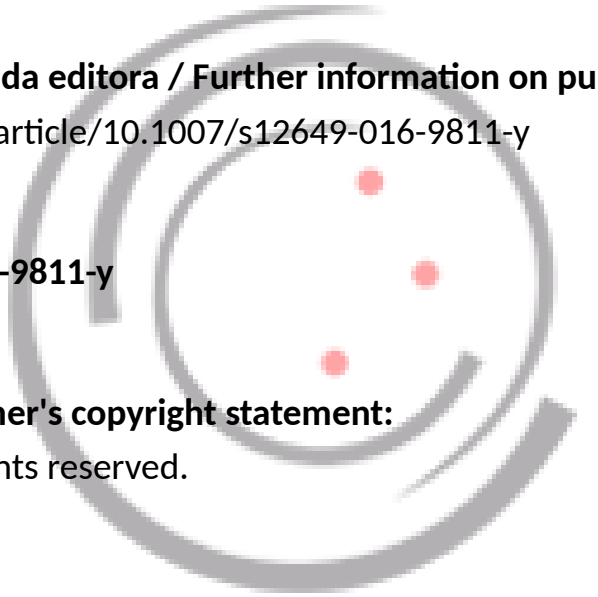

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ORIGINAL PAPER

Comparison of Growth and Lipid Accumulation at Three Different Growth Regimes with *Desmodesmus* sp.

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Abstract The production of third generation biofuel and use of microalgae as feedstock have recently gained increased attention in the research area in search of more sustainable processes. These microorganisms have characteristics that offer great potential to produce either feedstock to other process or even final use products. They have fast growth rates and accumulation of intracellular metabolites, including lipids. There are three basic types of cultures in which microalgae can develop: autotrophic, heterotrophic and mixotrophic. The objective of this work was to study the growth of the microalgae *Desmodesmus* sp. in these three types of cultivations in order to establish the most effective farming strain for lipids production. It was observed that the strain had higher yields of biomass and lipids in the mixotrophic cultivation with glucose (10 g L^{-1}) as the carbon source, obtaining 54.5×10^6 cells mL^{-1} with a growth rate of 0.28 ($days^{-1}$) and 25.1% of lipids in a 6 day cultivation time.

Keywords Microalgae · *Desmodesmus* · Mixotrophic growth · Lipids · Biodiesel

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Introduction

Due to environmental problems caused from the use of fossil fuels, the production of biofuels has become increasingly important [1]. There are three distinct generations of biofuels: first, second, and third generation. Recent research indicates that the third generations of biofuels offer great potential [2]. This category of biofuels is usually produced using microorganisms as feedstock: yeast, fungi, bacteria, macroalgae, and microalgae that have the ability to accumulate a substantial variety of compounds with potential to be precursors to fuels [3–5]. Among the advantages of third generation biofuels is the lack of competition for arable cropland [6, 7], simple cultivation in a wide variety of water environments, low land usage, and, for some feedstock, high CO_2 uptake capability [8]. The feasibility of biofuels production from microalgae of the whole process depend on the biomass viability which includes amount and cost. These microorganisms grow from photosynthesis and their growth rate is exponential. In principle, they can attain a high concentration of biomass in just 2 days. Microalgae have various advantages, including CO_2 consumption from flue gasses, the use of wastewater as cultivation medium (bioremediation) [9], and the wide variety of biofuels and bioproducts (feeding fish, pigments, and biofuels) that can be obtained from biomass [10]. Microalgae has a much higher productivity than oleaginous plants, which makes microalgae cultivation extremely appealing for various industries. Of course, many challenges have to be faced especially that one referent to biomass production rate.

Three varieties of cultivation are used to stimulate the microalgae growth and accumulation of metabolites in cells. However, each has an inherent advantage and disadvantage. Autotrophic cultivation uses light and CO_2 for

growth, heterotrophic cultivation uses organic carbon and no light for growth, and in mixotrophic cultivation, which is a mixture of the previous two regimes, light energy, and organic carbon are combined for cell growth [11]. Autotrophic growth is primarily used for large-scale microalgae production, as these cultures do not need to be axenic and their growth is usually carried out in open ponds. This is more economical than closed photobioreactors and fermenters, commonly used equipment for mixotrophic and heterotrophic cultivation, due to the greater control over the growth of variables [12].

In a comparison of the amount of biomass obtained, autotrophic culture leads to less than both mixotrophic and heterotrophic cultures. Mixotrophic cultures can generate up to fourfold more biomass than autotrophic cultures [13].

Several species of microalgae that accumulate large amounts of metabolites in their cells are currently used for the production of biodiesel. In these cases, the metabolite of interest are lipids, which is accumulated intracellularly for species like *Chlorella Vulgaris, Botryococcus braunii*, and *Desmodesmus*, among others [14].

Many strains require restricted conditions for growth, among others, specific temperature, pH, light, CO_2 feeding and, agitation. *Desmodesmus sp*. is a strain that is flexible in different operations conditions: it can survive and be efficient in a wide range of temperatures, light, CO_2 and pH levels [15]. Recent studies with *Desmodesmus sp*. have shown that it can reach up to 0.412 g L^{-1} in 14 days of growth using wastewater as culture medium [16].

According to Ho et al. [17], *Desmodesmus sp*. can tolerate temperatures up to 35 °C, while obtaining a lipid productivity of 263 mg L^{-1} day^{-1}. This microalga also has high lipid productivity when it is subjected to stress of nutrient, in this case nitrogen [18]. Accordingly, *Desmodesmus sp*. was selected to be used in this work and its behavior was observed during three types of growth in order to evaluate their impact on its development, biomass production and lipid accumulation.

This work was designed to study the growth of the microalgae *Desmodesmus sp*. in three types of cultivation: autotrophic, mixotrophic and heterotrophic, with the purpose to establish the most effective for biodiesel lipid production using the strain reported by Rios et al. [18].

Materials and Methods

Figure 1 illustrates the process of microalgae isolation and cultivation growth in the different regimes.

Generation of a *Desmodesmus* sp. Axenic Culture

A non-axenic sample containing a microalga *Desmodesmus* sp. strain was provided by the Laboratory of Research on Aquatic Organisms (LAPOA) of the Integrated Group on Aquiculture and Environmental Studies (GIA) of the Federal University of Paraná (UFPR), Brazil.

Bacterial staining was carried out through microalgae isolation to identify Gram positive and negative bacteria that were present in the original source of the microalgae. Up to six serial dilutions were performed using a sterile saline solution (NaCl 0.85% w/v) in order to determine the number of bacterial contaminants. All dilutions were carried out through vigorous agitation in a vortex mixer for 1 min, and 100 µL of diluted samples were spread out on Petri dishes containing Luria-Bertani-Agar (see Table 2) (20 g L^{-1}) medium and incubated for 4 days at

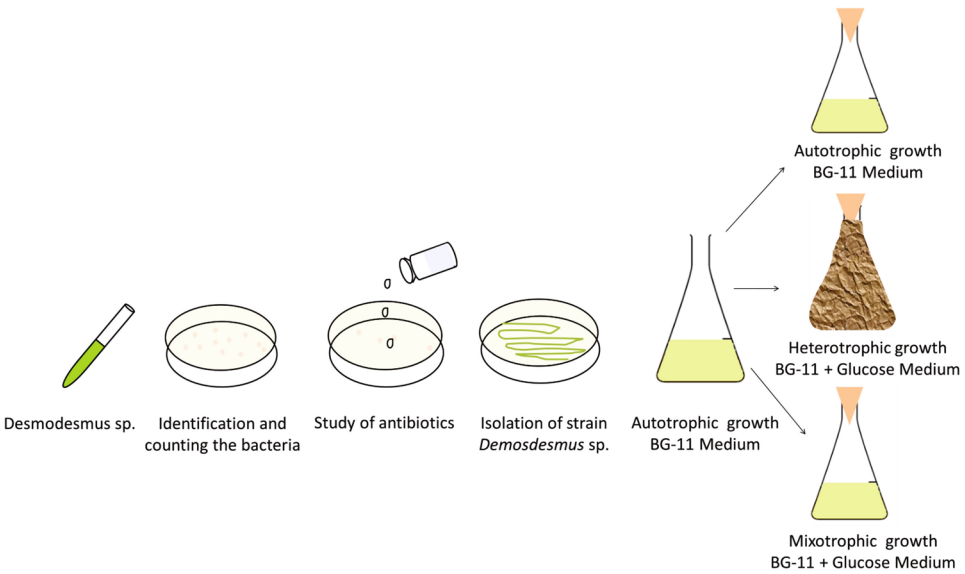

Fig. 1 Outline for microalgae isolation and inoculum development under different growth regimes

Table 1 Antibiotics tested for the removal of bacterial contaminants

Antibiotic	Concentration used
Chloramphenicol (Cm)	10 mg mL^{-1}
Kanamycin (Km)	10 mg mL^{-1}
Carbenicillin (Cb)	5 mg mL^{-1}
Amoxicillin/clavulanic acid	(500 mg/125 mg) mL^{-1}

Table 2 Culture media for bacteria control

Medium	Composition	pH
Luria Bertani	10 g L^{-1} of tryptone 5 g L^{-1} of yeast extract 10 g of NaCl	7.0
BG-11 rich	BG-11 supplemented with 5 g L^{-1} of yeast extract 5 g L^{-1} of peptone	7.5
BG-11 + glucose	BG-11 supplemented with 10 g L^{-1} of glucose	7.5

37 °C. After growth, bacteria were counted and the number of colony-forming units (CFU) was determined.

To compare the performance of the *Desmodesmus* sp. microalgae grown under phototrophic, mixotrophic and heterotrophic conditions, it was necessary to isolate the strain in order to avoid consumption of carbon sources by other microorganism in the medium. Cultivations were carried out in carbon-rich media with yeast extract (5 g L^{-1}), peptone (10 g L^{-1}) and glucose (10 g L^{-1}). Four antibiotics were tested in the aforementioned media in order to remove bacterial contaminants. Table 1 shows the antibiotics used and their concentrations.

After the elimination of bacteria, the microalgae strain was cultivated in streaks over Petri dishes in BG-11 medium with agar (20 g L^{-1}) in order to isolate pure microalgae colonies.

The BG-11 medium [19] employed in the cultivations of the microalgae under either phototropic, mixotrophic or heterotrophic conditions, contained (in mg L^{-1}): $NaNO_3$ (1500), K_2HPO_4 (40), $CaCl_2{\cdot}2H_2O$ (30), Na_2CO_3 (19), $MgSO_4{\cdot}7H_2O$ (8), $C_6H_8O_7.H_2O$ (7), ammonium ferric citrate (6), H_3BO_3 (3), $MnCl_2{\cdot}4H_2O$ (2), $Na_2EDTA{\cdot}2H_2O$ (0.7), $Na_2MoO_4{\cdot}2H_2O$ (0.4), $ZnSO_4{\cdot}7H_2O$ (0.2), $CuSO_4{\cdot}5H_2O$ (0.1) and $Co(NO_3)_2{\cdot}6H_2O$ (0.05). Culture medium pH was adjusted to 7.5 and was autoclaved at 121 °C for 15 min. In mixotrophic and heterotrophic cultivations, antibiotics for the inhibition of bacterial growth were added at room temperature after medium autoclaving. In the essays of bacterial elimination with antibiotics, carbon-rich culture media were employed (in both liquid and agar versions), as specified in Table 2.

Microalgae Growth

Three distinct growth regimes were tested in *Desmodesmus* sp. growth: autotrophic, mixotrophic and heterotrophic. Autotrophic and mixotrophic cultivations were carried out in 250 mL Erlenmeyer flasks with culture medium volume of 100 mL, a light flux of 79 µmol m^{-2} s^{-1} and a photoperiod of 24 h, agitation of 250 rpm in a shaker controlled at a temperature of 30 ± 1 °C. Heterotrophic cultivations were performed in the same conditions, although in absence of light. All batches were maintained for a period of 6 days.

Analytical Methods

Cell counting with a Neubauer chamber was employed as the biomass growth monitoring method, which yields results in millions of cells per mL. Images of the cultivations were taken at ×100 transmitted amplification with an optical microscope (Nikon).

Glucose concentration in the medium was quantified with an enzymatic analyzer (YSI 2700 SELECT Biochemistry Analyzer) on daily samples taken from the cultivations.

Chlorophyll and carotenoids were determined using a spectrophotometric method. In the last day of each batch run, aliquots from the cultivation were centrifuged at 16,000×*g* for 6 min and the supernatant was discarded. The biomass was washed with deionized water and the process was repeated. Methanol (99.9%) was added to the biomass samples, incubated for 12 h at 4 °C and then were centrifuged. The supernatant sample was then submitted to spectrophotometric analyses (BECKMAN DU 650) at different wavelengths. Equations 1–4 were used to calculate the amount of pigments in the biomass, where the index on the absorbance *A* represents the wavelength of the measurement [20, 21]:

$$Chlorophyll\ a\left(\frac{\mu g}{mL}\right) = -8.3\,A_{650} + 16.5\,A_{665} \quad (1)$$

$$Chlorophyll\ b\left(\frac{\mu g}{mL}\right) = 33.8\,A_{650} - 12.5\,A_{665} \quad (2)$$

$$Chlorophyll\left(\frac{\mu g}{mL}\right) = Chlorophyll\ a + Chlorophyll\ b \quad (3)$$

$$Carotenoids\left(\frac{\mu g}{mL}\right) = 4\,A_{480} \quad (4)$$

Lipid extraction was carried out through the Bligh & Dyer method [22] with the biomass produced within 6 days of cultivation.

Fig. 2 CFU from the original broth containing *Desmodesmus* sp. **a** Broth without dilution; **b** 10^4 dilution; **c** 10^5 dilution

Table 3 Tested antibiotics and primary results

Antibiotic	Result
Chloramphenicol (Cm)	Inhibited microalgae growth and reduced the bacterial load
Kanamycin (Km)	Allowed microalgae growth and reduced the bacterial load
Carbenicillin (Cb)	Inhibited microalgae growth and allowed the growth of different bacteria species
Amoxicillin/clavulanic acid	Allowed microalgae growth and no bacterial load reduction

Results and Discussion

Bacterial Identification, Quantification, and Removal

Initially, the original broth containing the microalgae was used to perform bacterial Gram staining and cultivation on solid media (Fig. 2). A high amount of bacteria was found by the two methods, including Gram negative and Gram positive bacteria. The amount of CFU mL^{-1} that could growth in agar-rich media was above 3×10^6.

Based on this outcome, several antibiotics needed to be tested in order to eliminate microorganisms other than the *Desmodesmus* sp. strain. In view of this, the mixture of microorganisms and microalga were cultivated in BG-11 medium (with and without glucose) and treated with the different antibiotics. Table 3 illustrates the results for each antibiotic. As expected, none of the antibiotics were able to reduce all contaminants. However, kanamycin (Km) eliminated most of the bacterial population and enabled microalgae growth.

The original broth was cultivated in Petri dishes in BG-11 medium with Km and glucose to obtain an axenic microalgae culture. As an example, one of the plates is pictured in Fig. 3. The most aseptic microalgae colonies were those found in the edges of the streaks. After several rounds of re-streaking green colonies in agar plates with Km, the monitoring through microscopic analysis confirmed first the reduction and then the elimination of the bacterial load, as shown in Fig. 4. Figure 4a presents a contaminated cultivation, where voids in the microalgae cells were believed to be caused by bacteria, which feed on microalgae carbon. In turn, Fig. 4b shows a cleaner cultivation with full, entirely healthy cells.

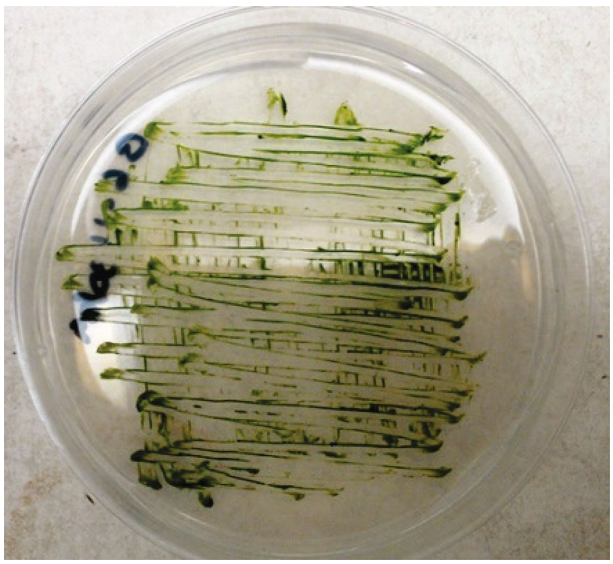

Fig. 3 Streak plate growth of *Desmodesmus* sp.

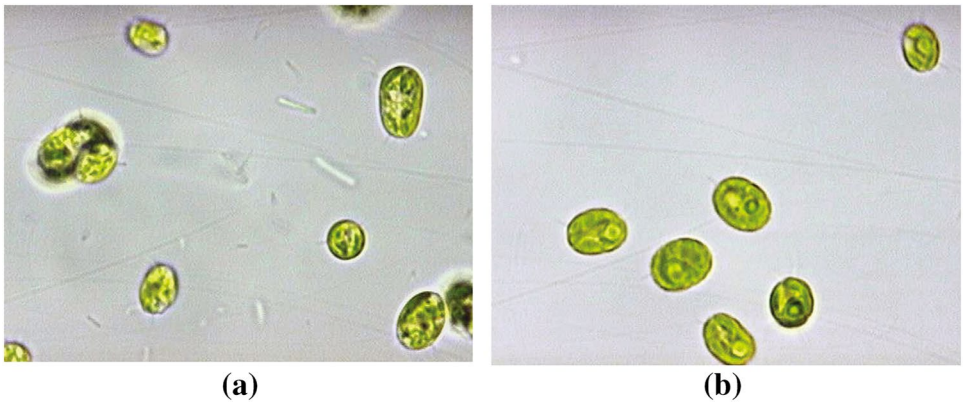

Fig. 4 State of *Desmodesmus* sp. inoculum before (**a**) and after (**b**) elimination of bacteria

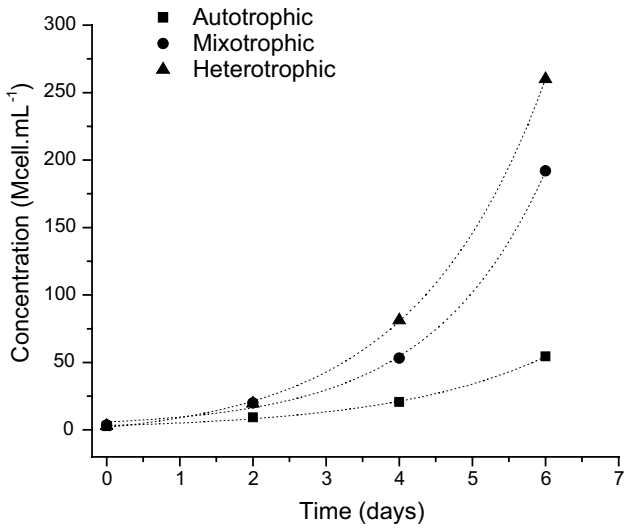

Fig. 5 *Desmodesmus* sp. growth profiles under phototrophic, mixotrophic and heterotrophic conditions in BG-11 medium for 6 days

Table 4 Doubling time (t_d) in phototrophic, mixotrophic and heterotrophic cultivations of *Desmodesmus* sp.

Growth	t_d ($\sigma = 0.45$) days
Phototrophic	3.23
Mixotrophic	2.46
Heterotrophic	2.16

Mixotrophic and heterotrophic were performed with 10 g L^{-1} of initial glucose

Desmodesmus sp. Growth

In order to study the autotrophic, mixotrophic and heterotrophic growth of *Desmodesmus* sp., an inoculum was collected from a 6-day photoautotrophic cultivation, from the isolated microalgae, and used to seed the cultures under three different regimes. Previous studies show that cultivations on the sixth day of growth are in the late exponential growth phase [17], which is the ideal condition for inoculation of higher-volume reactors. Figure 5 shows the growth profile of the batches, carried out in duplicate. The mixotrophic and heterotrophic growth batches in BG-11 medium were carried out with added kanamycin. It can be observed that a lag phase appears in the first 2 days of cultivation for all regimes (Fig. 5). The exponential phase is clearly defined in the mixotrophic and heterotrophic cultivations, since the slope of the curve is higher that the autotrophic cultivation. For the autotrophic cultivation, the cells in the exponential phase grow slower than in other regimes, resulting in a lower final cell count at 6 days of cultivation. On the sixth day of cultivation, an average of 260×10^6 cells mL^{-1} were obtained by heterotrophic growth, a value fivefold higher than the one obtained with the autotrophic regime (54.5×10^6 cells mL^{-1}). Mixotrophic reached intermediate values, with an average of 192×10^6 cells mL^{-1} (3.8 fold higher than the autotrophic batches) (Fig. 5).

Table 4 shows the average of the doubling time calculations for each of the tested growth regimes. It can be observed that heterotrophic growth doubling time occurs 7.35 h and 25.71 h faster than under mixotrophic and autotrophic conditions, respectively.

Desmodesmus sp. Glucose Consumption

Glucose consumption during mixotrophic and heterotrophic cultivation of *Desmodesmus* sp. was also evaluated in order to assess the required level of this organic carbon source (since its utilization in industrial cultivations would add significant operational costs). Figure 6 presents the glucose concentration profiles during the 6 days of cultivation. *Desmodesmus* cells grown under mixotrophic or heterotrophic conditions were not able to consume the 10 g L^{-1} of glucose in 6 days. During the 6 days of cultivation time, the microalgae cultures under heterotrophic growth consumed 4.45 g L^{-1} of glucose, while the mixotrophic growth consumed 4.8 g L^{-1}.

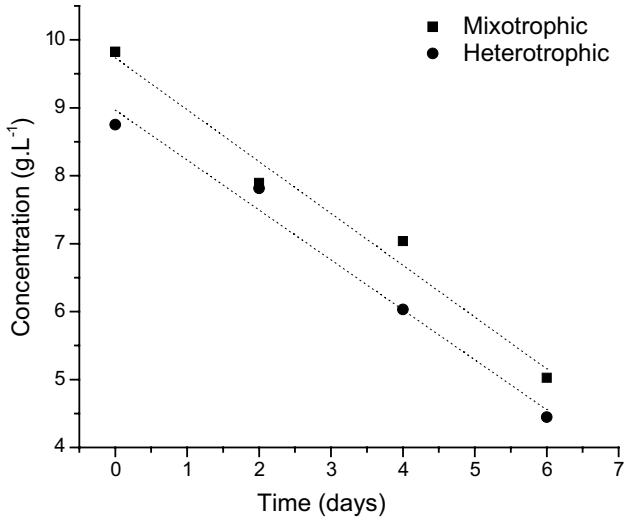

Fig. 6 Glucose concentration in BG-11 medium of *Desmodesmus* sp. grown in mixotrophic and heterotrophic conditions

Table 5 Lipid composition obtained for *Desmodesmus* sp. at 6 days of cultivation, in phototrophic, mixotrophic and heterotrophic mode

Growth regime	Lipid composition ($\sigma = 3.22$) %
Phototrophic	21.92
Mixotrophic	25.09
Heterotrophic	17.26

Desmodesmus sp. Lipid Accumulation

Lipid content in microalgal biomass obtained in each type of cultivation was evaluated, yielding the results shown in Table 5. The higher value obtained in the mixotrophic cultivation with 25.09% of lipid composition. This value was 4.17% higher than autotrophic and 7.83% higher than the heterotrophic cultivation. In these terms, the best option in growing and lipid accumulation was the mixotrophic cultivation. Worthwhile was possible used the glucose to obtained more lipids and more cells in the cultivation.

Desmodesmus sp. Chlorophyll Content

On the sixth day of cultivation, microalgae presented different coloring, as can be seen in Fig. 7. Heterotrophic-grown biomass has the lightest green hue, far less than in the mixotrophic or autotrophic cultivations. The explanation lies in the growth mechanism of heterotrophic cultivations, which do not rely on photosynthesis pigments such as chlorophyll and carotenoids. The color of the autotrophic and mixotrophic cultivation is similar, therefore, the intense green color of the mixotrophic cultivation is a result of higher cell densities. Results on chlorophyll measurements for each metabolic regime are shown in Table 6. Assays were performed using a fixed cell density of 5.38×10^7 cells mL^{-1}. Chlorophyll content in autotrophic cultivation resulted in 8.08 μg mL^{-1}, thus representing 90% more chlorophyll than in heterotrophic and 85% more than in mixotrophic batches. Results for carotenoids content follow a similar pattern, although in lower concentrations.

Conclusions

Experiments comparing the three regimes for *Desmodesmus* sp. growth showed that mixotrophic conditions enhance microalgae growth and lipid accumulation. The growth rate was 0.28 ($days^{-1}$) with 54.5×10^6 cells mL^{-1} and high lipid accumulated (25.09%) in 6 days of cultivation. In addition, mixotrophic cultivation of selected microalgae showed lower chlorophyll yields. This would facilitate pigment removal from biomass prior to microalgal

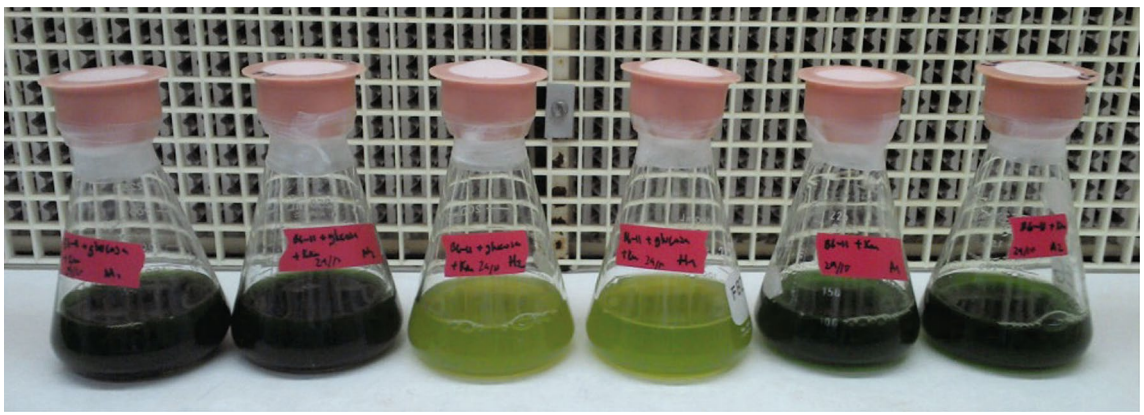

Fig. 7 Coloring in *Desmodesmus* sp. growth under phototrophic (*right*), mixotrophic (*left*) and heterotrophic (*middle*) conditions

Table 6 Chlorophyll and carotenoid content in phototrophic, mixotrophic and heterotrophic growth of *Desmodesmus* sp.

Growth	Chlorophyll ($\sigma = 3.36$) $\mu g\ mL^{-1}$	Carotenoid ($\sigma = 0.73$) $\mu g\ mL^{-1}$
Phototrophic	8.08	1.85
Mixotrophic	1.17	0.43
Heterotrophic	0.75	0.19

biodiesel production, which is required since chlorophyll is an oxidant agent to biofuel [23].

Techno-economic assessments of microalgal biomass production should be performed in order to determine whether this type of cultivation is economically feasible. Microalgal biodiesel production is still marginally economically feasible; therefore, studies such as this, aimed at researching the most feasible alternatives for the cultivation of *Desmodesmus* sp., are needed to aid the development of microalgae processes.

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