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Title: Lipid profile in a strain of the Dunaliella tertiolecta microalgae and its potential for biodiesel production

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Introduction

In Mexico, despite the efforts to reduce polluting atmospheric emissions and promote sustainability and sustainability through the generation of energy from renewable sources, the national energy balance published in 2019 by the Secretary of Energy (SENER, the acronyms in Spanish) reports that the country ranks 12th in imports of gasoline and diesel, which shows that each year it costs more to achieve energy independence. Regarding total consumption, the most intensive sector in the use of energy is transport followed by industrial, residential-commercial-public and finally agriculture, with diesel being the oil that is consumed in three of the four sectors (agriculture, livestock, transportation and industrial, according to consumption order).

Sulfur as such is a pollutant, much more if it is combined with oxygen, such as the sulfur dioxide generated in the combustion that occurs in vehicle type diesel, therefore the global strategy is to achieve a significant reduction in the air pollution from low sulfur content in fossil fuels and from there, the considerable reduction in emissions of other pollutants (carbon oxides, nitrogen oxides, non-methane hydrocarbons, particles, etc.) and avoid not only damage to health but also to terrestrial and aquatic ecosystems due to the effects of acidification of the soil and water.

In bioenergy, Mexico proposes obtaining biodiesel, with guidelines established in the complement to the Law for Promotion and Development Bioenergetics, in this sense, many species of microalgae such Chlorella, Nannochloropsis, Dunaliella, among others, have become the new raw materials for research in biodiesel production given the characteristics they possess in comparison with energy crops and residual animal fats such as not depending on a season of the year for their production, biomass production in a relatively short time and the lipid fraction containing in the cell wall that allow a profile of monounsaturated and polyunsaturated fatty acids Transesterifiable to achieve a diesel fuel with high impact physicochemical parameters in an engine such as oxidative stability, cloud point, cetane number, viscosity (Moreira, 2015) and from there, then generate pilot projects for future installations that can be established in Mexico from microalgae.

Introduction

In this work, the fatty acid profile of a strain of the microalgae *Dunaliella tertiolecta* is shown and from there its energy potential for the production of biodiesel in the region of Mazatlán, Sinaloa, Mexico is described, since said microalgae has characteristics such as growth adaptation in saline, sweet or brackish environments and high adaptability to extreme temperatures that are common characteristics in the region, in addition, most of the literature refers to its use for the biotechnology area and few have carried out research for biodiesel production.

Methodology

1.- Microalgae: to carry out the tests, an inoculum taken from a strain of the microalgae *Dunaliella tertiolecta* provided by the Live Food Laboratory of the Mazatlán Aquarium, Sinaloa, Mexico was used.

2.- Culture Medium:

N°	Soluciones stock	Medio de cultivo	Solución stock (g/L)	Cantidad agregada para 1.0 L de solución substock	Volumen agregado al medio de cultivo (mL)
1	Agua salada	Guillard-Erdschreiber	34.0	------	1000
Macronutrientes				------	
2	$NaNO_3$	Guillard	75.0	------	1.0
		Erdschreiber	59.5	------	3.3
3	$NaH_2PO_4 \cdot H_2O$	Guillard	5.0	------	1.0
	$Na_2HPO_4 \cdot 7H_2O$	Erdschreiber	5.4	------	3.3
4	$Na_2SiO_3 \cdot 9H_2O$	Guillard	30.0	------	1.0
Metales trazas		Guillard			1.0
		Erdschreiber			12.0
5	$Na_2EDTA \cdot 2H_2O$	Guillard	------	4.36 g	
		Erdschreiber	0.750	2.0 mL	
6	$FeCl_3 \cdot 6H_2O$	Guillard	------	3.15 g	
		Erdschreiber	0.097	2.0 mL	
7	$MnCl_2 \cdot 4H_2O$	Guillard	180.0	1.0 mL	
		Erdschreiber	0.041	2.0 mL	
8	$ZnSO_4 \cdot 7H_2O$	Guillard	22.0	1.0 mL	
	$ZnCl_2$	Erdschreiber	0.005	2.0 mL	
9	$CoCl_2 \cdot 6H_2O$	Guillard	10.0	1.0 mL	
		Erdschreiber	0.002	2.0 mL	
10	$CUSO_4 \cdot 5H_2O$	Guillard	9.8	1.0 mL	
11	$Na_2MoO_4 \cdot 2H_2O$	Guillard	6.3	1.0 mL	
		Erdschreiber	0.004	2.0 mL	
Vitaminas		Guillard			1.0
		Erdschreiber			1.0
12	B12	Guillard-Erdschreiber	1.0	1.0 mL	
13	H	Guillard	1.0	1.0 mL	
14	B1	Guillard	------	200 mg	

Methodology

3.- Microalgae propagation

The propagation of the Dunaliella tertiolecta microalgae was carried out using a fed batch type culture system, that is, once the cell growth had started, dilutions were made through a volumetric transfer of the nutrient medium prepared with the intention of preserving the strains in phase. growth and reach a high concentration of biomass (Coutteau, 2013).

The volumetric transfer of the culture medium was calculated using equation 1 (Arana, Orruño, Barcina, 2012) in which *Vi* represents the volume of inoculum that is needed for the next culture, *Vf* the final volume that will be obtained when diluting and *NCi*, *NCf* represent the number of cells present in the inoculum and those that are finally desired to have in the culture after dilution.

$$\boldsymbol{V_i = \frac{NC_f V_f}{NC_i}}$$

The propagation of the microalgae in the described culture media was evaluated from the cell growth kinetics that were constructed by performing a cell count in triplicate every 24 hours of the microalgae samples that were had, taking 1.0 mL of that sample that It was fixed with 50 µL of 10% Lugol solution that was placed on a Neubauer counting chamber (hemocytometer) with a 0.1 mm deep double bright line and for its observation, optical microscopy was used at 10 X and 40 X.

To quantify the efficiency of microalgal propagation, the specific growth rate (μ) was determined where *Xi* represents the initial concentration of the biomass and *Xf* the respective final concentration of the biomass with respect to time and from there the average doubling time was determined. (*td*) of microalgae growth (Godoy-Hernández, Vázquez-Flota, 2006).

$$\boldsymbol{\mu = \frac{lnX_f - lnX_i}{t};\ \ t_d = \frac{ln2}{\mu}}$$

Methodology

4.- Extraction of biomass

In this work, at the end of the cell growth period of Dunaliella tertiolecta in the two-culture media described, the recovery of the dispersed biomass throughout the volume was carried out by sedimentation-flocculation followed by centrifugation and lyophilization.

The flocculation process was carried out with three flocculating agents added under fixed chemical concentration (1.0 mol/L), pH variation of the microalgal biomass and fixed resting time (24 h) with the intention of promoting the auto flocculation of the biomass. A NaOH solution was used to increase the initial pH of the wet microalgal biomass from 7.8 (Ummalyma, Mathew, Pandey, Sukumaran, 2016), a H2SO4 to decrease the initial pH of the humid microalgal biomass to pH values between 4.0-5.0 (Liu et al., 2014) and finally, a solution of [Al2(SO4)3] in which case the concentration did not work established chemistry and a sweep with solutions of 0.25, 0.50 and 0.75 mol/L under the same rest time was decided (Andía-Cárdenas, 2000).

Centrifugation was carried out to obtain the highest separation of microalgal biomass from the aqueous medium in which it was found. Centrifugation tubes of 50 mL capacity were used, at 5000 rpm speed and 5 min of centrifugation (Chen et al., 2011).

Lyophilization was carried out to achieve the highest dehydration of the centrifuged biomass, for this a lyophilizer connected to a vacuum pump was used whose first stage was carried out at -50 °C temperature and 53 KPa of pressure, to subsequently subject the sample to the second drying stage with a temperature of 39 °C and 39 KPa (SP industries, 2009). At the end of the process, the dry biomass rested for 1 h under environmental laboratory conditions (± 25 ° C), was weighed and stored in the freezer in identified sterile bags.

At the end of the methods carried out, the microalgal biomass obtained remained in the form of a powdery solid, green color and a characteristic marine odor.

Methodology

5.- Extraction and characterization of lipids

For the extraction of the lipids present in the dry biomass obtained from the *Dunaliella tertiolecta microalgae*, was carried out using ultrasound extraction (sonication) (Suarsini, 2011; King, 2014).

The sonication and the modified Bligh & Dyer method was used, which consisted as a first step in the homogenization of all the dry biomass in a chloroform-methanol and water mixture (Archanaa, Moise, Suraishkumar, 2012). Then 20 g of this sample were taken and a chloroform-methanol mixture (2: 1) was added and stirred for 15 min and 200 rpm (Soto et al., 2014). Next, the sample was placed in an ultrasonic bath at a fixed frequency of 40 KHz for 20 min, ending the process with the addition of chloroform-methanol-water (2: 2: 1.8) at 5000 rpm for 5 min. (El Arroussi, Benhima, Bennis, El Mernissi, Wahby, 2015) with the aim of improving the formation of the two phases (upper: methanol-water and lower: biomass-lipids-chloroform). The recovery of the lipids was done by decanting the phases, removing the solvents by evaporation under vacuum at 60 rpm and 10 min. The samples were kept in amber flasks refrigerated at 5 ° C for later characterization of the fatty acid profile.

The characterization of the profile of the fatty acids of the microalgae was carried by injecting 1.0 mL of sample into a gas chromatograph coupled to an Agilent DB column. -5ms with temperature programmed between 50-180 oC, 300 ° C for the injector and 320 oC for the FID detector. This profile was characterized only in the microalgal biomass cultivated in Guillard's nutrient medium.

The fatty acid methyl esters were identified by comparing their retention times with the fatty acid standard (37 FAME compounds, SupelcoTM Mix C4-C24; trophic markers). Quantification was performed by integrating the area under the curve in the chromatographic traces using CHROMQUEST 4.1 software with calibrations derived from standard fatty acids.

Results

1. Evaluation of microalgal propagation: Cell growth kinetics

Graph 1 Cell growth kinetics of a strain of the microalgae *Dunaliella tertiolecta* in the Guillard and Erdschreiber culture media

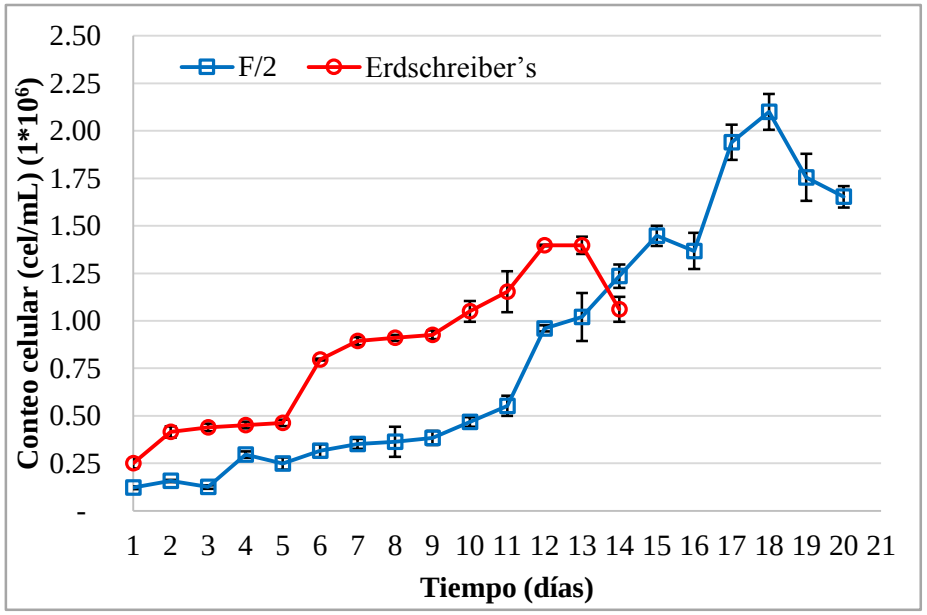


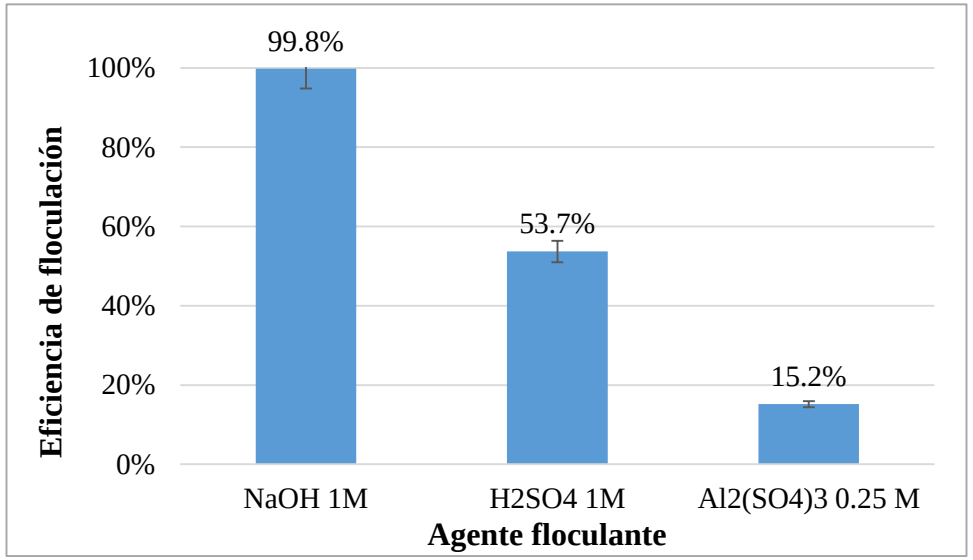
Table 4 Efficiency of cell growth of Dunaliella tertiolecta using two culture media

Parámetro determinado	Guillard culture media	Erdschreiber culture media
μ (d^{-1})	0.21 ± 0.02	0.15 ± 0.01
td (d)	3.34 ± 0.29	4.60 ± 0.33

Results

2. Biomass extraction

Graph 2 Efficiency of flocculation to obtain biomass from the microalgae *Dunaliella tertiolecta*



3. Lipid extraction and fatty acid profile

Table 4 Fatty acids profile presents in the microalgae *Dunaliella tertiolecta*

Fatty acids	Nomenclatura	%
SATURATED		
Hexadecanoico (Palmítico)	C16:0	21.2 ± 0.3
Octadecanoico (Esteárico)	C18:0	11.2 ± 0.1
Heptadecanoico	C17:0	8.9 ± 1.7
Pentadecanoico	C15:0	2.9 ± 0.9
Dodecanoico (Láurico)	C12:0	1.6 ± 0.2
Total:		**45.8 ± 0.6**
INSATURATED		
9,12,15-octadecatrienoico (Linolénico)	$C18:3^{9,12,15}$	37.2 ± 1.1
9,12-octadecadienoico (Linoleico)	$C18:2^{9,12}$	8.0 ± 0.1
9-octadecenoico (Oleico)	$C18:1^{9}$	6.6 ± 0.1
Cis-10-Heptadecenoico	C17:1	2.4 ± 0.0
Total:		**54.2 ± 0.3**

Conclusions

We determined that Guillard's nutrient medium is ideal for the *Dunaliella tertiolecta* microalgae strain since it presented cell density, absorbance, growth rate and doubling time within the ranges published in reference research, in addition to providing a very good quantity biomass. The Erdschreiber's medium is also recommended according to the results obtained, but possibly it provides a higher growth rate as a function of time by modifying some fundamental parameters such as light intensity and photoperiod in such a way as to study the propagation behavior of the microalgae more beyond the eleventh day or that in that time the average cell density improves.

Flocculation with sodium hydroxide turned out to be an excellent chemical agent for the extraction of microalgal biomass, which represents a contribution of this research, since the literature only reports it for microalgae of the genus Nannochloropsis and the recovery methods of the generated biomass. by Dunaliella and other genera require more time which causes energy expenditure to increase. From the implemented methodology, a high percentage of microalgal biomass recovery was achieved in a fairly short time, in addition, sodium hydroxide is a fairly inexpensive and easily acquired chemical compound.

Obtaining the microalgal biomass led to the extraction of the cellular lipid fraction using sonication combined with an extraction with solvents from the Bligh & Dyer method and achieving in a time of twenty minutes that all the exposed biomass was completely dry.

Conclusions

The profile of the lipid faction obtained was compensated by the presence of saturated fatty acids and, to a greater extent, by unsaturated fatty acids, which coincides with investigations referring to the same species of microalgae of this work (Dunaliella tertiolecta), of other species of the same genus such as Dunaliella salina, other genera of microalgae and plant crops such as soy and residual animal fats. It contains very acceptable percentages of the ideal fatty acids (C12 to C18) for transesterification and obtaining biodiesel, in which case the next step is to carry out said production and characterize the product through the fundamental physicochemical parameters established in the Mexican guidelines. Annexes to the Law for the promotion and development of bioenergetics to then know the influence of linolenic acid (the one with the highest proportion in the profile obtained) on oxidative stability, which is the one with the greatest impact on the use of fuel, either pure or through compensation if mixed with fossil diesel.

From the results, the strain of the microalgae Dunaliella tertiolecta analyzed has energy potential for the production of third generation biodiesel under the described working conditions, however, this would be fully established once the oxidative stability has been analyzed, either from the characterization of lipids or the production of biodiesel. Finally, and based on the analysis, the viability of the pilot scale can be determined under environmental conditions and the use of solar energy in the Sinaloa region to reduce costs in mass production.

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