP and B4 with water from the public distribution network, respectively. Lin & Lin (2011) obtained a consumption rate of 1.01 ± 0.17 mg $L^{-1}$ $d^{-1}$ of $NH_4^+$ in cultures of *Scenedesmus rubescens*, during the logarithmic growth phase with dry biomass production of 0.4 g $L^{-1}$ for 10 days of culture.

The concentrations of nitrate changed with lower intensity during the 24 days of cultivation (Figure 5) in relation to the ammonium concentrations (Figures 3 and 4).

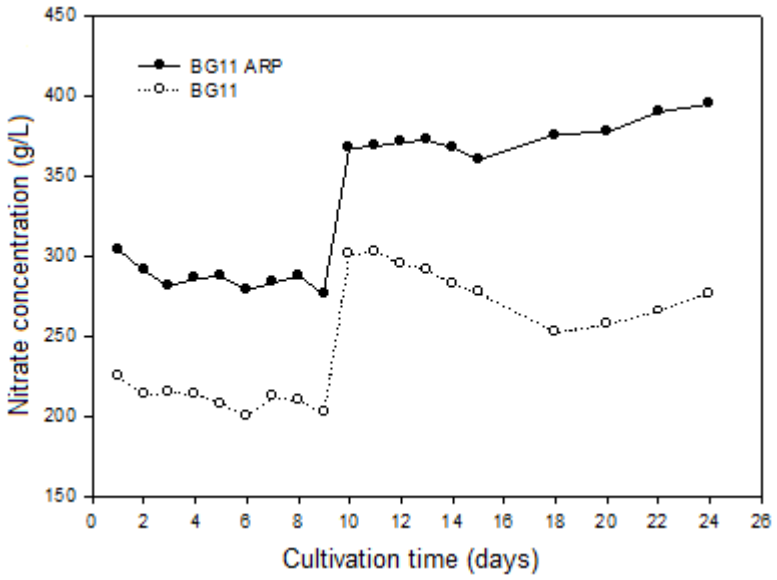



FIGURE 5. Nitrate concentration during cultivation of *Scenedesmus obliquus* BR003 in BG11 medium.

Xin et al. (2010b) found a dry biomass production of *Scenedesmus* sp. of 0.7 g $L^{-1}$ in 15 days of culture using BG11 medium modified to 25 mg $L^{-1}$ of nitrogen in the form of nitrate and 1.3 mg $L^{-1}$ of phosphorus in the form of phosphate, corresponding to a reduction of approximately 88% and 76% in N and P concentrations, respectively. Nigam et al. (2011) obtained a dry biomass production of 0.315 g $L^{-1}$ in 15 days of cultivation, using nitrate as a nitrogen source (55.4 mg $L^{-1}$ of N) in the cultivation of *Chlorella pyrenoidosa*, with total lipid content of 18%. When reducing the nitrogen concentration to 6.9 mg $L^{-1}$, the authors reached a production of biomass and lipid content of 0.127 g $L^{-1}$ and 26%, respectively.

Carbohydrates in microalgae are considered as a promising and low-cost raw material for the production of biofuels by fermentation, especially ethanol. The main obstacle of the process is pretreatment and carbohydrate extraction from the microalgae cell (Zhao et al., 2013). Figure 6 shows the variation of the carbohydrate contents of the four treatments on the 18th, 20th, 22nd, and 24th days of cultures in the culture media studied.

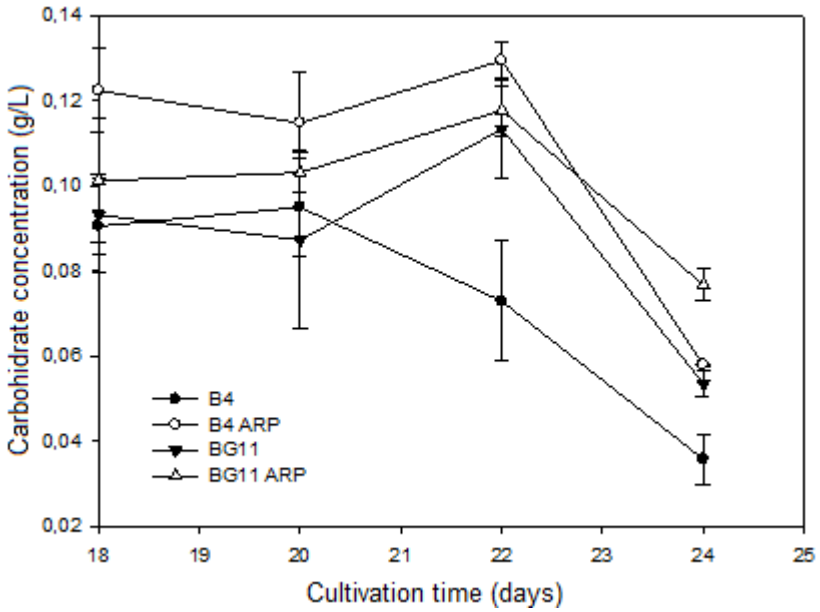



FIGURE 6. Variation in the concentration of total carbohydrates during the cultivation of *Scenedesmus obliquus* BR003.

On the 18th, 20th, and 22nd days, the carbohydrate contents for the four treatments did not differ among themselves by Tukey's test at 5% probability. However, on the 24th day, a statistically significant difference was found between the four treatments (Figure 7).

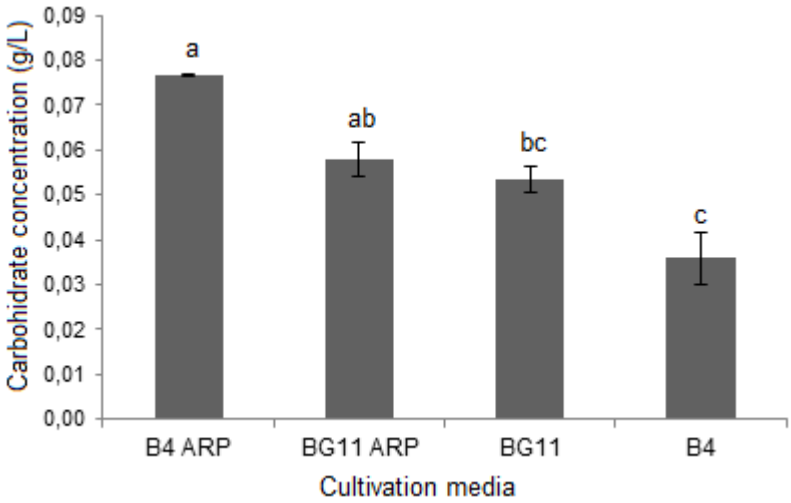



FIGURE 7. Total carbohydrate concentration of *Scenedesmus obliquus* BR003 in the different media on the 24th day of culture. Error bars with different letters indicate significant differences by Tukey's test with 5% probability.

The B4 culture medium in ARP presented a higher concentration of total neutral carbohydrates (0.076 ± 0.00018 mg $mL^{-1}$) among the treatments studied (Figure 7). The total neutral carbohydrates obtained are in the range of 3-18% of the ash-free dry mass of *Scenedesmus obliquus* BR003. Singh & Gu (2010) reported that the carbohydrate content for the strain of *Scenedesmus* sp. is in the range of 10-17%.

According to Griffiths & Harrison (2009), lipid productivity is a critical variable to evaluate the oil and biodiesel productivity of microalgae species. In industrial production, there is a direct relation between the lipid productivity of the strain and the oil productivity and its conversion to biodiesel in the cultivated areas; thus, species with low productivity of lipids will not be competitive with other sources to produce oil and biodiesel. The lipid concentration can be calculated by the product of the dry biomass concentration (grams of dry mass per liter per day) and the lipid content (decimal). The lipid concentrations for each treatment on the 18$^{th}$, 20$^{th}$, 22$^{nd}$, and 24$^{th}$ days of culture are shown in Figure 8.

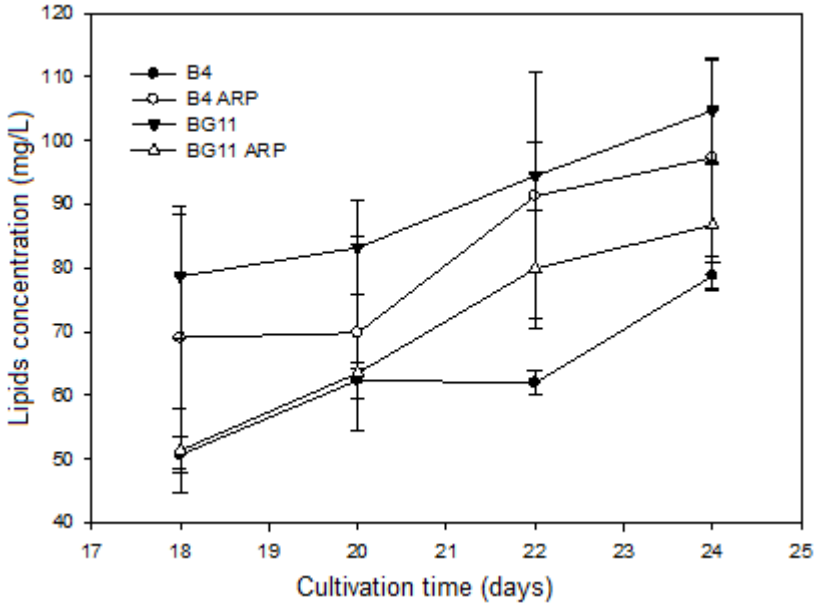



FIGURE 8. Variation in lipid concentration during the cultivation of *Scenedesmus obliquus* BR003.

It is observed from Figure 8 that the lipid concentration was increasing for all treatments in the period studied. The highest concentration was obtained for BG11 medium, which increased from 78 mg $L^{-1}$ on day 18 of culture to 104 mg $L^{-1}$ on day 24. The results obtained were more pronounced than those reported in the literature. Ho et al. (2010) found a lipid content and productivity for *Scenedesmus* sp. equal to 12.3% and 35.1 mg $L^{-1}$ $d^{-1}$, respectively. Mata et al. (2010) presented a bibliographic review of the various aspects associated with the production of biodiesel from microalgae. The authors showed that the values reported in the literature for the lipid content and productivity for *Scenedesmus* sp. are in the range of 19.6–21.1% and 40.8–53.9 mg $L^{-1}$ $d^{-1}$, respectively. Table 4 presents the lipid contents in relation to ash-free dry mass.

TABLE 4. Lipid content (% ash-free on dry basis) for *Scenedesmus obliquus* BR003 cultures.

| Day | B4 | B4 ARP | BG11 | BG11 ARP |
|---|---|---|---|---|
| 18 | 10.37 ± 0.63 b | 7.30 ± 1.15 a | 13.65 ± 1.53 b | 8.84 ± 1.02 b |
| 20 | 11.37 ± 0.30 b | 7.04 ± 0.96 a | 10.00 ± 1.94 b | 10.30 ± 0.15 b |
| 22 | 10.61 ± 0.55 b | 8.91 ± 0.68 a | 12.06 ± 0.83 b | 11.10 ± 1.33 b |
| 24 | 10.60 ± 0.30 b | 7.09 ± 0.56 a | 11.75 ± 0.21 b | 10.21 ± 0.97 b |

For each response variable, the averages followed by the same lowercase letter on the same row do not differ statistically from the Tukey test at 5% probability.

The culture media B4, BG11, and BG11 in ARP did not present significant differences in the total lipid content of the ash-free dry mass of *Scenedesmus obliquus* BR003 on any of the days studied (Table 4). The measured total lipid contents were between 7–13% in dry mass.

The success of biodiesel production of microalgae depends on the content of triglycerides, which generally corresponds to more than 70% of the lipid content. Thus, selection of species

capable of producing a high lipid content can support an efficient and sustainable production chain (Suali & Sarbatly, 2012). It has been reported that lipid extraction protocols that employ chloroform and methanol mixtures are used in many studies (Phukan et al., 2011; Nigam et al., 2011; Moazami et al., 2012). This mixture of solvents extracts not only neutral lipids but also polar lipids and pigments, which overestimates the lipid content that can be converted into biodiesel (mixtures of methyl or ethyl esters of fatty acid). The extraction protocol used in this work is more selective because it uses a mixture of ethanol, ethyl ether, and petroleum ether, basically extracting the neutral lipids and justifying the lower contents in relation to other studies reported in the literature for the same genus.

The genus *Scenedesmus* sp. has been considered by many authors as one of the most promising species of microalgae for biodiesel production. This is not because of their high lipid content but rather their productivity, ease of cultivation, and tolerance to high temperatures and contamination by other species (Xin et al., 2010; Jena et al., 2012; Chen et al., 2013). Xin et al. (2011) studied the effect of temperature on growth and lipid accumulation in *Scenedesmus* sp. and found that the strain can grow over a wide temperature range (10 to 30 °C). In this sense, several efforts are being directed towards the limitation of nutrients to increase the accumulation of lipids in the cells of microalgae, especially of *Scenedesmus* sp. Li et al. (2008) noted that the high lipid content reached under stress conditions, usually with nutrient limitation, is generally associated with a relatively low biomass production and therefore low lipid yield. Griffiths et al. (2011a) studied the effect of nitrogen limitations on the increase of lipid content in chlorophyta species. The nitrate concentrations evaluated in that study were 1500 mg $L^{-1}$ and 150 mg $L^{-1}$. In the condition of 1500 mg $L^{-1}$, all cultures still contained enough nitrate for growth at the end of the 14$^{th}$ day, while nitrate was completely used between the 3$^{rd}$ and 5$^{th}$ day of cultivation in the 150 mg $L^{-1}$ condition. The difference found in the lipid content of *Scenedesmus* sp. was significant, being increased from 9% to 43% from the first to the second condition, respectively. Ho et al. (2010) studied the nutrient deficiency approach to increase the lipid content of microalgae cells. The authors obtained a lipid content of 38.9% for *Scenedesmus* sp., which equates to a yield of 78.73 mg $L^{-1}$ $d^{-1}$ in 9 days of culture. Given this, it was verified that the binomial rate of growth and lipid content should be studied in consonance, aiming to achieve high productivity in lipids.

## CONCLUSIONS

The growth of *Scenedesmus obliquus* BR003 cultures was expressive but more pronounced from the 18$^{th}$ day of cultivation onward. This is due to the decrease in culture depth, showing the inhibition of growth caused by self-shading in outdoor growing conditions. For the same reason, the values of dry biomass for all treatments also increased after the 18$^{th}$ day of cultivation. The use of petroleum residue water (ARP) as a culture medium showed a positive effect on biomass production (dry mass) (B4-ARP 0.66 g $L^{-1}$ and B4 0.30 g $L^{-1}$).

There was an increase in the lipid concentration because of the increase in the ash-free dry biomass concentration, since the lipid contents remained constant during the period of the evaluated culture. The total lipid contents were close to 7–13% in dry mass. The highest lipid concentration was obtained for BG11 medium, which was 104 mg $L^{-1}$.

The B4 ARP culture medium had a higher concentration of total neutral carbohydrates (0.076 ± 0.00018 g $L^{-1}$) among the treatments studied. The total neutral carbohydrates obtained were in the range of 3–18% of the ash-free dry mass of *Scenedesmus obliquus* BR003.

The determination of the available phosphate, ammonium, and nitrate contents were useful in quantifying the nutrient consumption throughout the culture, where it can be verified that there was no growth limitation due to the availability of nitrogen and phosphorus. It can be concluded that the strain of *Scenedesmus obliquus* BR003 is tolerant to petroleum wastewater and could be used for its treatment. Future studies are suggested to optimize the concentrations of nitrogen and phosphorus salts to reduce nutrient costs and stimulate lipid production without limiting biomass production.

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