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# Omega fatty acids production in an isolated native *Chlorella* sp. from northeast Mexico with improved growth using urine as nutritive medium

# Producción de ácidos grasos omega en *Chlorella* sp. nativa del noreste de México con crecimiento mejorado en un medio nutritivo de orina

J.C. Canino-Gómez[1], B. Limón-Rodríguez[2], P. Rivas-García[1,3], C. Escamilla-Alvarado[1,3], J.R. Morones-Ramírez[1,3*], M.M. Alcalá-Rodríguez[1,3*]

[1] *Universidad Autónoma de Nuevo León, Facultad de Ciencias Químicas. Av. Universidad S/N, Cd. Universitaria, 66455, San Nicolás de los Garza, NL, México.*
[2] *Universidad Autónoma de Nuevo León, Facultad de Ingeniería Civil, Av. Universidad S/N, Cd. Universitaria, C.P. 66455, San Nicolás de los Garza, Nuevo León México.*
[3] *Centro de Investigación en Biotecnología y Nanotecnología, Facultad de Ciencias Químicas, Universidad Autónoma de Nuevo León. Parque de Investigación e Innovación Tecnológica, km. 10 autopista al Aeropuerto Internacional Mariano Escobedo, Apodaca, Nuevo León. 66629.*

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## Abstract

The production of essential fatty acids (Omega 3, 6, 9) from microalgae consortia and from *Chlorella* sp., using urine as a culture medium, was analyzed in this study. These microorganisms require a supply of nutrients and light to stimulate their growth; and through variations of these factors, it is possible to improve lipid synthesis, fundamental elements for the production of value-added products. The BG11 culture medium was replaced with a urine-based medium (MOB) to cultivate a microalgae of the genus *Chlorella* sp. native to the State of Nuevo Leon. The growth of the microalgae in MOB was increased when compared to its growth in BG11. An improvement was also observed in the production of fatty acids and proteins, where the cultivation of *Chlorella* sp. with the urine medium (MOB) showed an average production (%) of 17.22% saturated fatty acids (SUFA's), 22.03% of monounsaturated fatty acids (MUFA's) and 60.73% of polyunsaturated fatty acids (PUFA's), demonstrated also in the high concentrations of mainly linoleic and linoleic fatty acids in the microalgae. This work shows that human urine as a culture medium, provides enough nutrients to increase the production of biomass and oils; therefore, the use of this organic residue as a nutritive medium can be considered a suitable source for the production of essential fatty acids.

*Keywords*: Microalgae, urine, lipid accumulation, essential fatty acids, sustainable processes.

## Resumen

La producción de ácidos grasos esenciales (Omega 3, 6, 9) a partir de consorcios de microalgas y de *Chlorella* sp., utilizando orina como medio de cultivo, se analizó en este estudio. Estos microorganismos requieren un suministro de nutrientes y luz para estimular su crecimiento; y a través de variaciones de estos factores es posible mejorar la síntesis de lípidos, elementos fundamentales para la producción de productos de valor agregado. Se sustituyó el medio de cultivo BG11 con un medio a base de orina (MOB) para cultivar una microalga nativa del Estado de Nuevo León del genero *Chlorella* sp. El crecimiento de la microalga en MOB mostró un incremento al compararse con su crecimiento en BG11, así como en la producción de ácidos grasos y proteínas. En el cultivo de *Chlorella* sp. con el medio de orina (MOB) se obtuvo una producción promedio (%) de ácidos grasos saturados (SUFA's) de 17.22%, de ácidos grasos monoinsaturados (MUFA's) de 22.03% y de ácidos grasos poliinsaturados (PUFA's) de 60.73%, lo cual se demuestra en las altas concentraciones de ácidos grasos principalmente linoleicos y linolínicos en las microalgas. La orina humana como medio de cultivo, proporciona suficientes nutrientes para aumentar la producción de biomasa y aceites; por lo tanto, el uso de este residuo orgánico como medio nutritivo, puede considerarse como un cultivo adecuado para la producción de ácidos grasos esenciales.

*Palabras clave*: Microalgas, orina, acumlación de lípidos, ácidos grasos esenciales, proceso sustentable.

* *Corresponding author. E-mail*: jose.moronesrmr@uanl.edu.mx; monica.alcalard@uanl.edu.mx
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# 1 Introduction

Microalgae are considered a promising biological system for the production of value-added products, since these unicellular microorganisms can produce a wide range of complex molecules such as fatty acids, proteins, and carbohydrates; these characteristics makes each species unique due to its ability to modify its biochemical composition as a function of the assimilated nutrients (Spolaore *et al*., 2006; Lim *et al*., 2012).

The parameters that regulate the growth and production of fatty acids from microalgae are the light source and its intensity, temperature, and the composition and concentration of N and P in the nutrient medium (Atta *et al*., 2013). It is of particular interest that microalgae are capable of using nitrogen and phosphorus from organic and inorganic sources, such as agro-industrial contaminants (Zhou *et al*., 2014), wastewater (Cai *et al*., 2013), and human urine. However, the potential benefits and economic savings of using urine and wastewaters have been rarely exploited due to bacterial contamination and possible alterations in the obtained biomass. Furthermore, high concentrations of free ammonia have shown to inhibit microalgae growth, therefore the use of nutritious media from waste sources and urine is still under development.

Despite the advantages of using microalgae in the production of complex molecules, their use in production processes is low since only around 50 species, from the more than 30,000 species found, have been studied in detail from a physiological and biochemical perspective. Furthermore, less than a dozen microalgae species have been explored in biotechnological applications (Feng and Wu, 2006).

The use of microalgae biomass as a food product has provided nutritional supplements rich in carotenoids and polyunsaturated fatty acids with great properties that benefit health with antioxidant and anti-inflammatory effects (Batista *et al*., 2013). Microalgae are recognized as an excellent source of natural coloring and it is expected that they will surpass synthetic dyes as well as other natural dye sources(Batista *et al*., 2013). Regarding the production of fatty acids, microalgae have been analyzed and used mainly for the production of biodiesel since they have greater photosynthetic efficiency, are more effective in the assimilation of $CO_2$ and other nutrients compared to plants, accumulate between 20 and 80% of triglycerides (Chisti, 2008), do not require arable land, demand fewer consumption of renewable water and can be cultivated on brackish water (Feng and Wu, 2006).

The variable composition of microalgae when exposed to different factors is a highly appealing line of research (Atta *et al*., 2013). Therefore, this work was focused on the study of the production of essential fatty acids in the microalgae *Chlorella* sp. evaluating the effect of the composition and concentration of two nutritive media to assess a possible increase in biomass generation and an increase in the synthesis of essential fatty acids using urine as the only nutritional medium. This work demonstrated the ability to obtain value-added products, such as essential fatty acids, which are important for a wide variety of applications, including the health care and cosmetics fields.

# 2 Materials and methods

## 2.1 Microalgae sampling

Microalgae were obtained from a body of water designed to supply drinking water in Nuevo Leon, Mexico (Rodrigo Gomez "La Boca" dam). The sampling points were chosen in accordance with the provisions of the standard methods for manual sampling of plankton. The samplings were located near the banks of the discharge areas and the water inlets that can influence the physical and chemical composition of the dam's water. The UTM coordinates of the sampling points were 3860 38.81 E 2817 07.58 N; 3847 84.71 E 2812 851.14N; 3864 49.09 E 2812 821.22N. The sampling depth was determined between 0-1 m because the microalgae of the Chlorophyta division inhabit surface waters and to ensure that the sample is homogeneous (vertically mixed). Samples were taken in February and April 2017; and were processed and separated in 1-liter containers. The total characterization of nitrogen, phosphorus and pH of the water samples was carried out following the current regulations: Kjeldahl Total and Ammoniacal Nitrogen (ASTM D3590), ammonia and protein; chemical oxygen demand (COD) (ASTM D1252) and total phosphorus [ASTM D515-88 (A)].

## 2.2 Strain and culture conditions

The microalgae were cultivated in 500 mL flasks containing 450 mL of any of three different liquid

cultures at 25 ºC, in a light intensity of 60-80 $\mu$mol photons $m^{-2}s^{-1}$, 12/12 h day/night cycle and 2.5 g/L of $CO_2$, which were inoculated with 50 mL of original sample from "La Boca" dam. Two nutritious media were used to cultivate (consortium and *Chlorella* sp.) and analyze the variation and composition in the microalgae biomass; specifically, its fatty acid composition. The composition of BG11 medium consisted of (in g per L): $NaNO_3$ (6.2), $K_2HPO_4$ $3H_2O$(0.04), $MgSO_4{\cdot}7H_2O$ (0.075), $CaCl_2{\cdot}2H_2O$ (0.036), citric acid (0.006), Fe-ammonium citrate (0.006), $MgNa_2$ EDTA (disodium salt) $H_2O$ (0.001), $Na_2CO_3$ (0.02); Trace metals: $H_3BO_3$ (2.86), $MnCl_2{\cdot}4H_2O$ (1.81), $ZnCl_2{\cdot}7H_2O$ (0.22), $CuSO_4{\cdot}5H_2O$ (0.079), $Na_2MoO_4{\cdot}2H_2O$ (0.39) and $CoCl_2{\cdot}6H_2O$ (49.4). To improve the rate of growth, photosynthesis and biomass production a higher salinity was applied to the medium by substituting the zinc sulfate with zinc chloride, and cobalt chlorine by cobalt nitrate as indicated in the literature (Yu *et al*., 2011). The pH average was 7.5 for these two media. The third medium was human urine, hereinafter referred to as MOB. Urine was diluted using distilled water until two concentrations were obtained: a concentration of 0.06 g/L as $MOB_L$ and in concentration 0.15 g/L as $MOB_H$. Urine was directly collected using sterile bottles from 3 male and 3 female healthy employees from the UANL environmental engineering department and homogenized by a magnetic stirrer at 1000 rpm for 10 minutes for subsequent analysis. The characterization of the MOB medium was made according to the current regulations described in the characterization of the water sample. We observed its composition in Table 1. The urine stock was kept in the dark at -4 ºC

In this sense, in a study carried out with a consortium of flocculating microalgae (BR-UANL-01), native to the state of Nuevo Leon; it was reported that in a treated residual water sample with an initial pH of 7.2, the final microbial load of bacteria on a nutrient agar plate was 62,000 CFU/mL; 8 days later the pH of the culture was chemically altered with the addition of 0.1N NaOH until obtaining a pH of 9.5 and 10, the plate count analysis was performed and 17,000 and 14,000 CFU/mL were obtained and a 77.4% reduction in immersed bacteria was reported (Fariz *et al*., 2018). Bacteria tolerance to pH changes is limited, abrupt changes produce the cell sample, because the stability of the plasma membrane and the internal pH of the bacteria are compromised (Franchino *et al*., 2013). Various investigations of microalgae cultivation in open lagoons have been carried out and some species can be kept in traditional open systems with contamination control using selective alkaline or saline environments. (Solis-Mendez *et al*., 2020). Regarding the presence of a microbial load on the MOB, it was periodically monitored for presence of microorganisms, and the results showed no presence of microorganisms.

Table 1. Composition of human urine taken from references and actual measurement of human urine as MOB medium used as nutrient in this project.

| **Component** | **Reference ranges of human urine[a]** | **Actual measurement from adults** |
|---|---|---|
| pH | 4.6-8 [10] | 7 |
| $N_{tot}$ (mg/L) | 4320-6800 [11] | 7500 |
| $P_{tot}$ (mg/L) | 355-670 [11] | 1000 |
| DOC(mg/L) | 2405-4830 [11] | n.a. |
| Fe (mg/L) | <1 [11] | n.a. |
| Mg (mg/L) | 50 [11] | n.a. |
| Ca (mg/L) | 77 [11] | n.a. |
| Na(mg/L) | 710 [11] | n.a. |
| K(mg/L) | 717 [11] | n.a. |

Note n.a. = not available.
*Value of an undiluited human urine.
*Actual measured value for this human urine in this study

## 2.3 *Identification, counting and culture of microalgae*

Identification of microalgae from the "La Boca" dam was carried out using light microscopy, with the guidance of different taxonomic keys. According to the taxonomic key proposed by Bungartz *et al*. (2002), the identification was carried out according to the morphological characteristics of these microorganisms (color of the protoplasm, presence/absence of chloroplasts with pyrenoids, status of individual or colonial cells, and morphology such as globose or filament). This method of classification was used using similar techniques reported to distinguish microalgae at the genus level, using similar reported techniques (Lee *et al*., 2014). The Sedgewick Rafter (S-R) method was used for cell counting and cell concentration calculation. Pipettes were used to take 1mL aliquots of the sample and deposit them in S-R cells. Once the microalgae were settled, the count was performed by the counting method using a Whipple micrometer.

## 2.4 *Insulation techniques*

Samples were first diluted to 10-6 to aid in the insulation process. Sterilized Petri dishes (100×15 mm) containing approximately 40 mL of BG11 solid medium (15 g/L agar) were used to inoculate with 1 mL of the diluted sample. The diluted sample was then spread evenly across the surface. Inoculated plates were incubated 14 days at 20-25 ºC. This method was repeated until uniform colonies were observed, and the insolated organisms were transferred to a BG11 medium.

## 2.5 *Quantification of biomass (dry basis)*

### 2.5.1 *Filter pretreatment*

To eliminate interference in the glass filter, a pretreatment was performed using filters according to the method of total suspended solids referred in Standard Methods for the Examination of Water and Wastewater. Whatman (934-AH, 1.5 $\mu$m pore size) glass fiber filters were washed and dried in an oven (Shel Lab 1350FX) at 100 ºC for 30 minutes. The filter was then placed in a desiccator and allowed to cool for approximately 15 minutes, to room temperature, to subsequently record the weight of the filter.

### 2.5.2 *Biomass dry weight concentration*

To determinate the biomass dry weight content as described in the literature (Pegallapati and Nirmalakhandan, 2011), the culture was homogenized and 50 mL (the recommended amount when the cell density is greater than $2 \times 10^6$ cells per milliliter being *Chlorophytes*) was filtered through the previously pretreated glass fiber filter using vacuum (Koblenz, model DGP-134, Mexico) (Richmond and Hu, 2013). The filters were placed in teaspoons of foil and transferred to the oven (Shel Lab) for 6 hours at a temperature of 65 ºC; after that time, they were placed in desiccators for an hour and weighed in the analytical balance.

The weight of the sample was calculated with the following equation:

$$Dry\ weight = (PFM - PF) * R \tag{1}$$

where: PFM is the Dry filter weight plus microalgae, PF is the dry weight of the filter, and R is the ratio l/mL filtered (1000 mL)

## 2.6 *Calculations*

Yields were defined as the ratio between the product obtained and the substrate consumed, and cellular performance is defined through the concept of limiting nutrients. A limiting nutrient is that substrate whose consumption controls the speed of biomass production. Through this limiting substrate concept, the performance of the process can be defined. To determine the percentage of the consumed substrate, it was necessary to determine the biomass/substrate yield $Y_{x/s}$; which it was determined by the following equation:

$$Y_{x/s} = \frac{\Delta X}{\Delta S} \tag{2}$$

where: $\Delta X$ is the biomass produced and $\Delta S$ is the substrate consumption in g.

## 2.7 *Fatty acids determination*

### 2.7.1 *Lipid extraction*

The Bligh and Dyer based total lipid extraction method was used. (Bligh and Dyer, 1959). 2 g of dry biomass were weighed and homogenized in tubes. 2 mL of C11:0 triumdecanoate internal standard was added at a concentration of 5.00 mg/mL. 6 mL of 2:1 methanol: chloroform (MeOH: $CHCl_3$) was added. It was stirred for a period of 2 min. 4 mL of CHCl3 and 4 mL of

distilled water were added and stirred for 2 minutes. It was left to stand until the organic and aqueous phases separated. The lower (organic) phase was collected with a Pasteur pipette and transferred to a previously weighed 50 mL Erlenmeyer. A second extraction was performed adding 4 mL of $CHCl_3$ and 4 mL of distilled water and they were stirred for two minutes. When observing the phase separation, the inner phase was taken and placed in the Erlenmeyer that it had in the first phase. $CHCl_3$ was evaporated in an oven (Shel Lab) at 70 ºC.

#### *2.7.2 Gas chromatography analysis*

Determination of fatty acids was carried out according to the official method AOAC 969.33 and methyl esters were prepared according to the boron trifluoride method, in which the glycerides and phospholipids are saponified and the fatty acids were released and esterified in presence of a $BF_3$ catalyst for their GC analysis. A GC analysis was conducted in a gas chromatograph coupled to flame ionizing detector (Hewlett Packard 5890) was employed; prepared standards were Sigma brand (FAME mix of 37 components).

## 3 Results and discussion

### *3.1 Characterization and analysis of "La Boca" dam*

The concentration of nutrients (N, P) in the water sample obtained from the Rodrigo Gomez "La Boca" dam, registered values of 0.0004 g/L of total nitrogen and 0.0007 g/L of phosphorus with an alkaline pH (7.6 − 7.7) and the temperature ranges between (12 − 18 ºC). In this study, these concentrations of N and P, indicate the low levels of these macronutrients present in the dam at the time of sampling, which influences but does not interfere with the presence of microalgae in the body of water. However, it shows the high degree of survival resistance of these microorganisms to the deficit and the abundance of nutrients. The nutrient variation affects the microalgae habitat, inducing competition for nutrients, which causes environmental stress and influences in the distribution and abundance of the different genera. (Dayton, 1971; Paine and Levin, 1981; Roughgarden, 1983). These variations are obtained as a result of the rainy seasons and the economic activities that take place in the body of water and in its surroundings; such as: aquatic, domestic, agricultural and industrial activities that are carried out, since it is one of the most representative bodies of water in the state. These activities cause eutrophication (organic and inorganic contamination of a body of water), promoting alterations in the availability of nutrients for microalgae. In a study carried out in China, it is mentioned that most urban lakes are hypertrophic due to their high concentrations of N and P, with an average of 5.38 and 0.49 g/L respectively (Lv and Chen, 2011).

From the samplings carried out, a microalgae consortium was obtained, and it consisted of 4 divisions of microalgae; being Chlorophyta the one that presented the highest cellular density, specifically in the spring season. The results showed that the divisions of Chlorophyta, Cyanophyta, and Euglenophyta, responded similarly at average temperatures above 20 ºC, so it was determined that their number increases during the spring season Nuevo Leon State. This influenced the results shown in Fig. 1, where an increase in cell density of 261%, 259% and 188% was observed for Chlorophyta, Cyanophyta, and Euglenophyta respectively, in relation to the winter season. On the other hand, the Bacillariophyta division showed a better growth in winter registering an increase of 293% in its cellular density compared to spring. The natural variation between the microalgae groups and the influence factor that the climatic conditions were observed, as well as the adaptation capacity of the Chlorophyta division since despite having a low growth rate at low temperatures (winter) it was still the second division that showed higher cell density at the two monitored stations. As a consequence, the Chlorophyta division has the potential to be considered as a raw material under variable conditions (Peñaranda *et al.*, 2013).

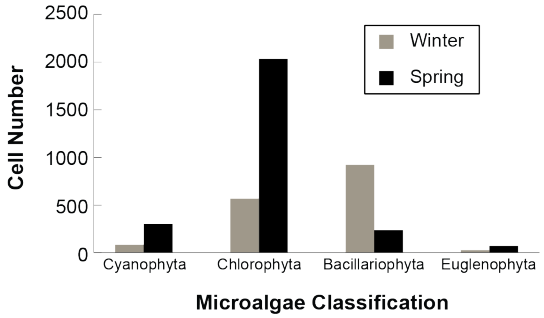


Fig. 1. Cell density by division of microalgae from the Rodrigo Gomez "La Boca" dam obtained in the winter and spring season.

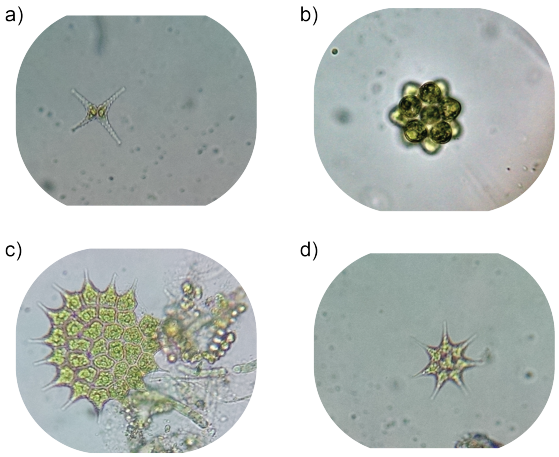


Fig. 2. Genera present in the sample of Rodrigo Gomez "La Boca" dam. a) *Staurastrum* sp., b) *Coleastrum* sp., c, d) *Pediastrum* sp.

Microscopy images were used to identify the genus of microalgae detected in the sample. These genera of microalgae have particular qualities, since the division, Chlorophyta has as the main characteristic the presence of chlorophyll a and b, which are the pigments that give them their characteristic green color (Batista *et al.*, 2013). Among the genera present in the sample are *Cosmarium* sp., *Chlorella* sp., *Staurastrum* sp., *Coleastrum* sp., *Pediastrum* sp. among others (Fig. 2 a-d).

### 3.2 *Isolation and characterization of Chlorella sp.*

A consortium of microalgae was obtained from the Rodrigo Gomez "La Boca" dam and subsequently, the genus *Chlorella* sp. (Fig. 3) was selected and isolated due to its ability to produce fatty acids (Gouveia and Oliveira, 2009). *Chlorella* sp. has been shown to be able to adapt to a wide temperature range, which provides advantages during the process of production of microalgae biomass (MBP). Afterward, the MBP and fatty acids production from the consortia and *Chlorella* sp. were further analyzed and characterized.

### 3.3 *MBP related to the exposure of different nutritive media*

The impact on the generation of microalgae biomass in response to the exposure of different culture media for essential fatty acids production can be observed from Figure 4.

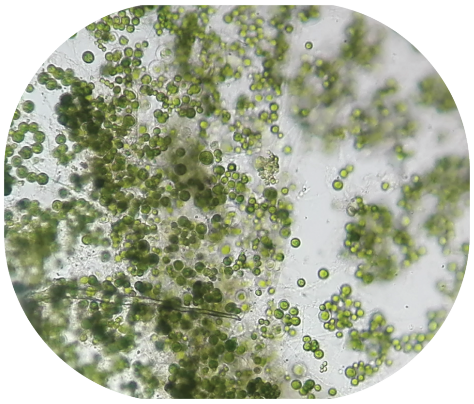

Fig. 3. Image of *Chlorella* sp. isolated from the microalgae consortium from Rodrigo Gomez "La Boca" dam.

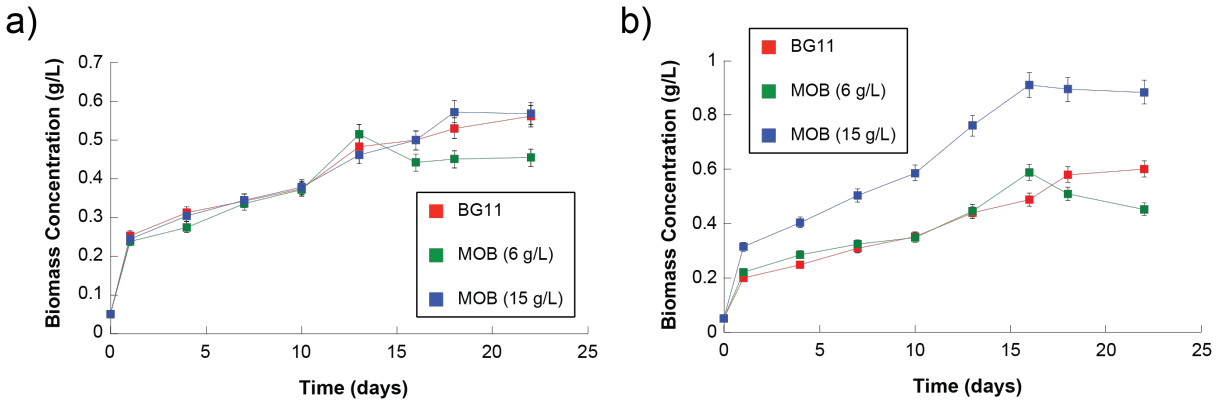


Fig. 4. Biomass production using three different media: BG11, urine medium (MOB). in two concentrations $MOB_L$ (low phosphorus concentration), $MOB_H$ (high phosphorus concentration) a) microalgae consortia, b) *Chlorella* sp.

Table 2. Content of nitrogen and phosphorus in a sample of water taken of The Rodrigo Gomez "La Boca" dam, BG11 and urine medium (MOB) in two concentrations: high ($MOB_H$) and low ($MOB_L$) phosphorus concentration.

| Nutrient | Original sample of water | BG11 | MOB | $MOB_L$ | $MOB_H$ |
|---|---|---|---|---|---|
| Nitrogen (g/L) | 0.0004 | 0.54 | 7.5 | 0.1 | 0.21 |
| Phosphorus (g/L) | 0.0007 | 0.006 | 1 | 0.06 | 0.15 |

The growing rate of microalgae is influenced by exposure to different nutrients, and biomass generation is a step in the process of production of essentials fatty acids. Growth analysis and MBP in the microalgae consortium and *Chlorella* sp., obtained from Rodrigo Gomez "La Boca" dam, was performed by growing them in two different nutrient media (BG11 and MOB). The first medium was BG11, and the culture showed an increment of over 100% in the concentrations of nutrients (N, P) than in the original sample of water (Table 2). In this case, our results for the culture of the consortia of microalgae and *Chlorella* sp. demonstrated that the high concentration of nutrients reflects an increase in MBP. This confirms the results reported from previous works that use BG11 and observed a 75% increment in the production of biomass compared to other media (Zhang *et al*., 2012; Kothari *et al*., 2017). The results shown here include a 25% higher biomass production in the consortia, compared to *Chlorella* sp. culture, just at the second day of growth. The difference in biomass production is reduced by day 16, where the difference between the consortia and the *Chlorella* sp. is just 2%. Subsequently, the cultivation of *Chlorella* sp. showed a rise from the $18^{th}$ to the $22^{nd}$ day, since the MBP was between 9 and 7% higher than the consortium.

In Figure 4a, being a consortium of microalgae, they present different growth peaks, therefore obtaining the highest concentration of biomass on day 13 (0.514 g/L) and a decrease of 17%. This variation could be correlated to the lack of nutrients or to the competition between the microalgae present in the consortium, since the following days the biomass concentration recovers registering increases of 2 and 0.9% on days 16 and 18.

In contrast to the microalgae consortium, the cultivation of *Chlorella* sp. with MOB medium increases its MBP. It is observed in Figure 4 b) that the highest yield in MBP was observed on day 16 in the two samples. On day 16 of cultivation, the use of the $MOB_H$ medium registered a production of 0.910 g/L of biomass and the final concentration obtained was 0.884 g/L on day 22, which means a decrease of 1.8%. With the $MOB_L$ medium, the behavior was similar in terms of maximum yield, which was registered on day 16 with a biomass concentration of 0.588 g/L and showed a decrease of 30% on day 22 of cultivation. Comparing the results obtained with the use of the BG11 and MOB medium, consistency was found in the variations in MBP; that is, using the BG11 medium, the maximum yield was increased on day 22 and MBP showed a decrease of 2% and 51% in the consortium and *Chlorella* sp. cultivated, respectively, in relation to the MBP by cultivation with the $MOB_H$ medium. The decline in growth and biomass concentration can be attributed to the decrease in the concentration of nutrients available in the culture medium. This behavior was observed in all the tests carried out.

Some species of microalgae increase their growth and have the capacity to modify their chemical composition when they are cultivated under diverse biotic and abiotic factors. These factors that affect growth and biochemical composition of the microalgae are light intensity, photoperiod intervals, temperature and added nutrients (Batista *et al*., 2013). This means that the use of a nutritious medium benefits the growth for shorter times since a greater concentration of biomass can be obtained. In terms of the content of fatty acids, their production is correlated directly with the concentration of biomass and oil content of the cell. It should be noted that under stress conditions such as nutrient limitation, cell growth decreases and lipid content increases (Yu *et al*., 2011).

In this work, the traditional microalgae culture medium was replaced by a culture made up of human urine, an organic waste, in order to analyze the effect of a lower cost culture medium on MBP and essential fatty acids production. The results showed high phosphorus content on the $MOB_H$ and in this culture *Chlorella* sp. had improved biomass growth at 22 days with 0.884 g/L of biomass production (Fig. 4b). According to an investigation (Chen *et al*., 2011), the effect of various nutrients on the growth of a green algae (Dunaliella tertiolecta) was analyzed; and the results showed that concentrations

decreased dramatically in the culture, up to 84% depletion during cultivation. This is attributed to the fact that phosphorus, in the form of phosphate, is involved in numerous metabolic processes in many organisms, including microalgae, therefore phosphorous is required in relatively high amounts, being a key element to biomass production. However, when nutrients are depleted, microalgae enter a state of stress, causing the production of fat reserves, and therefore increasing the concentration of fatty acid in the cells. It has been reported (May-Cua *et al*., 2019) that lipid production in *Coleastrum* sp. at the end of the exponential growth phase, under nitrogen limitation, increases by 83.33% and in photobioreactors with nitrogen limitation increases over 100% are achieved. Moreover, our results are also consistent with those of Jaatinen *et al*. (2016) where they report that a *Chlorella vulgaris* culture produces biomass using two different nutrient media, including diluted urine at different concentrations with the addition of trace metals; and they show that the highest percentage of biomass was obtained with 1:100 diluted urine with and without the addition of trace metals (0.73 and 0.60 g/L). They attributed this effect to the higher abundance in nutrients.

### *3.4 Relationship between the growth rate and substrate removal for biomass production consortium microalgae Chlorella sp.*

The MBP was incremented for the high concentration of nutrients in the culture; this was demonstrated since in the culture with $MOB_H$ medium, which corresponds to a high concentration of nutrients, the best MBP was obtained in both culture: in *Chlorella* sp. (0.910 g/L) and consortia (0.572 g/L), at the 16 and 18 days respectively. This amount of biomass corresponded to 1,044 and 1,720% production compared to the first day. These results show an increase of 70% when using a culture of *Chlorella* sp. in $MOB_L$ medium for producing microalgae biomass than using a consortium and a conventional medium. That behavior was associated with the human urine having high levels of nutrients, including N and P. Hence, the human urine can be considered as a good culture medium in the cultivation of microalgae. Comparing the results obtained in our research, we observed that in previous studies, biomass concentrations obtained with conventional nutritive media is less than the one we reported. One of the previous studies was carried out by Robles (2017), where a maximum biomass concentration of 0.13 g/L is reported in *Chlorella vulgaris* grown in the nutrient medium F/2; furthermore, Hosseini *et al*. (2015) worked with Scenedesmus sp. cultivated in the Basal Bold's nutritious medium and registered a maximum biomass concentration of 0.62 g/L. The correlation between biomass production and nutrient removal is an indicator of the importance of the concentration of nutrients in the culture medium (Aziz, 2016).

In relation to the consumption of nutrients for the production of biomass phosphorus consumption was analyzed during fatty acid biosynthesis. According to various investigations; it is mentioned that the cell density can be varied in response to the concentration of N and P (Lombardi and Maldonado, 2011); however, reference is only made to the relationship of the limitation of N in the production of biomass and lipids (May-Cua *et al*., 2018). Phosphorus is an essential macronutrient for growth and involves numerous metabolic processes such as: transfer energy (ATP), it is part of important metabolic intermediates, enzymes, a multitude of biochemical species, lipids, DNA and nucleic acid biosynthesis, etc. (Chen *et al*., 2011; Richmond and Hu, 2013), although phosphorus is not incorporated as such into the fatty acid molecule.

Therefore, the phosphorus was used because it is an element present in both BG11 and MOB media. Phosphorus was an indicator for measuring the ratio of substrate-consumption for the MBP in the culture of a microalgae consortia and *Chlorella* sp. for the essential fatty acids production based on previous work (Ahmad *et al*., 2016). His research shows that the availability of phosphorus in a microalgae culture regulates cellular proliferation (biomass production) and other processes such as signaling pathways, energy generation, and photosynthesis. Phosphorous is also involved in metabolism and development processes, such as the biosynthesis of ATP, nucleotides, phospholipids and other cellular components and fatty acid production.

Therefore, Figure 5 shows the variation in the concentration of P in the medium (BG11, MOB), and it can be observed that the percentage of nutrient consumption for the first 5 days in all the samples was greater than 80%; leading to a response of gradual growth or MBP after those 5 days. It should be noted that from the tenth day of cultivation, there was an increase in the generation of biomass in all cases, reaching its production of maximum biomass at day 15 to subsequently decrease; with the exception of the cultures with BG11 medium, which recorded their

Table 3. Comparison of biomass production, fatty acids and phosphorus removal in cultures of different microalgae using different nutrient media from the results obtained in this study with previously reported works.

| Reference | Microalgae | Strain | Results | | |
|---|---|---|---|---|---|
| | | | Biomass (g/L) | Phosphorus reduction percentage (%) | Fatty acids |
| Jaatinen S *et al*., (2016) | *Chlorella vulgaris* | Diluted human urine (1:100) | 0.6 | 80 | N.A |
| | | Urine+ TE | 0.73 | >99 | |
| | | CHU-10 | 0.53 | >99 | |
| Coopens et al., (2016) | *Arthrospira platensis* | Zarrouk | 1.3 | N.A | N.A |
| | | Nitrified urine | 0.84 | N.A | N.A |
| Chang et al., (2013) | *Spirulina platensis* | Synthetic human urine | 1.17 | 85-98 | N.A |
| Feng et al., (2006) | *Spirulina platensis* | Zarrouk | 3.74 | N.A | 11 |
| | | Synyhetic human urine | 2.46 | N.A | 17 |
| | | Diluted human urine | 2.32 | N.A | 20 |
| Solis-Méndez *et al*. (2020) | *Spirulina platensis* | Schlösser | 1.1 | N.A | N.A |
| In this study | *Consortium* | BG11 | 0.53 | 80 | 0.77 |
| | | MOB$_H$ | 0.5 | 78 | 1.6 |
| | | MOB$_L$ | 0.52 | 74 | N.A |
| | *Chlorella sp*. | BG11 | 0.5 | >100 | 0.2 |
| | | MOB$_H$ | 0.91 | >100 | 11.07 |
| | | MOB$_L$ | 0.59 | >100 | N.A |
| Zarei *et al*., (2017) | *Scenedesmus obliquus* | Guillard | 0.92 | N.A | SUFFA's- 25.9<br>MUFA's- 47.6<br>PUFA's-25.5 |
| | | Tamiya | 1.12 | N.A | SUFFA's- 25.9<br>MUFA's- 47.6<br>PUFA's-25.6 |
| | | Trenkenshu | 1.79 | N.A | SUFFA's- 25.9<br>MUFA's- 47.6<br>PUFA's-25.7 |

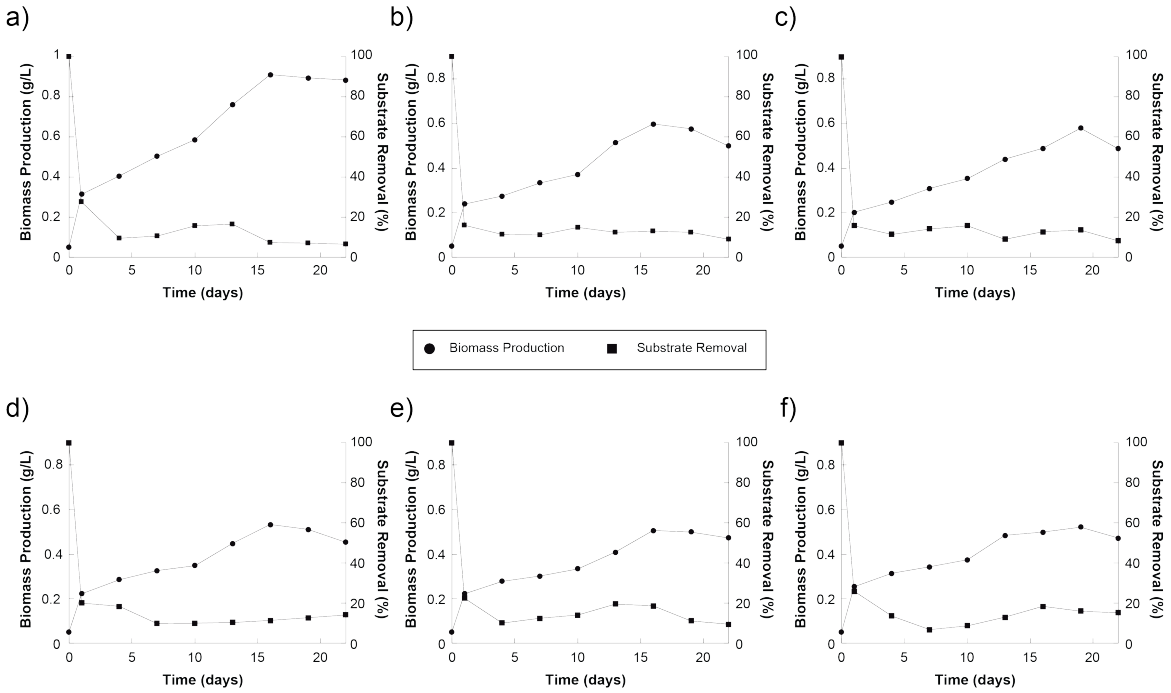


Fig. 5. Production ratio of microalgae biomass (MBP) and average nutrient removal in the cultivation of *Chlorella* sp. at three different media a) urine medium high phosphorus concentration ($MOB_H$) b) urine medium low phosphorus concentration ($MOB_L$) c) BG11 and in the microalgae consortium at three different media: d)$MOB_H$ e)$MOB_L$ f)BG11 medium.

maximum biomass concentration at day 19.Similar behavior was reported by Wang *et al*. (2011); he analyzed the removal of N and P in a residual water for the production of biomass in some green algae (Neochloris oleoabundans) and recorded that the increase of 0.028 – 0.047 g/L of phosphorus in the medium, increased the biomass production of 1.8 – 2.1 g/L; and a residual zero phosphate in the medium was obtained in 72 hours. It is possible that the observed phosphate removal is due to the combination of the biological assimilation of phosphate by the microalgae and the abiotic precipitation due to the high pH in late stages of the culture.

In addition to the aforementioned, Table 3 shows a comparison of the variables and the results obtained in this study against that reported in previous works, focused mainly on microalgae and nutrient medium used to obtain biomass, acids, fatty and/or phosphorus removal.

### *3.5 Cell alterations in Chlorella sp. after fatty acids extraction*

SEM was used to examine the morphology of *Chlorella* sp. after oil extraction treatment. In Fig. 6 it is observed the morphology of the cell surface, which shows damage caused by the combination of solvents (chloroform and methanol) after 8 hours in the oil extraction treatment. These solvents caused the cellular rupture of the microalgae (Mercer and Armenta, 2011), allowing the liberation of the oils contained in its structure and their recovery for further analysis.

### *3.6 Effects of use of different nutritive media on microalgae composition*

The biomass characterization in microalgae consortium and *Chlorella* sp. was performed to know the variation in its composition after the culture with BG11 and MOB medium.

Table 4. Bromatological analysis (%) of the dry biomass of microalgae consortium and *Chlorella* sp. cultivated in two media: BG11 and urine medium in high phosphorus concentration ($MOB_H$).

| Composition | Consortium | | | | *Chlorella sp.* | | | |
|---|---|---|---|---|---|---|---|---|
| | BG11 | SD | $MOB_H$ | SD | BG11 | SD | $MOB_H$ | SD |
| Fat | 0.77 | 0.09 | 1.6 | 0.11 | 0.2 | 0.04 | 11.1 | 0.03 |
| Protein | 6.26 | 0.14 | 3.47 | 0.30 | 4.1 | 0.08 | 43.2 | 0.36 |
| Total carbohydrates | 14.1 | 0.82 | 9.06 | 0.62 | 3.81 | 0.13 | 31.4 | 0.27 |

In the case of the MOB medium, for this analysis and onwards, the biomass cultivated with $MOB_H$ medium was used because the highest results of MBP (0.910 g/L) was obtained. It can be observed in Table 4 and Figure 7 that in carbohydrates and proteins, the consortium of microalgae produced a larger quantity of both components when it is grown in BG11 medium; this corresponds to an increase in the concentration of carbohydrates of 55% (14.09 – 9.06%) and proteins of 81% (6.26 – 3.475%) concerning the consortium cultivated with medium $MOB_H$. In the case of *Chlorella* sp., the opposite occurs. The use of $MOB_H$ medium in the culture increases all parameters measured (fatty acids, proteins, and carbohydrates) in concentrations of 5,435, 954 and 725% respectively about the culture of *Chlorella* sp. with BG11 medium.

The MOB medium allowed the production of a high concentration of biomass (0.910 g/L) at 16 days of culture without the incorporation of additional nutrients. Added to this, the increases in the concentrations of fatty acids, proteins, and carbohydrates were remarkable. Several investigations have been carried out to increase the composition of microalgae through the use of nutritive media, using synthetic medium and human urine to increment the production of biomass and proteins in addition to evaluating the removal of N and P from the medium. In the case of *Spirulina platensis*, a synthetic human urine medium was used to register the removal rates of nitrogen between 85 – 98% in 7 days and 96% of phosphorus in 9 days, producing a biomass concentration of 1.17 g/L (Chang *et al*., 2013). In a study made by Coppens *et al*. (2016), he increased the protein concentration in a culture of *Arthrospira platensis* using urine and Zarrouk as a nutrient medium; he working first with *Nitrobacter* sp. to nitrify the medium and then with the already nitrified medium it was used for the cultivation of the microalgae. *A. platensis* grown under a nitrified urine medium resulted in a high growth rate and biomass with high protein content (62%). Feng *et al*. (2006), work with *Spirulina platensis*

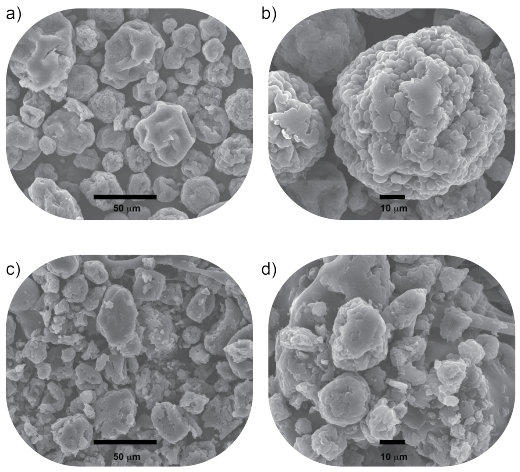


Fig. 6. (a, b) Scanning electronic microscopy (SEM) of surface morphology of *Chlorella* sp. after fatty acids extraction treatment, (c, d) SEM surface morphology of *Chlorella* sp. before fatty acids extraction treatment.

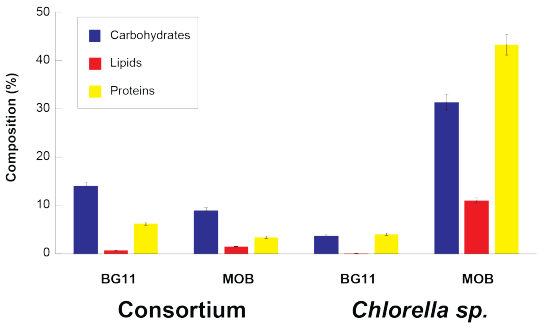


Fig. 7. Effect of two different media (BG11, *$MOB_H$) in biochemical composition of biomass in microalgae consortium and *Chlorella* sp.
*$MOB_H$ urine high phosphorus concentration medium.

to produce biomass; using three nutritive media: Zarrouk medium, a synthetic medium of human urine and human urine, reporting high concentrations of biomass, 3.7, 2.40, and 2.32 g/L respectively, in addition, the characterization of the biomass generated

was performed and reported concentrations of protein and lipids in the Zarrouk medium of 67 and 11%, in the synthetic medium of human urine of 35 and 17%, finally, in the medium of human urine of 32 and 20%; however, it was not possible to make a comparison with the results obtained in this study, because the characterization of the human urine medium was not reported.

In addition, according to published results, variations were found in the general composition of the microalgae according to the species used and the nutritional medium. Becker (2007) mentioned that *Chlorella vulgaris* has a concentration between 51-58% protein, 12-17% carbohydrates and 12 – 22% lipids; *Chlorella pyrenoidosa* 57% protein, 26% carbohydrates and 2% lipids; while *Spirogyra* sp. 6 – 20% protein, 33 – 64% carbohydrates and 11 – 21% lipids. It is important to mention that microalgae incorporate non-protein nitrogen ($NH_4^+$, $NO_3^-$, $NO_2$, and urea) to be subsequently converted into proteins, which is why, in the results obtained in this study, the culture of *Chlorella* sp. With the highest concentration of nitrogen ($MOB_H$) they recorded the highest protein values. These results were consistent with those of Mutlu *et al*. (2011), which grow *Chlorella vulgaris* under different concentrations of nitrogen; and obtained that the concentration of proteins under sufficient conditions of nitrogen was higher (50.8%), while the culture without the presence of nitrogen registered a protein concentration of 13.01%.

Therefore, according to these results, it is assumed that the protein concentration is a function of the initial nitrogen concentration in which the microalgae are grown.

The determining of the fatty acid concentration in a consortium of microalgae and *Chlorella* sp. was performed to understand the impact in the biochemical composition of microalgae by their cultivation with two different nutrient media (BG11 and $MOB_H$). The biomass produced by the microalgae consortium had very similar oil concentrations in the cultures using BG11 and $MOB_H$ media. The results in Table 5 showed a general raise of 7% in the concentration of fatty acids; it was observed the increase in the concentrations of polyunsaturated fatty acids (PUFA's), monounsaturated fatty acids (MUFA's), and saturated fatty acids (SUFA's) of 6, 9 and 7% respectively with the concentration of fatty acids in the biomass produced from the culture with $MOB_H$ medium.

Table 5. Total content (%) of saturated, monounsaturated and polyunsaturated fatty acid (SUFA's, MUFA's and PUFA's) in the dry biomass of the microalgae consortium of and *Chlorella* sp.

| Composition | Consortium | | *Chlorella* sp. | |
|---|---|---|---|---|
| | BG11 | $MOB_H$ | BG11 | $MOB_H$ |
| SUFA´s | 15.7 | 15.6 | 25.2 | 17.2 |
| MUFA's | 24.3 | 22.3 | 27.5 | 22 |
| PUFA's | 66.1 | 62.2 | 47.4 | 60.8 |

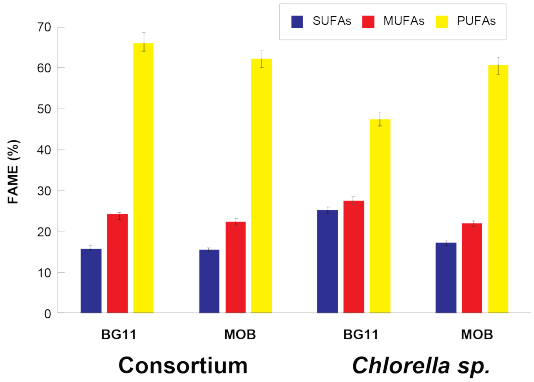


Fig. 8. Characterization of the composition of fatty acids biomass of microalgae consortium and *Chlorella* sp. exposed to two different media: [BG11 and high phosphorus urine ($MOB_H$) medium].
*__FAME__ – Fatty acids Methyl Ester

Concerning fatty acid composition in *Chlorella* sp. biomass; in Figure 8, we found an important variation in the results; the percentage of SUFA's concentration in the biomass of *Chlorella* sp. cultivated with the BG11 medium showed an increase of 46% concerning the use of the MOB medium; however, the concentrations of MUFA's and PUFA's in the biomass of *Chlorella* sp. cultivated in $MOB_H$ medium, they registered an increase of 25 and 28% respectively to the crop with BG11. That means, that microalgae can be considered a source of PUFA's when are cultivated in both media. Darki *et al*. (2017) mention that PUFA's are of the most important fatty acids and they are the key in animal nutrition. The conventional source of PUFA's is fish oil; however, its use represents significant limitations, such as undesirable odor, low oxidative stability and the presence of contaminants (Ohlrogge *et al*., 1995; Bellou *et al*., 2016). As for MUFAs, they are used for their health benefits; Mainly oleic acid, which is widely used as cooking

oil due to its monounsaturation benefits to reduce low-density lipoproteins, helps in reducing blood pressure and heart disease (Rohit *et al*., 2018). SUFAs can play a double role, as a deposit of saturated fatty acids to use them as an energy source and as a deposit of PUFA´s that are necessary to integrate into various metabolic processes and the synthesis of phospholipids and various membrane structures. Regarding its commercial importance, they are well known for their good properties, properties for the production of biodiesel and it has a higher caloric value due to the presence of long-chain hydrocarbons that release high energy (Darki *et al*., 2017).

### *3.7 Fatty acids analysis*

In this work, variations were observed in both the composition and the amount of fatty acids produced, these parameters depended on the culture medium used for growth. These variations were derived from the fatty acid composition; and were observed similar behaviors in the biomass of the microalgae consortium, since they recorded percentages of increase/decrease of less than 10%. This variation is important because the main fatty acids present in vegetable oils have 16 and 18 carbon atoms, and the most common are: C:16:0, C:18:0, C:18:1, C:18:2 and C:18:3 (Ohlrogge and Browse, 1995). In this project, the variations in the composition and the presence of fatty acids fluctuating according to the nutrient medium that they were exposed (Table 6).

In general, we found percentages of SFAs (C:16:0, C:18:0) higher in *Chlorella* sp., 25.15% and 17.22% cultivated in BG11 than in MOB medium; as for the MUFAs *Chlorella* sp. cultivated in BG11 contains C:16:1 and C:18:1 and the MOB was only found C:18:1 and finally the PUFAs are C:18:2, C:18:3 in both nutritive media and C:20:5 only in BG11.

In general, the biomass of the microalgae consortium showed a similar concentration of fatty acids in the cultures with BG11 and $MOB_H$ (Table 6); specifically, a higher concentration of PUFA's, with high values of C:18:2 (linoleic acid), MUFA's only with C:18:1 (oleic acid) and low concentrations of SUFA's. The biomass of *Chlorella* sp. culture with BG11 medium was the only biomass sample that recorded the presence of C:16:1 (palmitoleic acid); and increase the values of C:16:0 (palmitic acid) and C:18:3 (linolenic acid). On the other hand, the biomass of *Chlorella* sp. cultivated in the $MOB_H$ medium increased the concentration of oleic acid by 26% and linoleic acid by 139%, decreasing the same proportion of linolenic acid with respect to the biomass produced in the BG11 medium culture.

Table 6. Total Fatty acid composition (%) of microalgae consortium and *Chlorella* sp. grown in two media: BG11 and urine medium in high phosphorus concentration ($MOB_H$).

| Composition | Consortium | | *Chlorella* sp. | |
|---|---|---|---|---|
| | BG11 | $MOB_H$ | BG11 | $MOB_H$ |
| (C:16:0) | 11.4 | 10.6 | 20.2 | 11.6 |
| (C:18:0) | 4.2 | 5.07 | 4.96 | 4.96 |
| (C:16:1) | ND | ND | 10.1 | ND |
| (C:18:1) | 22.3 | 24.3 | 17.4 | 22 |
| (C:18:2) | 54.6 | 52.2 | 21.8 | 52.2 |
| (C:18:3) | 7.54 | 7.84 | 23.2 | 8.57 |
| (C:20:5) | ND | ND | 2.31 | ND |

The linolenic acid (C:18:3) and linoleic acid (C:18:2) are in the group of PUFA's because of their high saturations number. They commonly are named Omega 6 and 3. These acids play an important role in the cerebral function and many other benefits for health. This study showed that the consortium of microalgae and *Chlorella* sp. are an option for production essential fatty acids because both cultures have high concentrations of PUFA'S presented an average of 60% in its composition. Nowadays tests are being carried out to increase the concentration of fatty acids in microalgae through genetic engineering. However, in the results of their tests, they showed a concentration of PUFA's after the culture of *Chlorella* sp. in BG11 medium of 41.32% and after the treatment through the use of an enzyme, they managed to increase their concentration to 54.2% (Xue *et al*., 2016). In our investigation, were got values of PUFA's of 47.35% cultivating *Chlorella* sp. in BG11 medium and of 60.75% cultivating it in $MOB_H$. That represents a greater concentration without exposing microalgae to genetic engineering, reducing costs and avoiding exposure to genetic alterations.

## Conclusions

From the present study, it was concluded that human urine at a concentration of 0.15g/L ($MOB_H$) has a potential to be used as a nutrient medium since *Chlorella* sp. showed an increased production of fatty

acids (5,435%) compared to their production in other media. Regarding the production of saturated and monounsaturated fatty acids (SUFA's, MUFA's), the use of MOB induced a decrease of 31 and 20%, respectively, in relation to the use of the BG11 medium in the *Chlorella* sp. culture. The opposite effect was observed for the concentration of polyunsaturated fatty acids (PUFA's) which increased by 29%. Regarding biomass production in *Chlorella* sp. it was increased 51% in relation to the culture with BG11 medium, and the removal of P during the cultivation was 100%.

Furthermore, organic waste such as human urine induces the growth of microalgae with high concentrations of carbohydrates, proteins, and fatty acids; therefore, it can provide a valuable solution for the recovery of nutrients from wastewater and produce high-value biomass for the production of various products. It may be possible that the nutritive medium MOB can replace the BG11 medium in the cultivation of microalgae, achieving economically viable and environmentally friendly processes for the production of high value products.

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### *Nomenclature*

| | |
|---|---|
| TAG | Triacylglycerols |
| FAMEs | Methyl esters of fatty acids |
| BG11 | Nutritive medium |
| MOB | Urine medium |
| MBP | Micoalgae biomass production |
| V/Vm | Volume of air per volume of medium in one minute |
| SFAs | Saturated fatty acids |
| MUFAs | Monounsaturated fatty acids |
| PUFAs | Polyunsaturated fatty acids |
| ND | Not detected $BF_3$ |
| $BF_3$ | Boron trifluoride |

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