Vol. 19, No. 1 (2020) 133-146
Revista Mexicana de Ingeniería Química

OPTIMIZATION FOR THE PRODUCTION OF *Verrucodesmus verrucosus* BIOMASS THROUGH CROPS IN AUTOTROPHIC AND MIXOTROPHIC CONDITIONS WITH POTENTIAL FOR THE PRODUCTION OF BIODIESEL

OPTIMIZACIÓN PARA LA PRODUCCIÓN DE BIOMASA DE *Verrucodesmus verrucosus* A TRAVÉS DE CULTIVOS EN CONDICIONES AUTOTRÓFICAS Y MIXOTRÓFICAS CON POTENCIAL PARA LA PRODUCCIÓN DE BIODIESEL

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Received: January 11, 2019; Accepted: March 14, 2019

Abstract

In the present work *Verrucodesmus verrucosus* was evaluated in mixotrophic and autotrophic growth conditions as a suitable source of oil for biodiesel production, for this purpose synthetic media were evaluated and lixiviated coming from a biodigester. The evaluation of the kinetic parameters of growth was carried, so to, the nitrogen-lipid ratio was determined and lipid extraction. Finally, the fatty acid profile of the total pooled lipids was determined out using gas chromatography mass spectroscopy (GC-MS). The results demonstrate a cell density of 6.0×10^4 cells / mL with an autotrophic condition. On the other hand, the production of lipids of the microalgal biomass was 24.3% in the BG11 medium. The Fatty acid profile shows that the highest concentrations of fatty acids in *V. verrucosus* were palmitic, oleic, stearic, palmitoleic and linoleic with a concentration of 34.9, 22.8, 9.3, 7.0 and 6.8% respectively, indicating the suitability of this species for biodiesel production.

Keywords: Microalgal biomass, biodiesel, mixotrophic condition, autotrophic condition, fatty acids.

Resumen

En el presente trabajo *Verrucodesmus verrucosus* se evaluó en condiciones de crecimiento mixotrófica y autotrófica, como una fuente adecuada de aceite para la producción de biodiesel; para este propósito se evaluaron medios sintéticos y lixiviados de un biodigestor. Se realizó la evaluación de los parámetros cinéticos de crecimiento, así pues, se determinó la relación nitrógeno-lípidos y la extracción de lípidos. Finalmente, se determinó el perfil de ácidos grasos de los lípidos totales mediante cromatografía de gases con espectroscopía de masas (GC-MS). Los resultados demuestran una densidad celular de $6{,}0 \times 10^4$ células / ml en condición autotrófica. Por otro lado, la producción de lípidos de la biomasa microalgal fue de 24.3% en el medio BG11. El perfil de ácidos grasos muestra que las concentraciones más altas de ácidos grasos en *V. verrucosus* fueron palmítico, oleico, esteárico, palmitoleico y linoleico con una concentración de 34.9, 22.8, 9.3, 7.0 y 6.8% respectivamente, lo que indica la aptitud de esta especie para la producción de biodiesel.

Palabras clave: biomasa microalgal, biodiesel, condición mixotrófica, condición autotrófica, ácidos grasos.

1 Introduction

In recent decades, increasing efforts have been made to apply technologies based on microalgae for a more sustainable production of many different compounds used in the biofuel industries (Sacramento-Rivero *et al*., 2010; Barclay and Apt, 2013; Benvenuti *et al*., 2015; Gao *et al*., 2019). Effect of organic carbon to nitrogen ratio in wastewater on growth, nutrient uptake and lipid accumulation of a mixotrophic microalgae Chlorella sp. Bioresource Technology.), because the microalgae are an important feedstock due to they are organisms the fastest growing in the world, with up to 90% of their weight made up of carbohydrate, protein and oil (Richmond and Hu, 2013; Wen *et al*., 2019).

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https://doi.org/10.24275/rmiq/Bio463
issn-e: 2395-8472

Microalgae have the ability to grow and do photosynthesis with different sources of nutrients such as mineral salts, under autotrophic conditions and organic substances (such as manures and wastewater), under mixedotrophic conditions. Currently, efforts are being made for the non-phototrophic cultivation of microalgae (Chinnasamy, 2010; Zhan *et al*., 2017).

Additionally, some microalgae can grow under heterotrophic conditions, using organic carbon in the absence of light (Xu *et al*., 2006; León-Vaz *et al*., 2019), this metabolic plasticity allows them to adapt to different ecosystems and biotechnological processes, generating biomass that can be used in the production of biofuels, Studies have shown that many phototrophic microalgae can grow under heterotrophic or mixotrophic conditions (Chisti, 2007; Skorupskaite *et al*., 2015).

One of the main limitations of the industrial-scale cultivation of microalgae is the cost of the components of the culture medium, which generally contains inorganic fertilizers derived from petroleum. The increase in the prices of inorganic fertilizers and their negative environmental impact have resulted in the search for alternative sources of nutrients for photosynthetic organisms such as microalgae. Therefore, worldwide attention has turned towards the use of organic materials from various sources such as fertilizers (Ortiz-Moreno *et al*., 2012; Wang *et al*., 2017). Within the mixotrophic media are animal waste, in its liquid form, have a long history of use as a source of phosphorus, nitrogen and carbon, therefore they can be used as for microalgal growth, due to the mixotrophic medium provides necessary nutrients, such as nitrogen and phosphorus, and water for microalgal growth (Knud-Hansen, 1998; Venglovsky *et al*., 2006; Han *et al*., 2017).

The species *Verrucodesmus verrucosus* or *Snedesmus verrucosus* (Hegewald *et al*., 2013), a freshwater species has oval, elongated cells, forming cenobios 4 to 8 cells so far has not been evaluated in the area of bioenergy, it is because of that, in order to evaluate the production of lipids in an autotrophic and mixotrophic condition, in the present work we evaluated the efficiency of *Verrucodesmus verrucosus* (Roll) in Cultures medium enriched with lixiviated from biodigester and synthetic media such as: BG11, Suoeka and Guillard.

2 Materials and methods

The experiment was established in the Universidad Politécnica de Chiapas, located in the city of Tuxtla Gutiérrez, Chiapas, México. The geographic location was latitude 16° 45' 11" north and longitude 93° 06' 56", corresponding to tropical regions, with more than 1100 mm annual rainfall. During the experimental period the temperature of the greenhouse was maintained to 28 ºC, the air relative humidity was maintained at 60-65% with the objective of evaluating the efficiency of *Verrucodesmus verrucosus* (Roll) in Cultures medium enriched with lixiviated from vermicompost and synthetic medium such as: BG11, Suoeka and Guillard for the production of lipids destined for the production of biodiesel.

2.1 Algal sample

The algae sample *V. verrucosus* belongs to the collection of cultures of the laboratory of applied science UAM (Universidad Autonoma Metropolitana Iztapalapa). Immediately, it was brought to laboratory. A part of sample was observed with microscope for identification, then analysed to determine the size frequency of algal cells per ml of suspension using a FlowCam (Fluid imaging Technologies), the sizes were expressed as equivalent length (EL) (Sieracki *et al*., 1998). The algal species was morphologically same as *Verrucodesmus verrucosus*. Were identified microscopically according to Prescott, (1973); Sheath and Wehr, (1973). The microalgae species were morphologically validated; the latin scientific name and class were confirmed in the database of AlgaeBase (http://www.algaebase.org/) and database of NCBI (https://www.ncbi.nlm.nih.gov/pubmed/).

2.2 Culture mediums in autotrophic condition

The Suoeka medium is composed of the following elements: KH_2PO_4 8.3Mm; K_2HPO_4 5.3Mm; $MgSO_4.7H_2O$ 0.25 Mm; $CaCl_2.2H_2O$ 0.133Mm; Traces Hutner 1x; NH_4Cl 9.35Mm. Traces Hutner 200x: 12.7g of EDTA- $Na.2H_20$ in 250ml of water, were mixed one by one in the following order in 500ml of water at 100 ºC: 2.28g of H_3BO_3; 4.40g of $ZnSO_4.7H_2O$; 1.02g of $MnCl_2.4H_2O$; 1.00g of $FeSO_4.7H_2O$; 0.32g of $CoCl_2.6H_2O$; 0.32g of $CuSO_4.5H_2O$; 0.22g of $(NH_4)MoO_{24}.4H_2O$. The above solutions were mixed, brought to 100 ºC and

the pH adjusted to 6.8 with 20% KOH. (A temperature below 70 ºC) (Yang *et al*., 2016).

The Guillard medium contains: 1ml of solution containing 0.08g of $CoCl_2.6H_2O$ and 0.8g of $CuSO_4.5H_2O$ in 100ml of distilled water; which was added to a 800ml solution with: 0.2g of $FeCl_3.6H_2O$, 0.06g of $ZnSO_4.7H_2O$, 0.12g of $MnSO_4.H_2O$, 0.03g of $Na_2MoO_4.2H_2O$; 1ml of 1.2g EDTA-Na solution in 900ml of distilled water. The pH was adjusted to 7.0 and 10g of KNO_3 and 1.4g of KH_2PO_4 were added. It was brought to final volume of 1 liter at 100 ºC (De godos *et al*., 2016).

The BG11 medium contains: Na_2Mg EDTA 0.1g, $(NH_4)5[Fe(C_6H_4O7)2]$ 0.6g, $HOC(COOH)(CH_2COOH)_2.H_2O$ 0.6g, $CaCl_2.2H_2O$ 3.6g, $MgSO_4.7H_2O$ 7.5g, $K_2HPO_4.3H_2O$ 4g, K_2HPO_4 3.05g, H_3BO_3 2.86g, $MnCl_2.4H_2O$ 1.8g, $ZnSO_4.7H_2O$ 0.22g, $CuSO_4.5H_2O$ 0,07g, $COCl_2.6H_2O$ 0.05g, $NaMoO_4.2H_2O$ 0.39g, MoO_4 0.018g, Na_2CO_3 0.02g, and $NaNO_3$ 1.5g were added, the pH was adjusted to 7.5. It was brought to final volume of 1 liter at 100 ºC (Rippka *et al*., 1979).

2.3 Culture medium in mixotrophic condition: Preparation of the lixiviated

The lixiviated was provided by the Centro de Investigación y Desarrollo Tecnológico en Energía Renovables, located in the municipality of Tuxtla Gutiérrez, Chiapas, Mexico at 16 ° 45'11" north latitude and 93 ° 06'56" west longitude. The resulting of the liquid lixiviated was allowed to ferment for 8 days in an open container covered with a cloth, this is done to favor aerobic degradation by its accompanying microflora. The lixiviated obtained was filtered with the aid of a vacuum pump with Whatman filter paper number 1 of 0.45 μm. The filtrate was added in doses of 30, 50 and 80% in the culture medium (Suoeka, Guillard and Remital) and sterilized in an autoclave at 121 ºC for 20 minutes (Rosales *et al*., 2007).

2.4 Characterization of lixiviated

In preparation of samples for chemical analysis proximal Organic Carbon (OC), ash content and Organic matter (OM) was determined by a muffle furnace at 550 °C (TMECC 2002; Taipale *et al*., 2016; Yağci *et al*., 2016) Total nitrogen (N) was analysed by Kjeldahl method (AOAC 2000; Li *et al*., 2015). P contents were analyzed by colorimetric analysis in a spectrophotometer using the molybdate reagent ascorbic acid-ammonium (Shamim *et al*., 2015). Thus, the potential of hydrogen (pH) with the use of a potentiometer (HANNA HI 2211-01) was also determined, according to Konca *et al*. (2016). Ammonium was measured using Hach kits (Hach, Loveland, CO, USA), per standard methods 8155, total suspended solids (TSS) in water can be used to easily evaluate (Nasrabadi *et al*., 2018) and finally the turbidity was determined according to Momeni *et al*. (2018).

2.5 Growth kinetics and biomass estimation

The microalgal species under study were characterized for growth kinetics and biomass production potential to identify those which grew fast and accumulate higher biomass. The culture was aerated by air pump with 0.22 μm sterilized air filter to avoid settling and sticking to the surface of the flask. Cellular concentration was determined at the end of the experiments using a Neubauer hemacytometer (Hausser Scientific, Horsham, USA) and a Primo Star microscope (Carl Zeiss, Oberkochen, Germany). The cells were harvested by centrifugation and dried at 70 ºC. Growth rate parameters of each isolate were calculated on the basis of cell count during exponential phase of growth and cell biomass (Contreras *et al*., 2016). Following growth parameters were calculated from the observed data (Wang *et al*., 2010).

- Specific growth rate (μ): $\mu = \ln(Nt/No)/Tt - To$ where Nt is the number of cells at the end of log phase, No is the number of cells at the start of log phase, Tt is the final day of log phase and To is starting day of log phase.
- Doubling time: $Tt = 0.6931/\mu$.
- Biomass productivity (Pdwt): as the dry biomass produced per day (g l^{-1} day^{-1}).

2.6 Oil extraction from Verrucodesmus verrucosus

The algae was oven dried at 70ºC, after drying it was ground and sieved. Then 2g of dried algae was tied in filter bag and that filter bag was loaded in extraction chamber of Soxhlet apparatus. The extraction chamber was kept over a boiling flask containing 50ml of extraction solvent. Hexane was used as solvent. The solvent was allowed to reflux at 60 ºC. After a certain retention period, and many cycles the algal oil was concentrated in the boiling flask with hexane. After

the algal oil collection, hexane was removed from the oil by rotary evaporator. The flask with the oil and without oil was weighed to determine the amount of oil obtained (O'Fallon *et al*., 2007; Soto-León *et al*., 2014; Memon *et al*., 2016).

2.7 *Fatty acid profile of total lipids of Verrucodesmus verrucosus*

Fatty acid profile analysis of species wise pooled total lipids isolated from *Verrucodesmus verrucosus* was carried out using gas chromatography mass spectroscopy (GC-MS). Fatty acid methyl esters were prepared using following procedure: 30 mg of total lipid dissolved in 1 ml of methanol was mixed with 1 ml of 12% solution of KOH prepared in methanol. To this solution equal volume of 5% HCL in methanol was added and heated at 75 ºC for 15 min. This solution was allowed to cool and 1 ml of distilled water was added and shaken. Upper organic layer containing fatty acid methyl esters was carefully transferred to a new clean vial. GC-MS analysis of FAMEs was performed using diethylene glycol succinate capillary column (30m $\times$ 0.25 $\times$ 0.25 μm). 100 μl of methyl ester sample solution was injected for each analysis. Helium was used as a carrier gas. The injector temperature was 180 ºC and detector temperature was 230 ºC which was increased to 300 ºC at a temperature gradient of 15 ºC/min (Härtig, 2008).

2.8 *Statistical analysis*

To determine significant differences between the treatments evaluated the one way ANOVA test was carried out at p<0.05 level of significance; the statistical software used was the STATGRAPHICS PLUS (1999) for windows. For the first experiment, prior to statistical analysis, data were assessed three replicate experiments (Gao *et al*., 2018; Gonzalez-Fernandez *et al*., 2018; Kato *et al*., 2019), for equality of variance and normality. A Tukey test was carried out following to compare means of treatments.

3 Results and discussion

3.1 *Evaluation in autotrophic conditions: Analysis of the cell density and growth kinetic parameters*

The growth of *Verrucodesmus verrucosus* was evaluated in autotrophic conditions with the use of BG11, Guillard and Suoeka medium for 8 weeks with 12:12 light / dark cycles. In fig. 1 was observed that the Suoeka medium remains in an adaptation stage for 25 days unlike the other medium where the adaptation stage was less than 10 days.

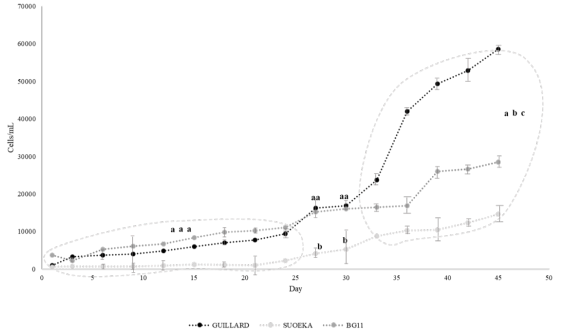

Fig. 1. Evaluation of the growth kinetics of *Verrucodesmus verrucosus* under conditions of autotrophic growth. The averages of three repetitions (± standard error) for each point, common letters do not differ significantly at $P < 0.05$.

Table 1. Kinetic growth parameters of *V. verrucosus* in different culture media under autotrophic conditions.

Culture mediums	Cell density at the beginning of the exponential phase (Cell/ml)	Cell density at the end of the exponential phase (Cell/ml$\times 10^4$)	Growth rate (Generations/Day)	Generation of time (Day)	Generation number
Guillard	1042 ± 0.02^b	*6.0 ± 0.01^a	0.90 ± 0.01^a	7.56 ± 0.11^a	6.81 ± 0.02^a
Suoeka	625 ± 0.01^c	1.4 ± 0.02^c	0.78 ± 0.02^b	6.52 ± 0.05^b	5.90 ± 0.01^b
BG11	3750 ± 0.12^a	2.8 ± 0.11^b	0.64 ± 0.04^c	5.37 ± 0.06^c	4.84 ± 0.03^c

* Mean of three repetitions. **The averages (± standard error) within each column without common superscript differ significantly at $P < 0.05$.

The culture medium evaluated showed the stage of exponential growth at 13 days, so before day 30 no significant statistical differences were demonstrated between the three culture medium evaluated, however, from day 33 the Guillard medium showed differences significant statistics producing a biomass of 6×10^4 cells / mL, as demonstrated by Yang *et al*. (2019), they evaluated Scenedesmus acuminatus who reached a population increase of 1.44×10^5 cell in a synthetic culture medium (Mahesh *et al*., 2019) (Table 1).

In this first test the growth kinetics of the culture medium that generate an autotrophic condition was analyzed, meaning that significant differences were identified between the treatments with a $P = 0.005$ value in the ANOVA. Dunnet's multiple comparison tests indicated that the Guillard medium outperformed the other nutrient sources (Table 1). The maximum cell density for the Guillard medium was 6.0×10^4 cells / mL with a generation time of 7.56 days and a specific growth rate of 0.90 generations / day surpassing the Sueoka and BG11 medium.

Verrucodesmus is a genus of *chlorophycea* microalgae that is characterized by rapid growth in cell culture, so it has the ability to use organic and inorganic compounds as a nutritional substrate (Wehr and Sheath, 2003; Xu *et al*., 2006; Chinnasamy, 2010).

Considering that during the autotrophic evaluation the Guillard medium was the one that reached the highest cellular density (Fig. 1), this efficiency is in agreement with that reported by Ortiz-Moreno *et al*. (2012) who evaluated the production of Chlorella sorokiniana in an autotrophic condition, and therefore recommended the use of Guillard in low concentrations (1g / L) as the best option for the mass production of microalgal biomass.

3.2 Characterization of lixiviate and mixotrophic growth condition: Analysis of cell density and growth kinetic parameters

The bromatological analysis of the lixiviate from a biodigester proved to be a source of nutrients that contributes 0.43% of nitrogen in the form of protein and 0.12% of minerals in the form of ash (Table 2). The physicochemical analysis of the leachate showed an acid pH (6.5), with a high content of total solids (1116 mg / L) and high turbidity (5720 NTU).

Table 2. Bromatological analysis of the biogas lixiviate.

Parameter	Units
Nitrogen	0.43%
Ammonium	0.03g/L
Phosphorus	0.02g/L
Ash	0.12%
O.C	7.40%
O.M	12.76%
C/N	17.22
Turbidity	5720 NTU
TS	1116mg/L
pH	6.5

NTU: Nephelometric Turbidity Units; O.C: Organic carbon; O.M: Organic matter; C/N: Carbon nitrogen ratio; pH: Potential of hydrogen; TS: Total solids.

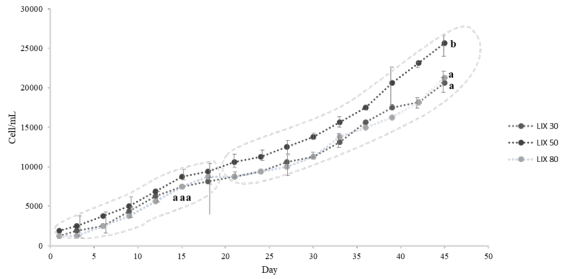

Fig. 2. Evaluation of the growth kinetics of Verrucodesmus verrucosus under mixed-trophic growth conditions. LIX 30: Lixiviate at 30%; LIX 50: Lixiviate at 50%; LIX 80: Lixiviate at 80%. The averages of three repetitions (± standard error) for each point, common letters do not differ significantly at $P < 0.05$.

The fig. 2 shows that lixiviate at a concentration of 50% achieves higher production of microalgal biomass (2.5×10^4 cells / mL), however, no significant statistical difference was shown with culture medium enriched with lixiviate at 30 and 80%. In the second trial, the growth kinetics of the culture medium that generate a heterotrophic condition were compared, identifying significant differences between the culture media with 30% and 50% lixiviate, as well as between the culture medium at a concentration of 80% and 50% of lixiviate. Dunnet's multiple comparison tests indicate that the medium enriched by the 50% lixiviate far outperformed to the others culture mediums (Table 3).

Table 3. Kinetic growth parameters of *V. verrucosus* in different culture media under heterotrophic conditions.

Culture mediums	Cell density at the beginning of the exponential phase (Cell/ml)	Cell density at the end of the exponential phase (Cell/ml×10^4)	Growth rate (Generations/Day)	Generation of time (Day)	Generation number
LIX 30	1250 ± 0.04^b	*2.06 ± 0.11^b	1.38 ± 0.10^b	5.10 ± 0.01^b	4.04 ± 0.01^b
LIX 50	1875 ± 0.01^a	2.56 ± 0.02^a	1.93 ± 0.04^a	7.64 ± 0.02^a	5.64 ± 0.02^a
LIX 80	1250 ± 0.02^b	2.12 ± 0.10^b	1.08 ± 0.03^c	3.63 ± 0.03^b	3.16 ± 0.01^b

* Mean of three repetitions. **The averages (± standard error) within each column without common superscript differ significantly at $P < 0.05$. LIX 30: Leached at 30%; LIX 50: Leached at 50%; LIX 80: Leached at 80%.

The maximum cell density for the medium with 50% lixiviate was 2.56 ×10^4 cells/ml with a generation time of 7.6 days and a specific growth rate of 1.9 generations/day, cellular density similar to that reported by León-Vaz *et al*. (2019). This may be due to the difference in the nutrient content of the lixiviate, since liquid manures have a high heterogeneity depending on their origin (Groeneweg and Schluter, 1981; Travieso *et al*., 2006).

In the industrial production of microalgae biomass it is necessary to use a culture medium that offers the necessary nutrients for the adequate growth of algae at low cost. Lixiviate are a viable alternative to reduce costs in the cultivation of microalgae, but these substrates must be suitably pretreated to obtain the desired results (Rosales *et al*., 2007; Kumar *et al*., 2016).

However a very low concentration of phosphorus was identified which limits the production of biomass as it is a fundamental element in many cellular processes, such as the formation of nucleic acids and energy transfer (Grobbelaar, 2004) their deficiency in the culture medium is one of the greatest limitations to growth. It is important to mention that *V. verrucosus* was adapted under mixotrophic conditions, so its metabolic plasticity allows it to adapt to different ecosystems and biotechnological processes, generating biomass (Chisti, 2007). Greater efficiency was observed in the chemical means of autotrophic condition, this is due to the point of photoinhibition since the saturation of solids in the medium is a condition that is harmful to the same algal cell causing even death, implying loss of efficiency photosynthetic and productivity of the crop in terms of biomass (Tomaselli and Richmond, 2004). So also Wen *et al*. (2019) mentions that other problem with de mixotrophic medium are heterotrophic bacterial contamination and inefficient conversion of organic carbon.

3.3 Interaction of autotrophic and mixotrophic medium: Analysis of cell density and growth kinetic parameters

The Guillard medium showed higher production of microalgal biomass (4.2×10^4 cells / mL) enriched with the lixiviate in the three concentrations (Fig. 3), however there is no significant statistical difference between the treatments that use the Guillard medium.

It is important to mention that when enriching the Guillard medium with the 50% lixiviate, microalgal biomass production increased by 28% compared to the culture medium that only includes the lixiviate, however, thus it was also observed that pure Guillard medium is more efficient (Table 4).

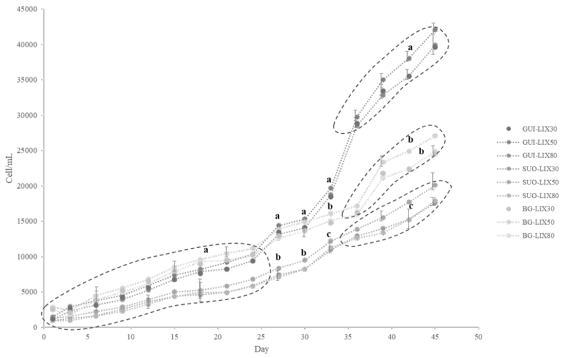

Fig. 3. Evaluation of the growth kinetics of Verrucodesmus verrucosus under mixed-tropic growth conditions. GUI: Guillard; SUO: Suoeka; BG: BG11; LIX 30: Lixiviate at 30%; LIX 50: Lixiviate at 50%; LIX 80: Lixiviate at 80%. The averages of three repetitions (± standard error) for each point, the different letters differ significantly at $P < 0.05$.

Table 4. Kinetic growth parameters of *V. verrucosus* in different culture media under heterotrophic and autotrophic conditions.

Culture mediums	Cell density at the beginning of the exponential phase (Cell/ml)	Cell density at the end of the exponential phase (Cell/ml×10^4)	Growth rate (Generations/Day)	Generation of time (Day)	Generation number
GUI-LIX30	1146 ± 0.08^b	*3.95 ± 0.10^a	0.94 ± 0.01^a	5.23 ± 0.02^{ab}	4.85 ± 0.04^{ab}
GUI-LIX50	1458 ± 0.12^b	4.20 ± 0.01^a	1.25 ± 0.07^a	5.55 ± 0.01^{ab}	5.11 ± 0.02^a
GUI-LIX80	1146 ± 0.01^b	3.98 ± 0.04^a	1.05 ± 0.02^a	4.45 ± 0.01b^c	4.12 ± 0.01^b
SUO-LIX30	937 ± 0.02^c	1.76 ± 0.10^c	0.83 ± 0.10b	3.68 ± 0.05^d	3.39 ± 0.07^c
SUO-LIX50	1250 ± 0.01^b	2.01 ± 0.02^c	0.95 ± 0.01^a	4.21 ± 0.10b^c	3.88 ± 0.10^{bc}
SUO-LIX80	937 ± 0.04^c	1.79 ± 0.11^c	1.06 ± 0.11^a	4.65 ± 0.11^{ab}	4.30 ± 0.01^b
BG-LIX30	2500 ± 0.17^a	2.45 ± 0.12 ^{c}	0.77 ± 0.10^b	3.41 ± 0.01^d	3.14 ± 0.10^c
BG-LIX50	2812 ± 0.01^a	2.70 ± 0.05^b	0.99 ± 0.01^a	4.38 ± 0.06^{bc}	4.04 ± 0.02^b
BG-LIX80	2500 ± 0. 13^a	2.48 ± 0.07^b	1.01 ± 0.01^a	4.47 ± 0.11b^c	4.12 ± 0.11^b

* Mean of three repetitions. ** The averages (± standard error) within each column without common
* superscript differ significantly at $P < 0.05$. GUI: Guillard; SUO: Suoeka; BG: BG11; LIX 30: Leached
* at 30%; LIX 50: Leached at 50%; LIX 80: Leached at 80%.

The aforementioned phenomenon is due to the physiological and metabolic adaptations that the microalga faces for the incorporation of organic compounds present in the environment (Xu *et al*., 2006; Chinnasamy *et al*., 2010).

Based on the results reported in the present research work, it was demonstrated that the means of synthetic crops allow the highest production of microalgal biomass, this is corroborated with the results obtained by Muñoz (2009) and Ortiz-Moreno *et al*. (2012) who, when evaluating *C. sorokiniana* found that the lixiviate of gallinaza did not allow an increase in cell density comparable to the inorganic medium Remital and Guillard. This may be related to the quality of nutrients provided by the culture medium (Moronta *et al*., 2006), as well as related to the availability of nutrients used in the mixotrophy and autotrophy, that is, if in the mixotrophic culture medium they use easily metabolizable substances and in the autotrophic one low levels of nitrogen and phosphorus, consequently the growth of microalgae will be favored in the mixotrophic culture medium or vice versa (Xu *et al*., 2006; Kumar *et al*., 2016).

This behavior is due to the ability to capture light to perform photosynthesis and develop different metabolic processes, as it proved Kato *et al*. (2019) Biomass yield under the LD (Light/dark) condition was significantly higher than that under the LL (Continuous light) condition, since, dynamic metabolic profiling showed higher levels of lipid/carbohydrate anabolism (including production of 3-phosphoglycerate, fructose 6-phosphate, glucose 6-phosphate, phosphoenolpyruvate and acetyl-CoA) from CO_2 under the LD condition, indicating higher CO_2 fixation than that of the LL condition.

3.4 Evaluation of total lipid production

In order to evaluate the potential of the culture medium for the production of lipids in the microalgal biomass, the results showed a significant statistical difference between the crop media evaluated, highlighting the BG11 medium as the best, since it can produce up to 24.3% of lipids, thus it is also observed in figure 6 that the medium enriched with 80% lixiviate is the most inefficient medium because producing 5.5% of lipids.

In order to evaluate the potential of the culture medium for the production of lipids in the microalgal biomass, the results shown in the present research work are corroborated with the work of Garibay *et al*. (2009), they get the average production of lipids by microalgae. The autotrophic condition allowed the highest accumulation of lipids as reported by Purkayastha *et al*. (2017) who analyzed several autotrophic media, Bold's basal medium (BBM), Wright's cryptophyte medium (WC), closterium medium (C) and blue-green medium (BG11). However, it was shown that the BG11 medium was the most efficient for the production of *C. ellipsoid* lipids with a concentration of 24.1%.

3.5 Evaluation of the lipid-nitrogen ratio

The nitrogen concentration in the different culture medium was evaluated, meaning that significant

statistical differences were identified by locating the highest concentration of nitrogen (0.42%) in the culture medium enriched with 80% lixiviate, followed by the Guillard medium with 0.37% nitrogen, it is important to mention that the media enriched with the 80% lixiviate showed a significant increase of nitrogen in the medium, however the BG11 medium showed the lowest concentration of nitrogen with 0.23% (Fig. 5).

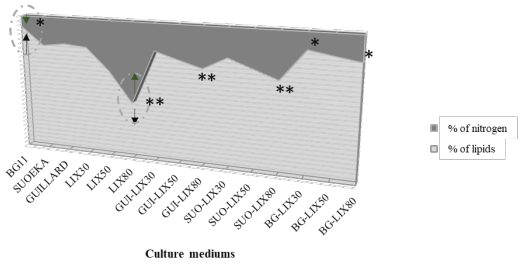

Fig. 4. Quantification of the lipid content in the microalgal biomass of V. verrucosus evaluated in mixotrophic and autotrophic conditions. The averages (± standard error) of three repetitions for each column, the different letters differ significantly at $P < 0.05$. GUI: Guillard; SUO: Suoeka; BG: BG11; LIX 30: Lixiviate at 30%; LIX 50: Lixiviate at 50%; LIX 80: Lixiviate at 80%.

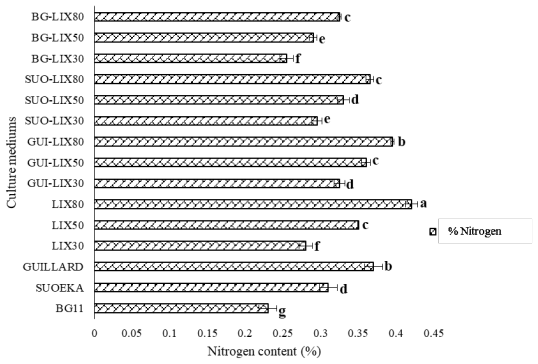

Fig. 5. Evaluation of the lipid-nitrogen ratio in the microalgal biome of V. verrucosus evaluated in mixotrophic and autotrophic conditions. The averages (± standard error) of three repetitions for each column, the different letters differ significantly at $P < 0.05$. GUI: Guillard; SUO: Suoeka; BG: BG11; LIX 30: Lixiviate at 30%; LIX 50: Lixiviate at 50%; LIX 80: Lixiviate at 80%.

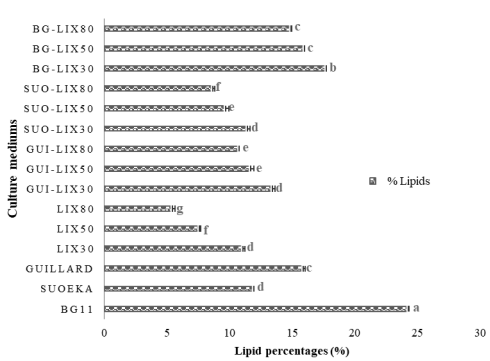

Fig. 6. Analysis of lipid-nitrogen interaction in the microalgal biomass of V. verrucosus evaluated under mixotrophic and autotrophic conditions. The averages (± standard error) of three repetitions for each column, the different letters differ significantly at $P < 0.05$. GUI: Guillard; SUO: Suoeka; BG: BG11; LIX 30: Lixiviate at 30%; LIX 50: Lixiviate at 50%; LIX 80: Lixiviate at 80%.

To identify the relationship between the content of nitrogen and the lipid content in the microalgal biomass, a correlation analysis was carried out between both variables, so it is observed that at lower nitrogen concentration, a greater accumulation of lipids is reported, such is the case of the culture medium BG11 with a nitrogen concentration of 0.23% and a lipid content of 24.30% (Fig. 4). It is also identified that at a higher concentration of nitrogen, less lipid accumulation, as demonstrated in the medium enriched with 80% lixiviate which contains a nitrogen concentration of 0.42% with a lipid accumulation of 5.54% (Fig. 5 and Fig. 6). It is now known that nitrogen is also a critical factor for regulating the lipid content of microalgae (Loera and Olguín, 2010).

So that the medium enriched with the 80% leachate showed a significant increase of nitrogen and a decrease in the production of lipids, this behavior was verified by Ho *et al*. (2012) and Ho *et al*. (2013) when corroborating that nitrogen being a limiting factor of the culture medium, the accumulation of lipid levels increases by more than 40%.

The data obtained in the present work agree with those established by Cobos-Ruíz *et al*. (2016) who evaluated the production of total lipids in five species of microalgae induced by the absence of nitrogen. The microalgae used were *Ankistrodesmus sp., Ankistrodesmus nannoselene, Chlorella* sp., *Scenedesmus* sp. and *Scenedesmus quadricauda*. The results indicated that the species with the

highest production of total lipids were *Ankistrodesmus* sp. (263.6 mg / g dry biomass), *A. nannoselene* (316 mg / g dry biomass) and *Scenedesmus* sp. (243.3 mg / g dry biomass) when grown in medium without nitrogen.

The results of this research work agree with that reported by Ho *et al*. (2011) and Radakovits *et al*. (2012) who conclude that limiting nitrogen concentrations allow to stimulate the accumulation of lipids in algae cells, however it impacts on the reduction of algae biomass, which suggests that the 2 conditions, high lipid content and high productivity, they can be mutually exclusive.

3.6 Fatty acid profile of total lipids of Verrucodesmus verrucosus

Furthermore, the neutral lipid evaluation on *Verrucodesmus verrucosus* allowed the retention of 11 fatty acid to further characterization (Table 5). The Fatty acid profile shows that the highest concentrations of fatty acids in *V. verrucosus* were palmitic, oleic, stearic, palmitoleic and linoleic with a concentration (34.9, 22.8, 9.3, 7.0 and 6.8% respectively), indicating the suitability of this species for biodiesel production. Finally, gas chromatography revealed that of the 11 fatty acid identified was characterized by a high level of saturated fatty acids (63.6%), higher amount of monounsaturated fatty acids (18.1%), lower level of polyunsaturated and triunsaturated fatty acids (9.0%), which would make it an interesting candidate for biofuel production (Fig. 7). In the present work, 11 fatty acids were identified, of which 63.6% corresponds to saturated acids, as demonstrated by Gnouma *et al*. (2018), when evaluating the production of fatty acids in three microalgae chlorophytes.

Also in the present work it is mentioned that the maximum concentration was of palmitic acid, saturated fatty acid with a 34.90% of presence, as demonstrated by Nayak and Mohanty (2016), who mention that, the microalga *Snedesmus* sp. contained a high percentage of saturated fatty acids from 46.2% to 49.5%, demonstrating to the palmitic acid as the highest concentration in *Snedesmus* sp., microalga that corresponds to the same family of *V. verrucosus*. The high saturated fatty acid me-thyl ester (FAME) indicated them to be a source of biodiesel with higher oxidation stability (Nascimento *et al*., 2013).

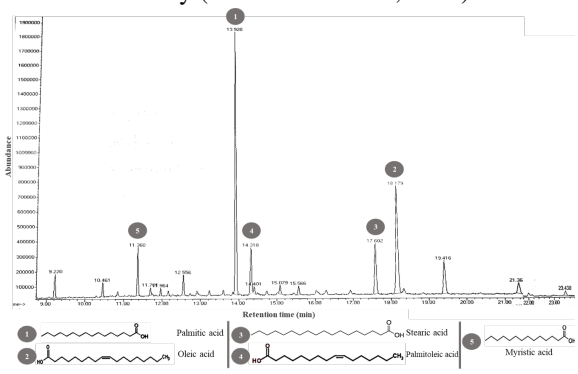

Fig. 7. Chromatogram of fatty acid profile analysis from *Verrucodesmus verrucosus* in medium BG11 using gas chromatography mass spectroscopy (GC-MS).

Table 5. Identification of the compounds present in the chromatogram of fatty acid profile analysis from *Verrucodesmus verrucosus* in medium BG11 using gas chromatography mass spectroscopy (GC-MS).

N.	Compounds	Chemical Formula	Parent ion (m/z)[2]	RT[1] (min)	Percentage of presence (%)	Fatty acid
1	Lauric acid	$C_{12}H_{24}O_2$	200,32	9.22	2.79	Saturated
2	Myristic acid	$C_{14}H_{28}O_2$	228,36	11.36	5.03	Saturated
3	Pentadecyl Acid	$C_{15}H_{30}O_2$	242,40	12.55	2.4	Saturated
4	Palmitic acid	$C_{16}H_{32}O_2$	256,43	13.92	34.9	Saturated
5	Palmitoleic acid	$C_{16}H_{30}O_2$	254,41	14.31	7.07	Monounsaturated
6	Enanthic acid	$C_7H_{14}O_2$	130.18	15.56	1.24	Saturated
7	Stearic acid	$C_{18}H_{36}O_2$	284,48	17.6	9.31	Saturated
8	Oleic acid	$C_{18}H_{34}O_2$	282.47	18.17	22.81	Monounsaturated
9	Linoleic acid	$C_{18}H_{32}O_2$	280,45	19.41	6.81	Polyunsaturated
10	α-Linolenic acid	$C_{18}H_{30}O_2$	278,44	21.36	2.4	Triunsaturated
11	Arachidic acid	$C_{20}H_{40}O_2$	312.53	23.43	1.09	Saturated

[1]RT: retention time, [2]Parent ion (m/z): molecular ions of the standard compounds (mass to charge ratio).

Conclusions

In the present research work the Guillard culture medium presented the best conditions for the growth of *V. verrucosus*, compared with the BG11, Sueoka (autotrophic condition) and leached (mixotrophic condition) media. The culture medium BG11 allowed the highest accumulation of lipids in *V. verrucosus* with 24.3% in a period of 60 days, identifying to the palmitic acid how the more abundant fatty acid with a 34.9%. Finally, the adequate concentration of nitrogen in a culture medium for the production of *V. verrucosus* with lipid purpose should be 0.23%.

Acknowledgements

The authors gratefully acknowledge to the 3IER, UPCH, for financial support.

Abbreviations

TAG	triacylglycerides
FAME	methyl esters of fatty acids
GC-MS	gas chromatography mass spectroscopy
NTU	nephelometric Turbidity Units
OC	organic carbon
OM	organic matter
C/N	carbon nitrogen ratio
pH	potential of hydrogen
TS	total solids
LIX 30	Lixiviate at 30%
LIX 50	Lixiviate at 50%
LIX 80	Lixiviate at 80%
GUI	Guillard
SUO	Suoeka
BG	BG11

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