Research, Society and Development, v. 9, n. 7, e823974882, 2020
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v9i7.4882

Bioremediation of urban river wastewater using *Chlorella vulgaris* microalgae to generate biomass with potential for biodiesel production

Biorremediação de águas residuais de rios urbanos usando a microalga *Chlorella vulgaris* para geração de biomassa com potencial na produção de biodiesel

Biorremediación de aguas residuales de ríos urbanos utilizando microalgas *Chlorella vulgaris* para generar biomasa con potencial la producción de biodiesel

Recebido: 25/05/2020 | Revisado: 27/05/2020 | Aceito: 30/05/2020 | Publicado: 16/06/2020

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Abstract

The production of biofuels through microalgae biomass represents a new generation of raw materials from renewable sources to meet society's clamors and growing insertion in the market of fuels from products that could grant the planet a sustainable future. The present study assesses the biomass obtained from microalgae *Chlorella vulgaris* when grown in urban wastewater, extracting the lipids from the biomass and performing Gas Chromatography analysis of Fatty acid Methyl Esters (FAME) composition after submitting the lipids through the transesterification process. The microalgae cultivation was monitored through chlorophyll

(a) analysis and the highest cell growth was 845.8 µg L^{-1} using urban wastewater as growth medium. The nutrients of interest were monitored for primary concentration of 8.06 ± 0.06 mg L^{-1} of ammoniacal nitrogen, 12.27 ± 0.27 mg L^{-1} of nitrate and 21.22 ± 0.85 mg L^{-1} of phosphate, reducing about 99% of ammoniacal nitrogen and nitrate, along with reducing 87% of phosphate. The lipid constitution extracted from 3.7 g of dry biomass of *Chlorella vulgaris* after cultivation using urban wastewater, was 7.7%. The lipids extracted from the *Chlorella vulgaris* biomass are suitable biodiesel production regarding the amounts of FAMEs identified, after the analysis carried out, the comparison of the results obtained with other studies and the hypotheses evaluation.

Keywords: Biodiesel; lipid contente; microalgae; urban river wastewater; renewable energy.

Resumo

A produção de biocombustíveis através da biomassa de microalgas representa uma nova geração de matérias-primas a partir de fontes renováveis para atender as necessidades da sociedade e a crescente inserção no mercado de combustíveis de produtos que possam conceder ao planeta um futuro sustentável. O presente estudo avalia a biomassa obtida da microalga *Chlorella vulgaris* quando cultivada em águas residuais urbanas, extraindo os lipídios da biomassa e realizando a análise por cromatografia em fase gasosa da composição dos Ésteres Metílicos de Ácido Graxo (EMAG) após a submissão dos lipídios pelo processo de transesterificação. O cultivo de microalgas foi monitorado através da análise de clorofila (*a*) e o maior crescimento celular foi de 845,8 µg L^{-1}, utilizando águas residuais urbanas como meio de crescimento. Os nutrientes de interesse foram monitorados quanto à concentração primária de 8,06 ± 0,06 mg L^{-1} de nitrogênio amoniacal, 12,27 ± 0,27 mg L^{-1} de nitrato e 21,22 ± 0,85 mg L^{-1} de fosfato, reduzindo cerca de 99% do nitrogênio e nitrato amoniacal, juntamente com a redução de 87% de fosfato. A constituição lipídica extraída de 3,7 g de biomassa seca de *Chlorella vulgaris* após o cultivo em águas residuais urbanas foi de 7,7%. Os lipídios extraídos da biomassa de *Chlorella vulgaris* são adequados para a produção de biodiesel em relação às quantidades de EMAG identificados, após a análise realizada juntamente com a comparação dos resultados obtidos com outros estudos e a avaliação de hipóteses.

Palavras-chave: Biodiesel; conteúdo lipídico; microalgas; águas residuais de rios urbanos; energia renovável.

Resumen

La producción de biocombustibles a través de la biomasa de microalgas representa una nueva generación de materias primas de fuentes renovables para satisfacer los clamores de la sociedad y la creciente inserción en el mercado de combustibles de productos que podrían otorgarle al planeta un futuro sostenible. El presente estudio evalúa la biomasa obtenida de las microalgas *Chlorella vulgaris* cuando se cultiva en aguas residuales urbanas, extrayendo los lípidos de la biomasa y realizando un análisis de cromatografía de gases de la composición de Ésteres Metílicos de Ácidos Grasos (EMAG) después de enviar los lípidos a través del proceso de transesterificación. El cultivo de microalgas se controló mediante análisis de clorofila (*a*) y el mayor crecimiento celular fue de 845.8 µg L^{-1} usando aguas residuales urbanas como medio de crecimiento. Los nutrientes de interés se monitorearon para determinar la concentración primaria de 8.06 ± 0.06 mg L^{-1} de nitrógeno amoniacal, 12.27 ± 0.27 mg L^{-1} de nitrato y 21.22 ± 0.85 mg L^{-1} de fosfato, reduciendo aproximadamente el 99% de nitrógeno y nitrato amoniacal, junto con la reducción del 87% de fosfato. La constitución lipídica extraída de 3,7 g de biomasa seca de *Chlorella vulgaris* después del cultivo con aguas residuales urbanas, fue del 7,7%. Los lípidos extraídos de la biomasa de *Chlorella vulgaris* son una producción adecuada de biodiesel con respecto a las cantidades de EMAG identificadas, después del análisis realizado, la comparación de los resultados obtenidos con otros estudios y la evaluación de hipótesis.

Palabras clave: Biodiesel; contenido de lípidos; microalgas; aguas residuales urbanas; energías renovables.

1. Introduction

The innovation of technologies for the development of new sources of renewable energy is of great relevance to meet the high demands of the whole society (Cardoso et al., 2020; Ramluckan et al., 2014; Moreira et al., 2013). The global concern to reduce the impact of greenhouse gas emissions, due to the serious environmental and human health consequences, as accentuate by the Paris Agreement signed at COP 21, further research is needed in the field of energy sustainability (Pragya et al., 2013), considering extremely important the generation of fuels through renewable sources, which would be biofuels.

New paths are opening up, mainly in the transport industry, where the increase in the generation of biofuels has intensified, such as biodiesel. Biodiesel is the result of the Transesterification process to which the oil from plants or lipids from extracted animals are subjected and mixed with a catalyst and alcohol (Knothe & Razon, 2017; Moreira et al., 2011).

As necessary feedstock for the generation of biodiesel are photosynthetic microorganisms such as microalgae and cyanobacteria, have a high growth rate in wastewater (whether industrial or domestic) and are rich in lipids (Dickinson et al., 2017). They belong to the third generation of raw materials for biofuels, with environmental advantages i. e., efficient land use, CO_2 biofixation and being crucial to resolving the recurrent food versus fuel conflict (Deviram et al., 2020; Cardoso et al., 2017; Moreira et al., 2014).

The use of microalgae biomass as energy was driven by researchers Howard Wilcox in 1968, due to the oil crisis in the 1970s, since then it has continued to develop research for the optimization of biofuels using microalgae biomass, proving to be a promising and future area (Andrade et al., 2020; Raheem et al., 2018).

An alternative that combines both lower costs and an application of a Clean Development Mechanism (CDM) technology is the microalgae cultivation in a medium with urban wastewater, which can be considered as a very promising strategy (Qin et al., 2016). CDM was established in during the creation of the Kyoto Protocol and it seeks investment from developed countries (annex I countries) in enterprises inside developing countries (non-Annex I countries) that would not increase greenhouse gases emissions (Zainuddin et al., 2017). This kind of sustainable implementation is an advantageous alternative specially for companies seeking the B certification, entering the global trend, when the private sector takes on the role of sustainable development agent, encouraged by banks like the Development Bank of Latin America (CAF, 2015; Moreira et al., 2016).

System integration of wastewater treatment through microalgae and bioproducts generation increases the economic viability (Wu et al., 2018) of microalgae usage for biofuel generation. That way, it is possible to carry out urban effluent treatment – which represents a major environmental problem regarding pollution of the increasingly scarce water resources, especially in large cities, – associating it with such a growing topic like biodiesel generation, and with carbon sequestration (Wang et al., 2019).

There is also the possibility to profit from integrated method application of microalgae cultivation using urban wastewaters by selling carbon credits. According to key IEA (International Energy Agency) indicators of 2017, Brazil presented an annual emission average of 428.79 Mt of CO_2, considering the fossil fuels emissions, increasing 132,61% from 1990. The last direct measurement of global emissions of CO_2 performed by NOAA (National Oceanic and Atmospheric Administration), held in September 2017, achieved the global mark of 403.38 ppm (parts per million) of CO_2 into the atmosphere only in this month.

According to Cheah et al. (2016), 1 T of Carbon Dioxide that is not emitted or absorbed, corresponds to a carbon credit that can be negotiated on the international market. Using the Carbon Equivalent concept, in which it is measured the GWP (Global Warming Potential) of each gas, the reduced emissions of other gases that add to the aggravation of greenhouse effect may further be converted into carbon credits. This way, these generated credits can be dealt in the emissions trading market, generating profit for deployments of sustainable projects. The carbon credit price in 2016 ranged from $\$0.50/tCO_2$ to more than $\$50/tCO_2$, having an average of $\$3,0/tCO_2$, totaling an amount in the transaction market of $76 million for the primary market, which is equivalent to the sale of project developers to end purchasers or intermediaries, and $107 million for the secondary market, which corresponds to the offset sales between intermediaries and final or intermediate buyers (Hamrick & Gallant, 2017).

Moreover, as the energy production through microalgae biomass have zero-emission of Carbon Dioxide and, regarding *Chlorella vulgaris*, has a rate of fixation of CO_2 of 251 mg L^{-1} to 865 mg L^{-1}, conditional to the type of cultivation (Pragya et al., 2013), the emission limit imposed by the Paris Agreement, which provides a means of limiting the increase in temperature on Earth under 2°C defined at COP 21 (available for reading on the UN Brazil website), would not be reached and would generate carbon credits to be sold to developed countries. In addition, low carbon circular economy strategies in industries have been embraced based on renewable energies. Biofuels generated from by-products of wastewater treatment process offer opportunities for circular bioeconomy systems that guide clean and sustainable manufacture (Kang et al., 2020).

In response to the world's demands and the increase of researches in renewable energies that can meet the requests of the global market and to sustainable development, this present study aims to evaluate oil potential from dry biomass of *Chlorella vulgaris* grown in urban river wastewater attending to its feasibility for generating biodiesel.

2. Material and methods

Microalgae strain

The species *Chlorella vulgaris* strains were acquired Canadian Phycological Culture Centre (CPCC) for the development of research. The microalgae was inoculated into culture medium Bold's Basal Medium (BBM), whose nutrient composition are: KH_2PO_4 8.75 g/500 ml, $CaCl_2 \cdot 2H_2O$ 12.5 g/500 ml, $MgSO_4 \cdot 7H_2O$ 37.5 g/500 ml, $NaNO_3$ 125 g/500 ml, K_2HPO_4

37.5 g/500 ml, NaCl 12.5 g/500 ml, $Na_2EDTA•2H_2O$ 10g/L, KOH 6.2 g/L, $FeSO_4•7H_2O$ 4.98 g/L, H_2SO_4 (concentrated) 1 ml/L, Trace Metal Solution 1 ml, H_3BO_3 5.75 g/500 ml, the pH was adjusted to 6.8. The culture medium was autoclaved at 120° C for 15 minutes for sterilization prior to inoculation of strain. After sterilization, serial propagation was performed following the methodology of Souza Andrade (2014). The initial volume was increased 15 ml to 4000 ml to transfer for photobioreactor built. Each photobioreactor received a volume of 250 ml of propagated strain (10% of the sample volume). Considering that 15 photobioreactors were used, totaling 3750 ml microalgae strains propagated.

Microalgae cultivation using wastewater

The urban area proposal for collection surface water the tropical River Camarajipe - Salvador, Bahia, Brazil. Coordinates demarcated: 12°58'53.1" (S) 38°27'09.1" (W). The samples were filtered using membrane filter glass fiber GF/A 47 mm with pore size of 0.45 µm, with the aid of a vacuum pump and compressor, for removal of particulate matter in suspension that can interfere in microalgal growth. Then, the filtrate was being analysed for nutrients of interest this research: phosphate (PO_4^{-3}), ammoniacal nitrogen (NH_4^+) and nitrate (NO_3^-), was made using an ionic chromatograph analysis of ions through the ASTM Method (2005).

The experiment was set up considering different wastewater gradients diluted in distilled water, resulting in: 0% urban waste water, 25% urban waste water (v/v), 50% urban waste water (v/v), 75% urban waste water (v/v) and 100% urban waste water.

The microalgae were grown in erlenmeyers with a total working volume of 3 L, planned in triplicates. The control reactor contains 0% of urban waste water, composed only of sterile distilled water. Each reactor has a volume of 3 liters, however only used to 2.5 liters. Aeration was carried out by automatic compressor pumps 3 L min^{-1} with 3 Watts power and air being filtered with filter Econofilter Nylon (25 mm) with 0.22 µm pore size. The lighting was done through one-off cold fluorescent lamps approximately 1400 lux each. Each erlenmeyers received 10% inoculum of cultivation of microalgae selected, being held a day/night simulation in light/dark photoperiod with 10/14 hours, during the 30 days experiment.

The temperature was controlled in a closed room keeping a small variation, remaining between 21°C at 25°C, over the period, considering a good growth track, since this species *Chlorella vulgaris* adapts well between 20 to 25° C (Posadas et al., 2015). The pH was monitored during the experiment, keeping between 7 and 8, a range that presents better

conditions for this species of microalgae (Lam et al., 2017). To analyses the efficiency of removal of phosphate, ammoniacal nitrogen and nitrate, was made through by Equation 1 and 2, whereas N_f refers to the final reading the total quantity of nitrogen in the sample and N_i the initial reading. P_f refers to the final reading of the total quantity of phosphorus and the P_i refers to initial reading (Nayak et al., 2016).

$$\mu N = (Ni - Nf)/Ni\ x\ 100 \quad (1)$$

$$\mu P = (Pi - Pf)/Pi\ x\ 100 \quad (2)$$

Measurement microalgae growth

The monitoring microalgae growth, was determined the pigment concentration (chlorophyll (*a*)) using spectrophotometer (Agilent UV-VIS) to record the absorbance values with selected 630, 647, 664 and 750nm wavelength by Standard methods - 10200 H (Jenkins, 1982; APHA, 2012). The monitoring was planned in six-time intervals being the 1st day, 6th day, 9th day, 15th day, 21st day and 30th day, because is the importance of monitoring the nutrients removal, nitrogen and phosphorus, to wastewater treatment and also check what day will have the microalgae growth.

Quantification microalgae cells is established through calculations of Standard methods - 10200 H through the pigment concentration chlorophyll (*a*), entering values optical density set by absorbance selected, as shown in the Equation 3. After you determine the concentration on the extraction of this pigment, it is estimated the total number of cells per unit volume as shown in Equation 4. After result converts from mg m^{-3} for µg L^{-1} (APHA, 2012).

$$C_a = 11{,}85(absorbance\ 664) - 1{,}54(absorbance\ 647) - 0{,}08(absorbance\ 630) \quad (3)$$

$$Chlorophyll\ a = (Ca * Volume\ extraction\ (L))/(Sample\ volume\ (m^3))\ (\text{mg m}^{-3}) \quad (4)$$

Collecting microalgae biomass

After the *Chlorella vulgaris* microalgae reached the stationary growth phase, the experiment was stopped, and the samples were centrifuged with a Baby I model 206 BL (Fanem) centrifuge at 2800 rpm for 15 minutes. The supernatant was removed using a Pasteur pipette for further analysis. The accumulated biomass collected in the test tubes was stored under refrigeration for freezing until the start of the cold drying phase.

Biomass drying

Using a L108 model (Liotop) lyophilizer, with a total capacity of 8.0 Kg and 5.0 Kg of ice/24h, the previously frozen samples were subjected to the process of freeze-drying during the period of 5 days, when, after removed from the freeze-dryer, were weighted, totalizing a mass of 3.7 g, and stored in a dissector for the lipid extraction step.

Oil extraction of dry biomass

Considering the methodology proposed by Ramluckan et al. (2014), lipid extraction from dry microalgae biomass was carried out. The equipment used was the Soxhlet (1879).

A sample of 3.7 g of biomass, previously dried in a lyophilizer and weighed, was properly packed in a glass tube of the Soxhlet extractor. The tube was then attached to the device inside an exhaust fan. 150 mL of Hexane (Merck) was used as the extraction solvent, being transferred to a 250 mL boiling capacity flask, coupled to the Soxhlet device and a Fisatom heating blanket, model 22E. To obtain a continuous flow of biomass washing with the reagent, a refrigeration system using condensers was used.

Using a temperature of 70 ºC, the extraction lasted 3 hours. After extraction, the flask was taken to a Buchi rotary evaporator, model R-210, to remove excess reagent.

After drying, the oil was collected and transferred to a 1.5 ml vial previously weighed and deposited in the hood, where it remained for 24 hours, so that any remaining reagent could be evaporated. At the end of 24 hours, the flask containing the extracted oil was weighed again to quantify the mass of the oil. After extraction, the lipid content was calculated using the following Equation 5:

$$C_{lipid} = (Weight\ of\ extracted\ lipid\ /Weight\ of\ dry\ biomass) * 100 \quad (5)$$

Analysis of lipid extracted

To evaluate the composition of the fatty acid methyl esters (FAME) was performed by gas chromatography. The oil extracted from the dry biomass sample, went through a transesterification process which uses as a catalyst a solution of 2 molar NaOH dissolved in methanol, creating the ion methoxide necessary for the reaction that allows in the production of biodiesel. The alcohol used in the reaction was Heptane (Pragya et al., 2013).

After the separation of the phases of biodiesel+heptane and glycerine by centrifugation of the samples, it was injected with a Mark Hamilton microsyringe 0.5 µL of the phase composed by biodiesel 50% diluted in a Varian gas chromatograph, model CP-3800, with split/splitless capillary injector, equipped with a Flame Ionization Detector (FID). A Carbowax GC 20 m capillary column 15 m long and 0.53 mm internal diameter was used. FAMEs analyzed concentrations were quantified by comparing the peak areas with the pattern with already known areas and identified by their retention times.

Statistical analysis

The experiment was carried out in triplicate with mean, standard deviation and one-way analysis of variance (ANOVA) with a significance level of $p<0.05$ was used for statistical analysis. The analyses were conducted using BioEstat 5.3 software. The chart was built in Microsoft Excel Software (2010)

3. Results and discussion

Nutrients removal of urban river wastewater

The values of the initial and final concentrations of nutrients phosphate, ammoniacal nitrogen and nitrate from urban rivers wastewater, analyzed with mean in triplicate and standard deviation (Table 1). Table 1 show that, at the end of the experiment, the concentrations of ammoniacal nitrogen and nitrate in urban wastewater diluted in distilled water reached the final concentration <0.05 mg L^{-1}.

Table 1 - Monitoring the concentration of nutrients in each treatment during the six days of experiment. Data shown as the mean ± SD, n = 3.

Phosphate concentration (mg L^{-1})

Time (days)	0% urban wastewater	25% urban wastewater	50% urban wastewater	75% urban wastewater	100% urban wastewater
1st day	14.54 ± 1,87	15.21 ± 0,40	17.10 ± 1,21	17.74 ± 0,92	21.18 ± 0,85
6th day	14.56 ± 0,33	14.17 ± 0,26	15.51 ± 0,39	16.19 ± 0,46	16.76 ± 0,57
9th day	12.83 ± 0,59	9.08 ± 0,32	8.91 ± 0,18	8.37 ± 0,51	7.33 ± 0,18
15th day	11.10 ± 0,45	4.54 ± 0,75	5.56 ± 1,58	5.14 ± 0,72	6.32 ± 0,46
21st day	12.46 ± 0,06	3.19 ± 0,83	4.27 ± 0,89	5.53 ± 0,35	4.69 ± 0,005
30th day	12.28 ± 0,91	0.13 ± 0,04	0.084 ± 0,006	0.27 ± 0,19	2.72 ± 0,70

Ammoniacal nitrogen concentration (mg L^{-1})

Time (days)	0% urban wastewater	25% urban wastewater	50% urban wastewater	75% urban wastewater	100% urban wastewater
1st day	0.125 ± 0,001	1.78 ± 0,02	3.99 ± 0,10	5.90 ± 0,17	8.06 ± 0,06
6th day	0.125 ± 0,001	0.72 ± 0,013	3.50 ± 0,22	5.24 ± 0,08	7.70 ± 0,21
9th day	0.125 ± 0,001	0.125 ± 0,001	0.24 ± 0,14	2.48 ± 0,25	4.21 ± 0,36
15th day	0.125 ± 0,001	0.125 ± 0,001	0.125 ± 0,001	0.125 ± 0,001	0.125 ± 0,001
21st day	0.086 ± 0,001	<0.05± 0,001	<0.05± 0,001	<0.05± 0,001	<0.05± 0,001
30th day	0.050 ± 0,001	0.050 ± 0,001	0.050 ± 0,001	0.050 ± 0,001	0.050 ± 0,001

Nitrate concentration (mg L^{-1})

Time (days)	0% urban wastewater	25% urban wastewater	50% urban wastewater	75% urban wastewater	100% urban wastewater
1st day	13.21 ± 0,21	13.31 ± 0,28	13.27 ± 0,05	13.33 ± 0,63	12.27 ± 0,27
6th day	6.48 ± 0,22	12.83 ± 0,08	12.91 ± 0,07	12.78 ± 0,42	12.00 ± 0,31
9th day	0.73 ± 0,15	1.46 ± 0,29	9.81 ± 1,98	14.08 ± 0,29	9.03 ± 1,34
15th day	0.125 ± 0,001	0.125 ± 0,001	0.40 ± 0,001	0.125 ± 0,001	0.125 ± 0,001
21st day	<0.05 ± 0,001	<0.05± 0,001	<0.05± 0,001	0.67 ± 0,001	0.125 ± 0,001
30th day	<0.05± 0,001	<0.05± 0,001	<0.05± 0,001	<0.05± 0,001	<0.05 ± 0,001

Source: Authors, 2020.

The highest initial value found for the nutrient phosphate in urban wastewater was 21.18 ± 0.85 mg L^{-1}, and at the end it was 2.72 ± 0.70 mg L^{-1}, evaluating the removal efficiency in

87%, where about 18.46 mg L^{-1} in total was removed (Figure 1). The highest rate of phosphate removal was observed in the concentration of 50% of urban wastewater, where the initial concentration was 17.10 ± 1.21 mg L^{-1} and the final was 0.084 ± 0.006 mg L^{-1}, and perceive noted that the removal was approximately 17.02 mg L^{-1} in total and that it corresponds to 99% efficiency in removal.

The initial phosphate values found for the concentrations of 25% urban wastewater and 75% urban wastewater were 15.21 ± 0.40 mg L^{-1} and 17.74 ± 0.92 mg L^{-1}, corresponding to the removal rate of this nutrient it was verified in 99% and 98% respectively. The lowest removal efficiency rate was assessed at 16% in distilled water containing 0% urban wastewater, as it has the lowest initial phosphate concentration containing only 14.54 ± 1.87 mg L^{-1} and the end was reduced to 12,28 ± 0.91 mg L^{-1}, removing about 2.25 mg L^{-1} of total phosphate, corresponding to 15% removal efficiency (Figure 1.). This can be justified by the absence of ammoniacal nitrogen that was not verified for this concentration of urban waste water (0%) (Figure 2), so the increase in efficiency is related to the necessary balance between nutrients.

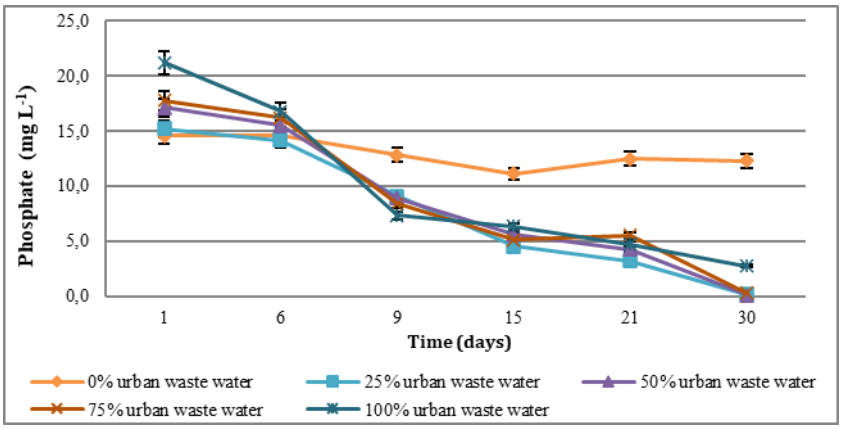
Phosphate is of the essential macronutrients for microalgae, as it participates in the energy generation process in phosphorylation (ATP and NADP), also influencing the formation of lipids (phospholipids) that contribute to the generation of biodiesel. However, for this process to occur, there needs to be a balance between nitrogen (N) and phosphorus (P) absorption (Khanzada, 2020). Determined species of microalgae, such as *chlorella*, have been isolated from sewage and have shown that they can accumulate phosphorus in the form of polyphosphate. This process can influence the generation of lipids where the accumulation can reach an increase of around 24.9% in lipid yield (Wang et al., 2019).

This proportion was initially analyzed by Redfield (1958) who claims that there is an absorption ratio phosphate and nitrogen for microorganisms of 1 P: 16 N moles. Other researchers have evaluated the variation of this proportion for a good treatment of urban wastewater using microalgae, contribute to a variation between 1 P: 9 N and 1 P: 18 N moles (Xin et al., 2010). In this research, where the phosphate presented higher concentrations in relation to nitrogen, 1 P: 0, 58N was evaluated, a proportion well below the recommended which explains the non-removal of 100% phosphate. The research carried out by Khanzada (2020) highlights that it is required to have a balance between the concentrations of nutrients and synthetic forms can be added, this process significantly improved the growth of microalgae in wastewater.

In the research by Álvarez-Díaz et al. (2017) seven different species of microalgae were grown in urban wastewater to assess the potential for nutrient removal and biodiesel generation.

The species *Chlorella vulgaris* was the first to completely remove the phosphate (about 1.5 mg L^{-1}) compared to the others. As phosphate concentrations were less than ideal, total nitrogen removal has not been achieved, so it is indicated that it can be complemented with other wastewater with higher phosphate concentrations (Álvarez-Díaz et al., 2017).

Figure 1 - Temporal monitoring of the removal phosphate with microalgae *Chlorella vulgaris* during 30 days of experiment and different amount urban waste water. Data shown as the mean ± SD, n = 3



Source: Authors, 2020.

The initial value of ammoniacal nitrogen in wastewater from contaminated urban rivers was 8.06 ± 0.06 mg L^{-1}, reaching a final result of <0.05 mg L^{-1}, which would be below the detection limit of the equipment. This removal of 100% ammoniacal nitrogen from the wastewater was achieved in just 9 days of cultivation with microalgae for concentrations of 0%, 25% and 50% of urban waste water. Already the concentrations of 75% and 100% of urban waste water, the ammoniacal nitrogen was completely removed in 15 days of cultivation (Figure 2). This removal was also observed in the prodution of Iasimone et al. (2018) cultivated microalgae in sewage and ammoniacal nitrogen removal has been reported to present as a result of removal in about 80% of an initial concentration of 30.2 ± 1.7 mg L^{-1}.

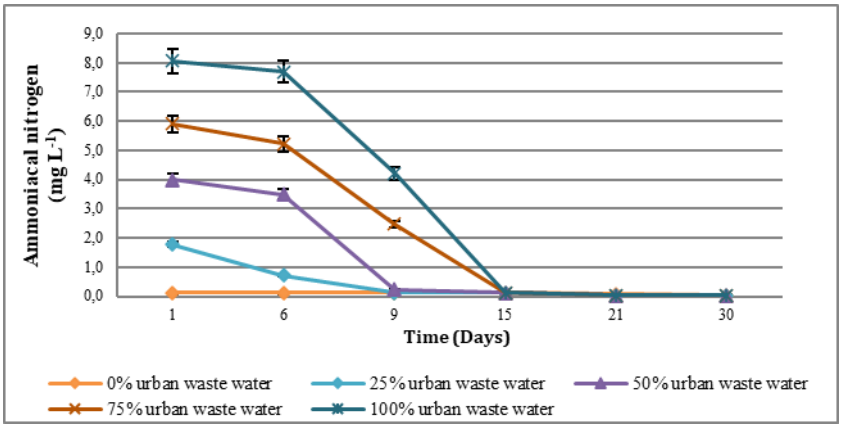
The absorption of ammoniacal nitrogen and nitrate occurs during the exponential growth phase and is not necessarily influenced by light intensity, but rather by the initial concentration of these nutrients (Iasimone et al., 2018). In the research by Mujtaba & Lee (2017), the efficiency of removing ammoniacal nitrogen from wastewater was around 80% where concentrations were between 13 to 21 mg L^{-1}, the same species was used for the treatment,

proving that this microalgae is capable of treating urban wastewater and consequently bioremediation of contaminated urban rivers.

The initial nitrate value for urban wastewater was 12.27 ± 0.27 mg L^{-1}. After 15 days of microalgae cultivation in urban wastewater, total removal of nitrate occurred, reaching <0.05 mg L^{-1} (Figure 3). Other studies have achieved nitrate removal around 67% in 11 days of microalgae cultivation in urban wastewater, where the need to increase the cultivation time to optimize removal efficiency has been observed (Kumar et al., 2019). The concentrations of 0% and 25% of urban wastewater, the nitrate removal efficiency was around 94% and 89%, respectively, in just 9 days of cultivation.

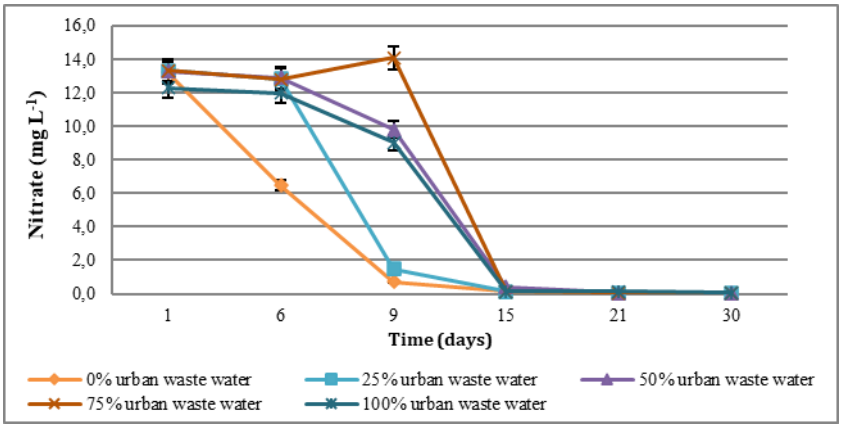
Microalgae have priority in the removal of ammoniacal nitrogen, as it is the reduced form of nitrogen and its assimilation is easier, which justifies the faster removal of ammoniacal nitrogen in relation to nitrate (Delgadillo-Mirquez et al., 2016). Figure 3 shows a slight increase in the concentration of nitrate in the experiment containing 75% of urban wastewater on the 9th day of cultivation. This increase is explained by the fact that microalgae in the intracellular metabolic process oxidize nitrogen to form nitrate (Sanz-Luque et al., 2015). Anyhow, shortly after, nitrate was completely consumed in 15 days. It is worth mentioning that the nitrifying bacteria that make this biological process in urban wastewater, however, all samples were sterilized in an autoclave, avoiding the existence of any type of bacteria, which highlights the exclusive action of microalgae.

Figure 2 - Temporal monitoring of the removal ammoniacal nitrogen with microalgae *Chlorella vulgaris* during 30 days of experiment and different amount urban waste water. Data shown as the mean ± SD, n = 3



Source: Authors, 2020.

Figure 3 - Temporal monitoring of the removal nitrate with microalgae *Chlorella vulgaris* during 30 days of experiment and different amount urban waste water. Data shown as the mean ± SD, n = 3



Source: Authors, 2020.

For treatments, experiments containing 0%, 25% and 50% of urban wastewater, this process was not evident, since all ammoniacal nitrogen had already been removed, leaving only the nutritive nitrate consumed. Other studies considered the removal of ammoniacal nitrogen and nitrate with efficiency in 11 days as well, but reinforce that to obtain a complete treatment with greater efficiency it is necessary to extend the cultivation process. The duration of treatment also depends on the type of microalgae species used and the specific growth rate (Samorì et al., 2013). In general, the microalgae were efficient in removing the nutrients proposed in this research, proving its use for the treatment of urban wastewater as a way of bioremediation and cleaning up urban rivers in cities that are important mainly for public health.

The response to the removal of nitrogen and phosphorus by the microalgae is already achieved in 7 days, presenting about 21% despite noting that the longer the cultivation time the better and more efficient is the removal of nutrients by the microalgae (Marques et al., 2017).

Microalgae growth and biomass production

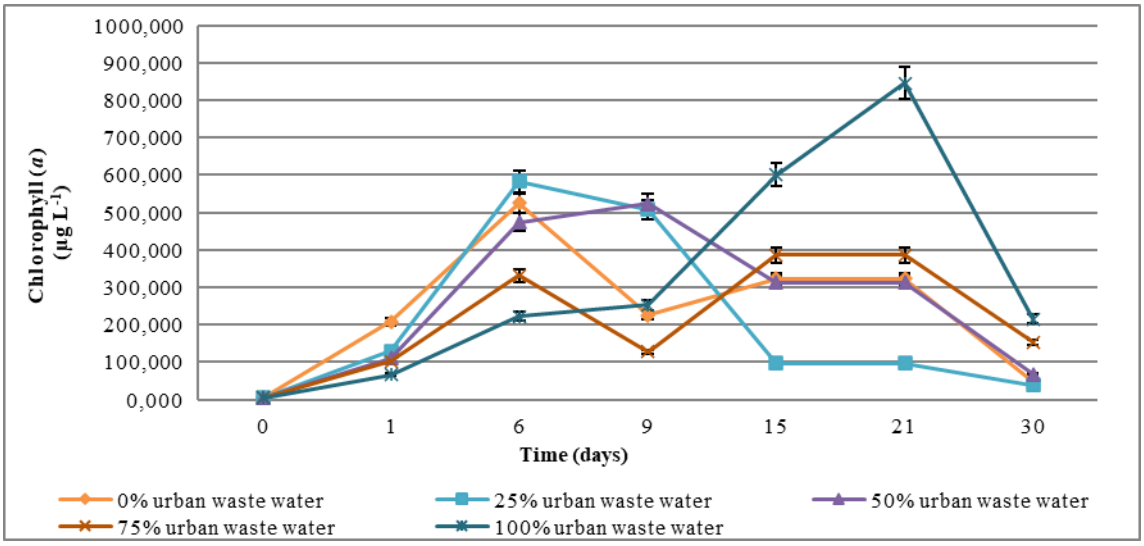
The growth monitoring of the microalgae *Chlorella vulgaris* was done during 30 days of experiment, through the analysis of the chlorophyll pigment concentration (Figure 4). The first days corresponds to the phase of acclimatization of the microalgae with the new culture medium, since when they were propagated it was in the stationary phase. The greatest growth observed was in 100% of urban wastewater reaching the maximum of cultivation with 845.82

µg L^{-1} after 21 days, decreasing to 216.20 µg L^{-1} after 30 days of cultivation, due to the lack of nitrogen in the medium.

The concentrations of 0% and 25% of urban waste water showed their maximum cell growth on the 6th day of cultivation with 524.74µg L^{-1} and 583.56 µg L^{-1} respectively, after observing continuous decline. Nutrients when less in the culture medium are consumed more quickly. The concentration of 50% of urban wastewater showed the maximum growth of microalgae cells on the 9th day of cultivation containing 523.99 µg L^{-1}. The maximum growth of the microalgae cells for the 75% concentration of urban waste water was achieved on the 15th day with 385.95 µg L^{-1}, however this was the one that presented the lowest cell growth in relation to the other photobioreactors, which can be explained because it occurred on the 9th day of cultivation the consumption of ammoniacal nitrogen and the oxidation process to nitrate, forcing the microalgae to a new adaptation to the culture medium (Delgadillo-Mirquez et al., 2016).

Other urban rivers have been investigated, proving that it is increasingly common for urban rivers to receive organic loads and are contaminated. The contaminants analyzed remain above what is permitted by environmental legislation, where microalgae growth with a maximum of 650 µg L-1 is also found (Marques et al., 2016).

Figure 4 - Cell growth monitoring of species *Chlorella vulgaris* during 30 days of cultivation in five different treatments. Data shown as the mean ± SD, n = 3



Source: Authors, 2020.

After cultivating the microalgae biomass was collected and dried for quantification, resulting in the generation of 3,7 g L^{-1} of dry biomass total. Souza Andrade (2014) states that the average generation per day for this microalgae species *Chlorella vulgaris* can reach between 0.02 to 0.20 g L^{-1}. In the research by Daneshvar et al. (2019) state that microalgae grown in urban wastewater can increase from 0.04 g L^{-1} initial amount of strain to 0.43 g L final amount of strain after 12 days of cultivation. Criterion such as the choice of species, concentration of nutrients, cultivation conditions such as luminosity, pH and temperature, increase the generation of microalgal biomass for the generation of clean energy such as biodiesel (Cheah et al., 2016). It is important to emphasize that this microalgae species studied in this research has the potential for the bioremediation of urban wastewater from urban rivers. Other research has identified that lighting is one of the essential parameters for the growth of microalgae, with sunlight being a contributing factor to the generation of biomass. In addition, it can influence the treatment of wastewater in the removal of organic carbon material (Nascimento et al., 2020).

Quantification of lipid

The result of the lipid content extracted from 3.7 g of the microalgal dry biomass grown in urban wastewater showed a 7.7% lipid yield. Algae produce lipids as a storage form of energy. In a compilation of several studies, an oil percentage range in *Chlorella vulgar* is ranging from 5 to 40% was found (Xaaldi Kalhor et al., 2017), depending on the species these percentages can vary a lot, reaching up to 77% with species *Schizochytrium sp.* (Carneiro et al., 2018).

It is believed that the use of the solvent changes the efficiency in the extraction of lipids since the hexane solvent is less efficient than the combination of chloroform and methanol when used under the conditions of this study, although hexane is less toxic and has a greater selectivity for neutral lipid fractions (Pragya et al., 2013). This is precisely due to the mixture of two solvents being a polar, which in this case corresponds to methanol and another non-polar, which corresponds to chloroform, allowing the extraction of polar and neutral lipids (Prommuak et al., 2012).

Moreover, considering that the microalgae were grown in wastewater from urban rivers, where nutrients may be more dissolved, it may have influenced the relative low extraction of lipids (Mubarak et al., 2015).

Analyzing the composition of Fatty acid Methyl Esters (FAMEs)

By analyzing the composition of Fatty acid Methyl Esters (FAMEs) in esterified lipids by gas chromatography, it is possible to observe that the composition of methyl esters of fatty acids from the microalgae *Chlorella vulgaris* cultivated in this study consists mostly of fatty acids C16:0 (~29.75%), C18:0 (~4.23%), C18:1 (~28.34%), C18:2 (~ 31.75%), C18:3 (~4.51%) and C22:0 (~ 1.42%) (Table 2). The profile obtained was compared with the fatty acid profile of soybean biodiesel so that they could be identified. The profile obtained from the microalgae *Chlorella vulgaris* appears to be sufficiently suitable for second generation biodiesel production, which corresponds to the biodiesel created from feedstock materials that are not used for food (Hoekman et al., 2012).

According to Mohd-Sahib et al. (2017), the feedstock material for biodiesel production must be rich in C16 to C18 fatty acids, called Palmitic Acid (16:0), Stearic Acid (18:0), Oleic (18:1), Linoleic (18:2) and Linolenic Acid (18:3). These fatty acids are usually identified in other feedstock materials such as other species of microalgae, soybean oil (Knothe & Razon, 2017), palm oil (Verma et al., 2016) and sunflower oil (Orsavova et al., 2015) (Table 3) which have already been confirmed to be suitable for biodiesel production (Lam et al., 2017).

Table 2 - Composition of fatty acid methyl esters (FAMEs) analyzed in the biomass of microalgae *Chlorella vulgaris*. * SFA: Saturated Fatty Acids; UFA: Unsaturated Fatty Acids

Fatty Acids	Nomenclature	Structure	Quantity (%)
Palmitic acid	Hexadecanoic acid	C16:0	29.7
Stearic acid	Octadecanoic acid	C18:0	4.2
Oleic	CIS-9-Octadecanoic acid	C18:1	28.3
Linoleic	CIS-9, cis-12-	C18:2	31.7
Linolenic acid	CIS-9, cis, cis-15-12	C18:3	4.5
Behênico	Benzoic	C22:0	1.4
Total SFA	-	-	35.3
Total UFA	-	-	64.5

Source: Authors, 2020.

Unlike studies such as Santos et al. (2016), which also used the species *Chlorella vulgaris*, it was not detected the existence of fatty acids C14:0 (Myristic), C16:1 (Palmitoleic) and C20:4 (arachidonic acid) in fatty acid composition (Table 3). In the study by Kialashaki et al. (2019), microalgae was grown to treat municipal wastewater with the aim of generating biodiesel. It was presented a small amount of C16: 3 was detected, different in the present study. Some others were not detected as C16: 1, C18: 3 (linolenic acid) C20: 4 and behenic (C22: 0). Although the FAME percentage in total product is a function of lipid content by algal strain, the conversion yield of transesterification technique is also determinative to the final FAME percentage in total product. Acid-catalyzed transesterifications are generally slower than alkaline-catalyzed reactions and have lower yields in similar reaction conditions (Kialashaki et al., 2019).

Table 3 - Comparison of data from quantities of FAME's present in the oil of *Chlorella vulgaris* and other types of raw materials. * ND: not detected; DNP: data not provided

	Quantity (%)					
Fatty acid	Present Study	Kialashaki et al. (2019)	Salama et al. (2017)	SANTOS et al. (2016)	Soybeans	Palm
C14:0	ND	ND	2.7	1.96	DNP	1.01
C16:0	29.7	15.0	21.0	17.57	11.8	40.2
C16:1	ND	ND	1.3	13.13	DNP	ND
C16:3	ND	2.7	ND	ND	ND	ND
C17:0	ND	ND	ND	ND	DNP	ND
C18:0	4.2	8.3	0.1	2.78	4.6	4.6
C18:1	28.3	15.4	9.1	ND	21.8	42.4
C18:2	31.7	9.3	7.2	28.53	53.1	9.9
C18:3	4.5	ND	56.8	1.2	8.0	0.47
C20:4	ND	ND	ND	1.38	DNF	ND
C22:0	1.4	ND	ND	ND	DNF	ND

Source: Authors, 2020.

In another study, where three different microalgae species were grown in medium containing phytohormone for biodiesel generation, present for the species *Chlorella vulgaris* where the amounts of polyunsaturated fatty acids increased significantly, but only C18: 3 had a higher value compared to this research (Salama et al., 2017). Although, the results are promising for the use of microalgae biomass with potential for biodiesel generation.

The oxidation rates in biodiesel depend on the types of fatty acid saturation, being unsaturated and poly unsaturated more likely to go through the process of oxidation (Knothe & Razon, 2017). The predominant characteristic of the FAMEs of this present study is the largest presence of Linoleic (~31.7%) and oleic (~28.3%) acids, which are unsaturated fatty acids, implying a lower resistance of the fuel produced to go through oxidation, but these are the fatty acids that help improve the flow properties of biodiesel (Knothe et al., 2015).

Although , there is a high amount of saturated Fatty Acid Palmitic Acid (~29.7%), that despite being less fluid, has more resistance to oxidation, which could bring a balance in terms of proportion to the results of unsaturated fatty acids, and may make the fuel more stable (Jose & Anand, 2016). Unsaturated fatty acids are considered good for the excellent cold flow properties of biodiesel fuel (Singh et al., 2016). The high concentrations of unsaturated fatty acids found in the extracted lipids and, finally, in the resulting fuel, are an important determinant of the quality fuel.

Although, it is necessary to consider the use of techniques to increase stability of biodiesel and increase its efficiency, such as the inclusion of antioxidants (Serrano et al., 2013), the withdrawal of an amount of unsaturated fatty acids or the increase of the quantity of saturated fatty acids.

This article presented possibilities in the bioremediation of urban rivers contaminated from the treatment of urban wastewater using microalgae, in addition to proposing the reuse of biomass, which would be a waste after wastewater treatment, for the generation of biofuels. This research contributes to the circular economy since it becomes more and more necessary to manage liquid waste and reuse energy, valuing in the production chain thus reducing environmental pressures in obtaining raw material as well as in the final destination of the product (Oliveira et al., 2019; Benedito et al., 2019).

4. Conclusion

In this present research it was possible to show that the use of microalgae in the production of biofuels brings some advantages since they are due to the consistency of productivity and the need for less use of clean water as they grow in contaminated water. Moreover, they present high rate growth, can perform biofixation of CO_2 and absorb nutrients from wastewater, without needing additional use of pesticides or herbicides. In addition, there is the fact that the use of microalgae biomass for biodiesel production has low environmental pollution throughout its process.

The best microalgae cell growth was presented in concentrated urban waste water from a tropical River, your maximum growth in twenty-one days containing 845.8 µg L^{-1} of microalgae cells. The generation of dry biomass was better presented in concentrated urban waste water, generating 0.604 g L^{-1}. The species of microalgae in this study featured a profile of FAMEs suitable for 2nd generation biodiesel production, in addition to presenting a high fatty acid content, although it had a low rate of lipids extracted, possibly due to the high amount of nitrogen availability in the mean, when compared with the phosphate and with the rate of absorption of these nutrients by microalgae, and the variation of concentrations of urban waste water in culture.

It is noticed that it is necessary, however, to assess more thoroughly the behavior of the lipid profile of biomass when subjected to stress conditions of nutrients, performing periodic collections for compilation of great rates. In addition, it is necessary to optimize the conditions and methods of extraction of lipid extraction to increase the amount of lipid extracted and lower costs of production of microalgae for biofuel that can present a greater advantage when

compared to fossil fuels and other existing biofuels which do not have the same rate of productivity.

Despite the optimizations necessary to be carried out, it can be concluded that the use of the species *Chlorella vulgaris* for generation of biodiesel is viable when cultivated in urban waste water, combining sustainable technologies in its process. These results will provide references for the production of renewable energy - microalgae biodiesel from wastewater and contribute to sustainable development. It could not only alleviate the energy crisis, but also reduce the cost of biodiesel production. And it also benefits the environment by efficient wastewater treatment utilization and reduced greenhouse gas emission.

Acknowledgements

This work was supported by the Coordination for the Improvement of Higher Education Personnel – Brazil (CAPES) [Funding code 001, 2014]. The authors thanks the laboratory LEPETRO – excellence in oil in Federal University of Bahia. Thanks Salvador University – UNIFACS for all technical support and recommendations provided.

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