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Autochthonous microalgae cultivation with anaerobic effluent: isolation of strains, survivorship, and characterization of the produced biomass

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ABSTRACT

Six Chlorophyta strains were isolated from the effluent of an anaerobic reactor treating municipal wastewater and identified as *Desmodesmus* sp. L02, *Chlorococcum* sp. L04, *Coccomyxa* sp. L05, *Chlorella* sp. L06, *Scenedesmus* sp. L08 and *Tetradesmus* sp. L09. The microalgae strains were cultivated in unsterilized wastewater under laboratory conditions to determine their potential to survive under non-sterile conditions. The strains were also cultivated in sterilized wastewater in order to analyze their nutrient removal potential and characterize the produced biomass. Amongst the isolated microalgae, *Chlorella* sp. L06 had the highest survivorship percentage (90%) for ten days of culture, whilst *Desmodesmus* sp. L02 had the lowest, not exceeding 1.8% after 24h of inoculation. The dried biomass of the isolates showed an average of 28.7% of protein, 15.4% of lipids and 14.8% of carbohydrates, with *Chlorococcum* sp. L04 reaching 29.3% of carbohydrates. In terms of nutrients, nitrogen removal varied from 59.2 to 93%, and phosphorus removal ranged from 79.1 to 95.4%, with *Tetradesmus* sp. L09 being the most efficient strain.

Keywords: anaerobic effluent, microalgae, survivorship.

Cultivo de microalgas nativas com efluente anaeróbio: isolamento de cepas, sobrevivência e caracterização da biomassa algácea

RESUMO

Seis cepas de Chlorophyta foram isoladas do efluente de um reator anaeróbio tratando esgoto sanitário e identificadas como *Desmodesmus* sp. L02, *Chlorococcum* sp. L04, *Coccomyxa* sp. L05, *Chlorella* sp. L06, *Scenedesmus* sp. L08 e *Tetradesmus* sp. L09. As cepas de microalgas foram cultivadas em efluente não-esterilizado em condições de laboratório para determinar os potenciais de sobrevivência sob condições não estéreis. As cepas também foram cultivadas em efluente esterilizado para avaliar os potenciais de remoção de nutrientes e caracterizar bioquimicamente a biomassa produzida. Entre as microalgas isoladas, *Chlorella*

sp. L06 teve a maior porcentagem de sobrevivência (90%) por dez dias de cultura, enquanto *Desmodesmus* sp. L02 apresentou o menor valor, não excedendo 1,8% após 24h de inoculação. A biomassa seca dos isolados apresentou uma média de 28,7% de proteína, 15,4% de lipídios e 14,8% de carboidratos, com *Chlorococcum* sp. L04 atingindo 29,3% de carboidratos. Em termos de nutrientes, a remoção de nitrogênio variou de 59,2 a 93%, e a remoção de fósforo variou de 79,1 a 95,4%, com *Tetradesmus* sp. L09 sendo a cepa mais eficiente.

Palavras-chave: efluente anaeróbio, microalgas, sobrevivência.

1. INTRODUCTION

Since their inception, wastewater treatment plants (WWTPs) have had a fundamental role in minimizing the impact on water environments on a global level. Until recent years, WWTPs have been implemented with the single objective of protecting the health of humans and the environment. Nevertheless, a paradigm change is occurring in the universe of wastewater treatment, changing the emphasis on the treatment to the focus on recovering the various resources within the wastewater, such as water and nutrients (Puyol *et al.*, 2017). Microalgae-based technologies are perfect candidates to fulfil the aforementioned objectives, considering that these microorganisms grow rapidly, can be cultivated on non-arable lands using wastewaters, promote CO_2 sequestration and can be used to polish several types of effluents.

However, the cultivation of microalgae in wastewater effluents is not a new subject, considering that Oswald and Gotaas (1957) first proposed it in the 1950s, and in the decades since, high-rate algal ponds (HRAPs) have been widely studied – only with the purpose of treating wastewater. The algal biomass produced in these systems has not been traditionally recovered to use as feedstock for bioenergy production, specifically due unsatisfactory productivities and the fact that there are no low-cost and effective harvesting technologies.

Conventionally, microalgae biomass production systems use chemical fertilizers and clean water from the distribution grid, and consequently the high costs for the cultivation are one of the main bottlenecks of microalgae-to-biofuel systems in real scale (Brasil *et al.*, 2017). For this reason, most of the scientific community assumes that to reduce the environmental impacts and improve the economic viability of microalgae-to-biofuel processes, alternative sources of water and nutrients must be used. In fact, numerous authors have already reached high biomass productivities when cultivating microalgae in wastewaters, as evidenced by a recent review on the subject (Lv *et al.*, 2017).

Cho *et al.* (2017) discuss that microalgae that are indigenous to the type of wastewater used as medium could display higher adaptability to grow and remove nutrients from it. However, most studies focus on strains from Microalgae Culture Collections, ignoring the microalgal diversity that is already present in the wastewater. Therefore, this study had the primary objective of isolating microalgae strains from the effluent of a UASB reactor treating municipal sewage, in order to cultivate them in non-sterile wastewater and analyze their survivorship potential. The strains were also cultivated in sterilized wastewater in order to determine their growth, nutrient removal potential and to analyze the biochemical properties of the produced biomass.

2. MATERIALS AND METHODS

2.1. Microalgae isolation and identification

To isolate each strain, borosilicate flasks were filled with the unfiltered effluent from an upflow anaerobic sludge blanket reactor (UASB) treating municipal wastewater, located at an experimental WWTP in the Federal University of Espírito Santo (UFES), closed with cotton

leads and exposed to indirect sunlight for 15 days to stimulate the growth of autochthonous microalgae and other microorganisms. After this period, the raw effluent turned green due to the multiplication of naturally present microalgae cells, which were favored by the environmental change from complete darkness (inside the reactor) to indirect sunlight. Samples from these mixed cultures were used for the isolation procedures, namely the micropipette method followed by the streak plating technique until unialgal colonies were obtained, according to Andersen and Kawachi (2005). The streak plates were incubated with the following conditions: temperature of 27 ± 3°C; luminosity of 80 μmol m^{-2} s^{-1}; and 12 h/12 h (light/dark) photoperiod. Isolated colonies were kept in liquid sterile effluent media and on agar effluent plates and incubated with the same conditions as the streak plates. Further information on the isolation, identification and maintenance procedures applied herein can be found in Pereira *et al.* (2018).

2.2. Effluent sampling and analysis

The effluent was sampled, filtered with glass fiber membrane filters (GF/C; 1.2 μm pore size) and analyzed for pH, volatile solids, chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and total phosphorus (TP), according to the Standard Methods for the Examination of Water and Wastewater (APHA *et al.*, 2005).

The main characteristics of the unsterilized filtered effluents (UFE) throughout the experiments (n = 3) were: 28.8 ± 1.5 mg L^{-1} for TKN; 5.0 ± 0.7 mg L^{-1} for TP; 208 ± 16 mg O_2 L^{-1} for COD; and pH of 7.6. On the other hand, the autoclaved filtered effluents (AFE) had: 23.5 ± 1.4 mg L^{-1} of TKN; 5.6 ± 0.6 mg L^{-1} of TP; 162 ± 10 mg O_2 L^{-1} of COD and pH of 10.

2.3. Survivorship of the isolated strains in unsterilized filtered effluent (UFE)

To verify the survivorship potential of the isolated strains, Erlenmeyer flasks (50 mL) were filled with 30 mL of UFE and 1 mL of each inoculum (n = 6), closed with sterile cotton leads, then incubated with a temperature of 27 ± 3°C; luminosity of 80 μmol m^{-2} s^{-1}; and 12 h/12 h (light/dark) photoperiod over 10 days. Fresh samples were taken daily to determine the cell density (N) with an Improved Neubauer Chamber and a microscope (Carl ZEISS Axioplan-2), using standard counting procedures (n = 3). The survivorship potential was determined according to the following Equation 1:

$$\%\, Survivorship\ = \frac{N\ of\ the\ isolate}{N\ of\ all\ microalgae} X\ 100 \quad (1)$$

Where the cell density (N) of a determined isolate was divided by the cell density of all the microalgae cells present in the counting fields and multiplied by 100.

2.4. Cultivation of the strains, characterization of the biomass and nutrient removal

Each isolated strain was cultivated in borosilicate glass flasks (22 L capacity) with 20 L of autoclaved filtered effluent (AFE) and its respective inoculum under the following conditions: temperature of 27 ± 3°C; luminosity of 80 μmol m^{-2} s^{-1}; 12 h/12 h (light/dark) photoperiod and continuous air bubbling (1.5 mL s^{-1}). The cultures were monitored daily with an *in vivo* fluorometer during 14 days in order to establish the growth curves. *In vivo* chlorophyll *a* measurements were previously correlated with results from the acetone extraction method 10200-H (APHA *et al.*, 2005) for each microalgae strain. The extraction method was performed with 10 mL (n = 3) samples and the readings were carried out with a spectrophotometer DR/2000 (HACH).

On the 14th day the biomass was harvested with 250 mg L^{-1} of $Al_2(SO_4)_3$, centrifuged, dried (60°C) and pulverized with a pestle and mortar. The liquid phase was filtered with glass

fiber membrane filters (GF/C; 1.2 µm pore size) and analyzed for all the parameters within Section 2.2. in order to determine the nutrient and COD removal potential of each strain. The dried biomass was used for the biochemical characterization of lipids, proteins and carbohydrates as in Pereira *et al.* (2018).

3. RESULTS AND DISCUSSION

3.1. Microalgae isolation and identification

Microscopic examination of the algal bloom (obtained by incubating natural UASB effluent) showed the presence of several types of green microalgae, diatoms and cyanobacteria, as well as fungi and protozoans. Six Chlorophyta strains were isolated as described in Section 2.1 and identified based on their morphology as *Desmodesmus* sp. L02, *Chlorococcum* sp. L04, *Coccomyxa* sp. L05, *Chlorella* sp. L06, *Scenedesmus* sp. L08 and *Tetradesmus* sp. L09. These six strains were maintained in laboratory according to Lorenz *et al.* (2005) and were used as inocula for both the survivorship and cultivation experiments.

3.2. Survivorship of the isolated strains in unsterilized filtered effluent (UFE)

After the daily cell density monitoring of the cultures, survivorship curves were made for each strain, and the results are depicted in Figure 1.

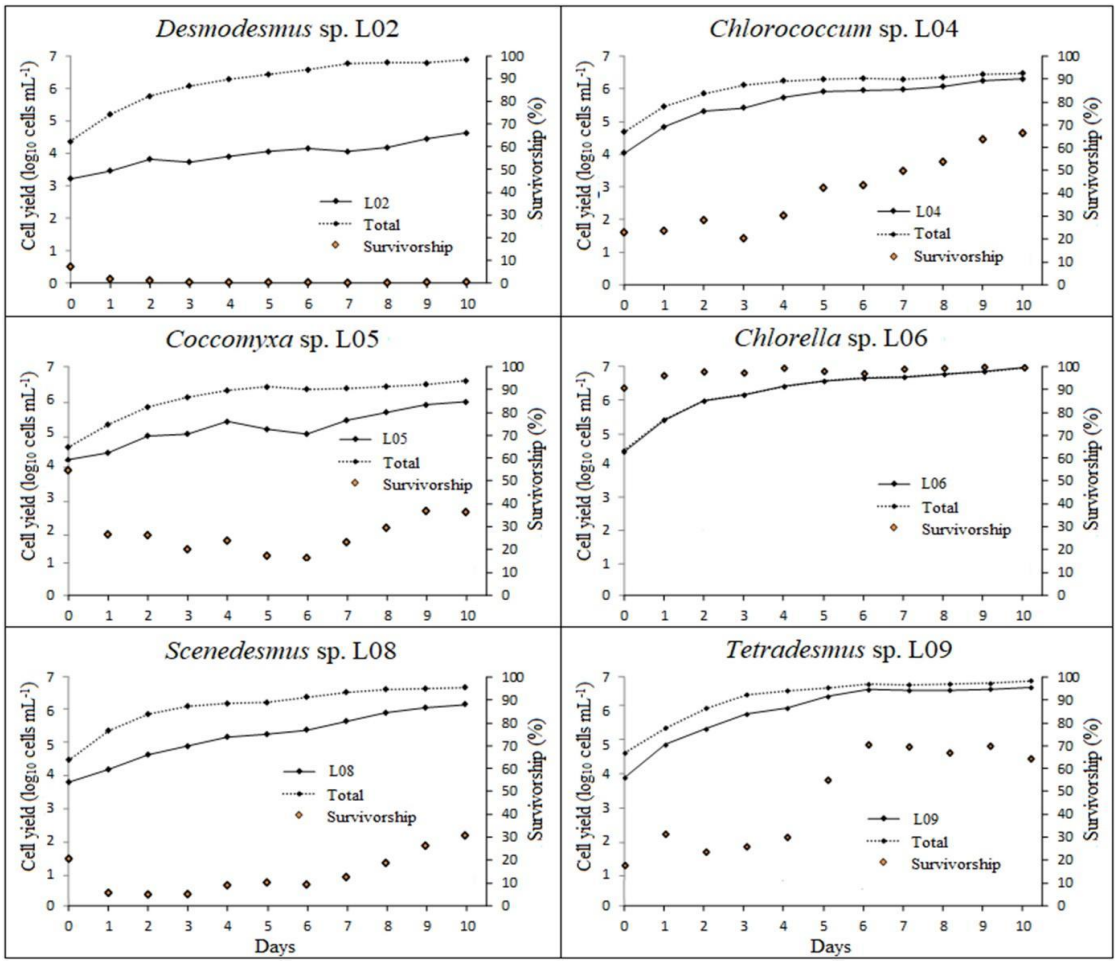

Figure 1. Growth and survivorship curve of six microalgae strains cultured in unsterilized filtered effluent (UFE) in laboratory at a temperature of 27 ± 3°C, luminosity of 80 µmol m^{-2} s^{-1} and 12 h/12 h (light/dark) photoperiod over 10 days. Dashed lines represent the total cell density, including all microalgae, continuous lines represents the isolate's cell density and the orange diamonds represent the survivorship of the isolate at a given time.

Results showed that *Chlorella* sp. L06 was the most adaptable strain, being able to develop and grow in non-sterile wastewater despite the presence of competitors and predators. During the ten days of experiment, this strain had a survivorship of higher than 90% for each day, with an average of 97.7%. As for the other strains, the mean survivorship value was 1.2% for *Desmodesmus* sp. L02, 40.3% for *Chlorococcum* sp. L04, 16.3% for *Coccomyxa* sp. L05, 14.9% for *Scenedesmus* sp. L08 and 47.5% for *Tetradesmus* sp. L09.

Although all the strains were isolated from the same wastewater as the one used to cultivate them, it is natural to assume that different strains have different capacities to thrive in a determined environment. Some of them can be weaker competitors, or more susceptible to environmental changes. This seemed to be the case for *Desmodesmus* sp. L02, due to its inability to develop properly in the UFE medium. On the other hand, *Chlorella* sp. L06 had the best survivorship results, which was expected, considering that species from the genus *Chlorella* have been widely used in studies on cultivation of microalgae biomass linked to wastewater treatment (Lv *et al.*, 2017). A study conducted by Mennaa *et al.* (2015) compared the growth of several microalgae species on non-sterile urban wastewater and obtained better growth results for the *Chlorella* species, when compared with *Scenedesmus* species.

Cho *et al.* (2011) state that the effluents from WWTPs can be used to produce microalgae biomass at a much lower cost, since it does not require nutrient addition, only a pretreatment method such as filtration or UV disinfection for the control of competing microorganisms. The capacity to grow in non-sterile wastewater is a trait that many researchers consider crucial for a microalgae strain, considering the cost reduction in the pretreatment of the wastewater used as media (Guldhe *et al.*, 2017).

3.3. Growth of the isolated strains in autoclaved filtered effluent (AFE)

The microalgae strains were cultivated in 22 L flasks according to Section 2.4. and the growth results, based on chlorophyll *a*, are depicted in Figure 2.

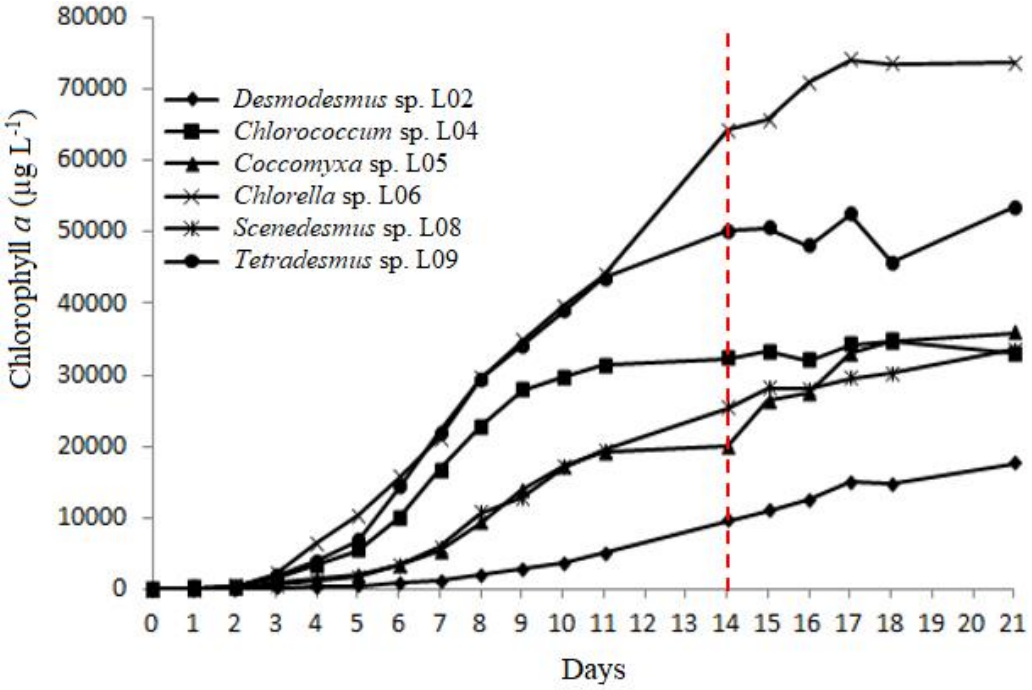

Figure 2. Growth curve, based on chlorophyll *a*, of six microalgae strains cultured in autoclaved filtered effluent (AFE) in laboratory at a temperature of 27 ± 3°C, luminosity of 80 µmol m^{-2} s^{-1}, 12 h/12 h (light/dark) photoperiod and air bubbling (1.5 mL s^{-1}) over 21 days. The dashed red line represents the day that biomass was harvested from a large sample of the cultures.

The growth results showed that all the isolated strains were capable of growing in AFE and reaching an exponential growth phase, indicating that this type of medium could support microalgae cultivation, despite the fact that its initial pH was 10. Microscopic examination showed that each microalgae culture was clearly dominated by the strain of its respective inoculum. Regarding each strain, *Chlorella* sp. L06 was not only the most adaptable one in UFE, it was also the strain with the best results on AFE with air bubbling. Following the same trend, *Desmodesmus* sp. L02 had the least satisfactory growth results, and *Tetradesmus* sp. L09 was the second best.

3.4. Biochemical characterization of the produced biomass

The dried biomass of each microalgae strain was characterized in terms of biochemical composition and the results are shown in Figure 3.

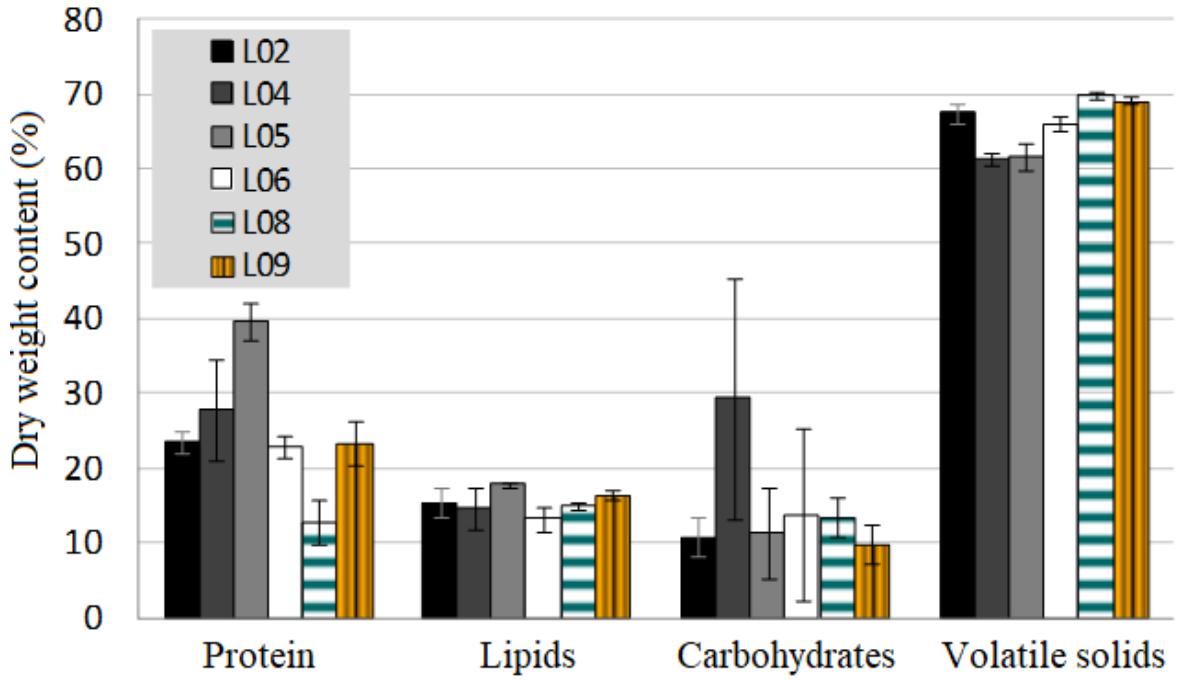

Figure 3. Biochemical composition of the dried biomass for each strain cultivated in autoclaved filtered effluent (AFE) in laboratory at a temperature of 27 ± 3°C, luminosity of 80 µmol m^{-2} s^{-1}, 12 h/12 h (light/dark) photoperiod and air bubbling (1.5 mL s^{-1}) over 14 days (bars represent SD and n = 3).

The average composition of the biomass was 25.0% protein, 15.4% lipids and 14.8% carbohydrates, all within 66.0% of volatile solids. It can be observed that the protein content was higher than the other fractions in every case (except for *Scenedesmus* sp. L08), followed by lipids and carbohydrates. *Coccomyxa* sp. L05 had the highest protein content amongst the strains (39.7%) and *Scenedesmus* sp. L08 had the lowest (12.9%). Regarding biomass-to-bioenergy processes, the anaerobic digestion of the biomass is an attractive alternative to generate biogas within WWTPs that already use anaerobic systems. However, high protein content on the biomass (low C:N ratios) is often unfavorable for anaerobic digestion, considering the inhibitory effects that NH_3 has over the methanoarchea community (Fricke *et al.*, 2007).

Mutanda *et al.* (2011) discuss that the co-digestion of the biomass with carbon-rich substrates and the pre-treatment of the biomass are both alternative strategies that can significantly enhance bio-conversion into methane. In fact, Passos *et al.* (2015) achieved higher methane production when the microalgae biomass was pre-treated with physical methods

(thermal, microwave and ultrasound). Other than the production of biogas through anaerobic digestion, the biomass can also be used in the production of ethanol through the fermentation of the carbohydrates. In this case, *Chlorococcum* sp. L04 deserves recognition due to the high carbohydrate content (29.3%).

3.5. Nutrient and COD removal efficiency

Table 1 shows the nutrient and COD removal efficiency from the liquid phase based on different microalgae cultures after the addition of the coagulant.

Table 1. Nutrients and COD concentration in the liquid phase of the microalgae cultures at the beginning of the experiment (in) and after the addition of the coagulant agent (out). The strains were cultivated in autoclaved filtered effluent (AFE) in laboratory at a temperature of 27 ± 3°C, luminosity of 80 μmol m^{-2} s^{-1}, 12 h/12 h (light/dark) photoperiod and air bubbling (1.5 mL s^{-1}) over 14 days (±SD and n = 3).

		Cultures/Strains					
Parameters	**Units**	**L02**	**L04**	**L05**	**L06**	**L08**	**L09**
TKN (in)	mg L^{-1}	26.5 ± 0.3	26.5 ± 0.3	26.5 ± 0.3	26.5 ± 0.3	26.5 ± 0.3	26.5 ± 0.3
TKN (out)	mg L^{-1}	10.8 ± 0.3	5.6 ± 1.0	3.9 ± 1.8	8.9 ± 0.8	8.7 ± 0.3	1.8 ± 1.3
TKN removal	%	59.2	78.9	85.3	66.4	67.2	93.2
TP (in)	mg L^{-1}	7.1 ± 0.2	7.1 ± 0.2	7.1 ± 0.2	7.1 ± 0.2	7.1 ± 0.2	7.1 ± 0.2
TP (out)	mg L^{-1}	1.5 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.1	0.8 ± 0.2	0.3 ± 0.0
TP removal	%	78.9	90.1	88.7	90.1	88.7	95.8
COD (in)	mg L^{-1}	184 ± 6	184 ± 6	184 ± 6	184 ± 6	184 ± 6	184 ± 6
COD (out)	mg L^{-1}	93 ± 0.0	101 ± 4.0	82 ± 8.0	64 ± 5.0	76 ± 11.0	58 ± 13.0
COD removal	%	49.4	45.3	55.3	64.9	58.7	68.3

Abbreviations: TKN, total Kjeldahl nitrogen; TP, total phosphorus; COD, chemical oxygen demand.

It can be observed that, at the end of the experiment, the TKN removal varied from 59.2% (*Desmodesmus* sp. L02) to 93.2% (*Tetradesmus* sp. L09). Regarding TP, removal efficiency was higher than 80% for all cultures (except *Desmodesmus* sp. L02), with *Tetradesmus* sp. L09 reaching 95.8%. In terms of COD, it is interesting to note that its concentration was already low at the beginning of the experiment (184 mg L^{-1}), as expected, since secondary treated effluents from anaerobic treatments are generally low in COD and BOD. However, the removal efficiency of this organic fraction still ranged from 45.3% (*Chlorococcum* sp. L04) to higher than 68.3% (*Tetradesmus* sp. L09). COD removal in this case may have been caused by the high photosynthetic O_2 generation inside the flasks, which could enhance the oxidation of organic matter by heterotrophic microorganisms. On the other hand, high COD removal can indicate microalgal mixotrophic nutrition, as discussed by Park *et al.* (2011). Adsorption by the biomass and or filter membrane may have also contributed to the COD removal, since a fraction of the COD is often recalcitrant, therefore difficult to assimilate biologically.

The results for removal efficiency obtained herein are similar to the ones obtained by Mennaa *et al.* (2015), and agree with the range of values reported in a recent review study on the subject of microalgae cultivation in secondary-treated wastewaters (Lv *et al.*, 2017). On the other hand, since the pH of all cultures reached values above 9.0, it might be reasonable to assume that at least part of the nitrogen was lost due to abiotic processes such as ammonia stripping, rather than assimilation by microorganisms (Nurdogan and Oswald, 1995). The same logic can be applied to phosphorus removal due to the chemical precipitation of this nutrient at pH levels higher than 9.0. Nonetheless, in terms of tertiary treatment, we can conclude that nitrogen, phosphorus and COD were efficiently removed from the liquid phase of the effluent in this experiment.

4. CONCLUSIONS

The results showed that *Chlorella* sp. L06 and *Tetradesmus* sp. L09 were able to survive and thrive in unsterilized UASB effluent after inoculation, indicating that they were more adapted to this medium than the other microalgae isolates, in terms of competition for nutrients and resiliency to protozoan grazers. The strains were also cultivated in autoclaved UASB effluent, and all of them were able to grow and reach the exponential growth phase.

The dry biomass of the isolates showed, on average, 66.0% of volatile solids, of which 25.0% was composed of proteins, 15.4% of total lipids and 14.8% of total carbohydrates. The microalgae cultures were able to remove up to 93% of nitrogen and 95.4% of phosphorus from the wastewater's liquid phase, improving the UASB effluent quality and converting these nutrients into biomass that can be used as feedstock for the production of biogas or syngas.

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