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THIRD GENERATION BIODIESEL PRODUCTION FROM MICROALGAE *Phormidium autumnale*

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Abstract - The aim of this work was to evaluate third generation biodiesel production by the microalgae *Phormidium autumnale* using sucrose as exogenous carbon source. The study focused on optimization of the different C/N ratios and on the analysis of biofuel quality. The results indicate that a C/N ratio of 40 improved the performance of the system, reaching single-cell oil productivities of 18.9 mg/L in steady-state conditions. This oil has a composition predominantly saturated (45.2%) and monounsaturated (34.7%) suitable for biodiesel synthesis (ester content of 99.8%, cetane number of 58.5%, iodine value of 67.2 $gI_2/100$ g, unsaturation degree of 71.3% and a cold filter plugging point of 6.7 ºC).
Keywords: Microalgae/cyanobacteria; Heterotrophic cultivation; Sucrose; Biodiesel 3G.

INTRODUCTION

The last decade has seen an emergence of biofuels due in part to social and political acknowledgement that fossil fuels are a finite resource. This is evidenced by a reduction in the discovery of new fossil fuel sources and the exploitation of more energy intense reserves such as shale gas and tar sands (Scaife *et al*., 2015). As a result of this reality, biofuel research and development has progressed through several stages globally and within Brazil.

Currently, the biofuels are classified from first to fourth generation (Harun *et al*., 2010; Martín and Grossmann, 2012). The third generation biofuels are obtained from microalgae biomass that possess high productivity of lipids, which after extraction are transesterified to obtain biodiesel, turning them into one of the most promising feedstocks for biofuel production (Wijffels and Barbosa, 2010).

There are two possible technological routes to microalgal biomass production: photosynthetic and heterotrophic cultivation. The photosynthetic culture of microalgae, based on CO_2 conversion, is limited by engineering related factors, since design and scale-up methodologies are poorly developed. Factors such as reactor configuration and material construction are considered the main difficulties, when closed photobioreactors are used. On the other hand, open pond technology is limited by biological factors such as organism survival, growth, CO_2 uptake, light utilization, seasonality, harvest and biosafety of transgenics. These questions are related mainly to photosynthetic oil production. An alternative process is based on heterotrophic metabolism of microalgae, in which

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the organic carbon source is converted in the absence of light. These processes can be conducted in conventional reactor configurations such as stirred tank and bubble column reactors, eliminating some disadvantages of the photosynthetic route (Queiroz *et al*., 2011).

The microalgae heterotrophic culture, however, is severely limited by organic carbon availability. The organic substrate is estimated at about 80% of the total cost of the process and economically may make it unfeasible. An alternative to the high costs is the replacement of certain sources of organic carbon by low-cost substrates, such as sucrose, which can reduce costs by up to 40% (Xu *et al*., 2006). According to Li *et al*. (2007), biodiesel 3G may in turn be produced by microalgae that use sucrose as the substrate, making it a more profitable alternative than ethanol from sugar cane. Additionally, Francisco *et al*. (2014), in a survey of potential carbon sources for microalgae production, identified sucrose as a suitable substrate to support heterotrophic microalgae cultivation. The heterotrophic microalgae possess structurally specific mechanisms for active transport of sucrose into the cell. This disaccharide is metabolized via the oxidative pentose-phosphate pathway after hydrolysis into monosaccharides (Knowles and Plaxton, 2003).

Thus, the aim of this work was to evaluate the third generation biodiesel production from heterotrophic cultivation of the microalgae *Phormidium autumnale* employing sucrose as exogenous carbon source. The study focused on optimization of the carbon/nitrogen ratio of the culture media, in the evaluation of different operational modes of the bioreactor and in the analysis of the biofuel quality.

MATERIAL AND METHODS

Microorganisms and Culture Media

Axenic cultures of *Phormidium autumnale* were originally isolated from the Cuatro Cienegas desert (26º59'N, 102º03'W-Mexico). Stock cultures were propagated and maintained in solidified agar-agar (20 g/L) containing synthetic BG11 medium (Ripka *et al*., 1979). The incubation conditions used were 25 ºC, a photon flux density of 15 µmol/m^2/s and a photoperiod of 12:12 h (light:dark). To obtain the inoculums in liquid form, 1 mL of sterile synthetic medium was transferred to slants, the colonies were scraped and then homogenized with the aid of a mixer tubes. The entire procedure was performed aseptically.

Bioreactor

Measurements were made in a bubble column bioreactor. The system was built of borosilicate glass and had an internal diameter of 15 cm and height of 20 cm, resulting in a height/diameter (h/D) ratio equal to 1.33 and a nominal working volume of 2.0 L. The dispersion system of the reactor consisted of a 2.5 cm diameter air diffuser located inside the bioreactor. The air flow was monitored by a flow meter (KI-Key Instruments®, Trevose-PA, USA) and the inlet of air and outlet of gases were filtered through filtering units made up of polypropylene membrane with a pore diameter of 0.22 µm and total diameter of 50 mm (Millex FG®, Billerica-MA, USA). The bioreactor including filtering units was previously sterilized by autoclaving at 121 ºC for 40 min and then for 30 min containing the synthetic medium.

Obtaining Kinetic Data in an Experimental Bioreactor

Initial experiments were performed in a bioreactor operating under a batch regime, fed with 2.0 L of culture medium. The experimental conditions were as follows: initial concentration of inoculum of 100 mg/L, temperature of 30 ºC, pH adjusted to 7.6, aeration of 1 VVM (volume of air per volume of culture per minute) and absence of light. The culture medium consisted of BG11 synthetic medium modified and supplemented with different concentrations of sucrose to obtain carbon/nitrogen ratios (C/N) of 20, 30, 40, 50, 60, 70 and 80. The concentration of sucrose was adjusted stoichiometrically (Francisco *et al*., 2014).

In the continuous culture, after 120 h of batch culture, feed culture medium was added to the bioreactor at the dilution rate D=0.02 h^{-1}. At the same time, equal volumes of cell suspension were withdrawn from the bioreactor. The steady-state was considered to have been established after at least three volume charges, with a variation of cell dry weight less than 5%.

The experiments were performed twice, and in duplicate for each operational mode. Therefore, kinetic data refer to the mean value of four repetitions.

Kinetic Parameters

Biomass data were used to calculate the biomass productivity [$P_X = (X_i-X_{i-1})(t_i-t_{i-1})^{-1}$, mg/L/h] and the lipid productivity [$P_L = P_X.L_C$, mg/L/h], in which X_i is the biomass concentration at the time t_i (mg/L) and X_{i-1} is the biomass concentration at the time t_{i-1} (mg/L), t is the residence time (h) and L_C is the lipid

content of the biomass (%). The concentrations of total organic carbon were used to calculate the substrate consumption rate (r_S=dS/dt, mg/L/h), and the biomass yield coefficient ($Y_{X/S}$=dX/dS, $mg_{cell}/mg_{substrate}$), where S_0 is the initial substrate concentration (mg/L), S is the substrate concentration (mg/L) and t is the time (h).

Sampling and Analytical Methods

Samples were collected aseptically in a laminar flow hood. The cell biomass, the pH dynamics and the consumption of organic carbon were monitored every 24 hours during the growth phase of microorganism.

The cell biomass was gravimetrically evaluated by filtering a known volume of culture medium through a 0.45 μm membrane filter (Millex FG®, Billerica-MA, USA), drying at 60 ºC for 24 h.

The organic carbon concentration was expressed in terms of chemical oxygen demand (COD) and analyzed according to the closed reflux colorimetric method (APHA, 2005).

The total lipid concentration of the biomass was determined gravimetrically by the Bligh and Dyer (1959) method.

The saponification and esterification (methylation reaction) by the modified method of Hartman and Lago (1976) was used with the dried lipid extract to obtain the fatty acid methyl esters (biodiesel). An amount of 250 mg of oil was added to 5.0 mL of 0.50 mol/L NaOH in methanol. The mixture was then heated under reflux for 5 min. After adding 15.0 mL of the esterification reagent (prepared from a mixture of 2.0 g of ammonium chloride, 60.0 mL of methanol, and 3.0 mL of concentrated sulfuric acid for *ca*. 15min), the mixture was heated under reflux for another 3 min and subsequently transferred to a separation funnel containing 25.0 mL of petroleum ether and 50.0 mL of deionized water. After stirring the mixture and phase separation, the aqueous phase was discarded. Then 25.0 mL of deionized water was added to the organic phase. This mixture was stirred and, after phase separation, the aqueous phase was discarded. This procedure was repeated. The organic phase was collected, the solvent was evaporated in a rotary evaporator and the residue was removed under nitrogen flow. The methyl esters were solubilized in *n*-heptane before injection in the gas chromatograph. The fatty acid composition was determined using a VARIAN 3400CX gas chromatograph (Varian, Palo Alto-CA, USA). The fatty acid methyl esters were identified by comparison of the retention times with those of the standard (Supelco, St. Louis-MO, USA) and quantified by area normalization.

The fuel properties of biodiesel (ester content, EC; degree of unsaturation, DU; cetane number, CN; iodine value, IV and cold filter plugging point, CFPP) were determined according to the methodology proposed by Francisco *et al*. (2010).

The cetane number of the mixture was estimated by empirical equations. The cetane number, saponification value and iodine value were calculated in accordance with Eqs. (1)-(3)

$$CN = 46.3\frac{5458}{SV} - 0.225IV \tag{1}$$

$$SV = \frac{\sum(560.N)}{M} \tag{2}$$

$$IV = \frac{\sum(254DN)}{M} \tag{3}$$

where CN is the cetane number, SV is the saponification value, IV is the iodine value, D is the number of double bonds, M is the molecular mass and N is the percentage of each fatty acid component.

The degree of unsaturation was calculated from empirical Eq. (4), taking into account the amount of monounsaturated and polyunsaturated methyl ester (wt%) present in the microalgae oil:

$$DU = (MUFA) + 2(PUFA) \tag{4}$$

where DU is the unsaturation degree (%), MUFA is the weight percentage of the monounsaturated fatty acids (wt%).

The long-chain saturated factor was obtained from empirical Eq. (5), taking into account the composition of fatty acids and assigning more weight to the composition of fatty acids with a long chain. This parameter was correlated with the cold filter plugging point, using Eq. (6):

$$LCSF = (0.1C16) + (0.5C18) + (1C20) + (1.5C22) + (2C24) \tag{5}$$

$$CFPP = 3.1417LCSF - 16.477 \tag{6}$$

where LCSF is the long-chain saturated factor; C16, C18, C20, C22, and C24 are the weight percentage of each of the fatty acids (wt%) and CFPP is the cold filter plugging point.

RESULTS AND DISCUSSION

The assessment of suitable concentration of the carbon sources for the production microalgal

biomass and bioproducts is a fundamental step in the consolidation of the process. The kinetic parameters using different C/N ratios for heterotrophic culture of *Phormidium autumnale* are shown in Table 1. The best results were evidenced in the range of C/N ratio between 30-50, with maximum kinetic performance at the C/N ratio of 40. In this condition, a maximum specific growth rate of 0.02 h^{-1}, generation times of 32.3 h, maximum cell density of 6170 mg/L, average biomass productivity of 40.7 mg/L/h, average rate of sucrose consumption of 42.3 mg/L.h and a biomass yield coefficient of 0.44 $mg_{cell}/mg_{sucrose}$ were obtained. This biomass had a lipid content of 20.7%, resulting in an oil productivity of 8.46 mg/L/h.

According to Fay (1983), in general cyanobacterial culture requires a minimum C/N ratio of 20. Specifically for sucrose, the results obtained indicate a bell-shaped curve pattern in the range between 20 to 80. Comparatively, the results obtained are higher than those reported by Markou and Georgakakis (2011) that indicate maximum cell densities of 1180 mg/L for heterotrophic culture of *Phormidium* sp. using sucrose as exogenous carbon source.

Sucrose is metabolized by microalgae through the pentose-phosphate pathway (Smith, 1982). These microorganisms possess structurally specific mechanisms for the active transport of sucrose into the cell membrane. The heterotrophic microalgae have an inducible active carbohydrate symport system responsible for uptake of these molecules from the culture medium. The induction of this transport is achieved by some specific sugars. In general, in cultivations with suitable concentration and type of sugars, the symport system is induced to promote the alkalinization of the culture media by a net movement of protons accompanied by sugar uptake (Figure 1). The rate of the increase in pH fundamentally depends on the concentration and type of sugar used (Hong and Lee, 2007). Additionally, the disaccharides such as sucrose are only used in the pentose phosphate pathway, after a previous hydrolysis that transforms this sugar in monosaccharides, particularly fructose and glucose. Several enzymes, specifically invertase, are involved in these reactions and have been identified in microalgae cultures (Fuchs *et al*., 1994; Gupta *et al*., 2011).

Table 1: Kinetic parameters for different C/N ratios using sucrose as substrate in batch cultures.

C/N	μ_{max} (1/h)	tg (h)	X_{max} (mg/L)	P_X (mg/L/h)	r_S (mg/L)	$Y_{X/S}$ ($mg_{cell}/mg_{sucrose}$)	L_C (%)	P_L (mg/L/h)
20	0.013±0.00	51.3±0.5	2775±56	14.7±0.4	30.9±0.8	0.47±0.00	8.9±0.2	1.32±0.03
30	0.020±0.00	34.1±0.3	5260±30	34.4±0.4	36.1±0.7	0.43±0.00	18.6±0.2	6.41±0.08
40	0.021±0.00	32.3±0.4	6170±28	40.7±0.4	42.3±0.4	0.44±0.00	20.7±0.3	8.46±0.15
50	0.024±0.00	28.8±0.1	5330±53	34.9±0.3	21.2±0.3	0.64±0.00	9.9±0.1	3.50±0.09
60	0.016±0.00	42.0±0.3	2925±18	18.2±0.2	25.0±0.2	0.72±0.00	8.1±0.1	1.40±0.05
70	0.014±0.00	48.8±0.3	1938±16	12.9±0.1	30.4±0.6	0.42±0.00	6.1±0.2	0.80±0.01
80	0.015±0.00	44.7±0.2	1683±22	10.3±0.1	53.1±0.7	0.18±0.00	5.1±0.1	0.53±0.01

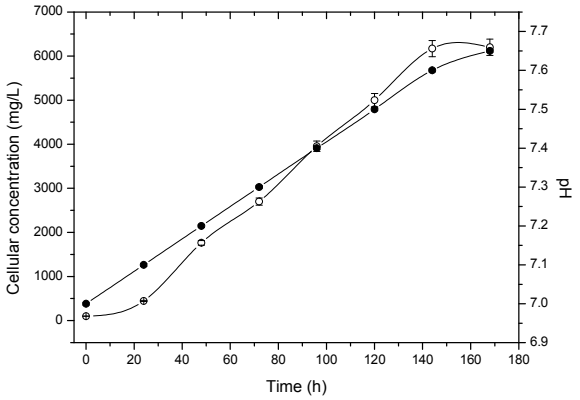

Figure 1: Variation of the pH (closed circle) and cellular concentration (open circle) *vs*. time at a C/N ratio of 40.

To further improve bulk oil production, continuous cultivations were performed (Table 2). Maintaining cells in the steady-state resulted in an oil productivity of 18.9 mg/L/h, an increase of 2.2-fold over batch cultivations. A faster product formation rate implies a higher productivity and corresponding reductions in plant operating time and operating cost, for an existing plant. On the other hand, for a new plant to be built, the increased rate implies, in addition to improved productivity, a smaller reactor and therefore a lower capital investment cost. Likewise, improved productivities imply a lower raw material cost and a lower capital investment for existing and new plants (Francisco *et al*., 2015).

Scale-up projections for these values in the present scenario are limited by the large-scale application of heterotrophic microalgal bioreactors. However, in comparison with the productivity that can be achieved with soybeans, the Brazilian feedstock commonly used industrially for biodiesel, a scale-down projection analysis indicates that, for the Brazilian harvest of 2014, the average lipid productivity of soybeans was $0.46 g_{oil}/m^2$.day, considering a production cycle of 120 days (CONAB, 2015). Each hectare of arable Brazilian soil produces an average of 2,700 kg of soybean, containing up to 20% oil. The data obtained for *Phormidium autumnale* indicate that a continuous bioreactor with 1.0 L/m^2 of working volume, operating on a cycle of 120 days/year, would yield the same amount of lipids as that produced by soybean. This comparison indicates that the oil productivity of *Phormidium autumnale* can be increased by several fold by associating bioreactor optimal design with an operating cycle of 330 days/year.

Finally, in terms of oil composition and fuel properties of biodiesel (Table 3), the lipid fraction of biomass indicated eight different compounds, with oleic acid (26.2%) being the major. The single-cell oil showed a profile predominantly saturated (45.2%) and monounsaturated (34.7%), which determine the fuel properties of microalgal biodiesel (Francisco *et al*., 2010). The biodiesel produced from microalgal oil had the following fuel properties: ester content of 99.8%, cetane number of 58.5, iodine value of 67.2 $gI_2/100$ g, degree of unsaturation of 71.3%, and cold filter plugging point of 6.7 ºC. All these parameters comply with the limits established by the US, European, and Brazilian standards (ASTM, 2002, UNE-EN, 2003, ANP, 2003), besides being comparable with soybean biodiesel (Knothe, 2005). These results indicate the potential for the exploitation of this feedstock for biofuel production.

Table 2: Kinetic parameters of the steady-state process.

Parameter	Value
X_{max} (mg/L)	4300±698
P_X (mg/L/h)	92.1±1.5
r_S (mg/L/h)	56.3±1.2
L_C (%)	20.5±0.3
P_L (mg/L/h)	18.9±0.4

Table 3: Fatty acid profile and fuel properties of biodiesel 3G.

Fatty acid profile								
Methyl esters (g/100g)	**Lauric (12:0)**	**Myristic (C14:0)**	**Palmitic (C16:0)**	**Palmitoleic (C16:1)**	**Stearic (C18:0)**	**Oleic (C18:1n9c)**	**Linoleic (C18:2n6c)**	**γ-Linolenic (C18:3n6)**
	4.9±0.1	7.3±0.1	22.5±0.5	8.5±0.1	10.5±0.2	26.2±0.8	17.8±0.5	2.10±0.03
Fuel properties								
Properties	**EC (wt %)**	**CN**	**DU (wt %)**	**IV (gI_2/100g)**	**LCFS (wt %)**	**CFPP (ºC)**		
	99.8±0.1	58.5±0.9	71.3±1.8	67.2±0.9	7.4±0.2	6.70±0.06		

CONCLUSION

The results obtained indicate that sucrose is an exogenous carbon source with the potential to produce bulk oil and biodiesel by *Phormidium autumnale*, enabling oil productivities of 18.9 mg/Lh. This oil had a composition of predominantly saturated (545.2%) and monounsaturated (34.7%) fatty acids, suitable for biodiesel synthesis (ester content of 99.8%, cetane number of 58.5, iodine value of 67.2 gl_2/100g, degree of unsaturation of 71.3%, and cold filter plugging point of 6.7 ºC.

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NOMENCLATURE

Acronyms

C/N	Carbon/nitrogen ratio
CFPP	Cold filter plugging point (ºC)
CN	Cetane number
COD	Chemical oxygen demand (mg/L)
D	Number of double bonds
DU	Degree of unsaturation (%)
EC	Ester content (%)
IV	Iodine value (gl_2/100g)
Lc	Lipid content of the biomass (%)
LCSF	The long-chain saturated factor
M	Molar mass (g/mol)
MUFA	Weight percentage of the monounsaturated fatty acids (%)
N	Percentage of each fatty acid component (%)
PUFA	Weight percentage of the polyunsaturated fatty acids (%)
SV	Saponification value (%)
VVM	Volume of air per volume of culture per minute

Symbols

μ_{max}	Maximum specific growth rate (1/h)
P_L	Lipid productivity (mg/L/h)
P_X	Average cellular productivity (mg/L)
r_S	Substrate consumption rate (mg/L/h)
S	Substrate concentration (mg/L)
S_0	Initial substrate concentration (mg/L)
T	Residence time (mg/L)
tg	Generation time (h)
$X_{máx}$	maximum cell biomass (mg/L)
$Y_{x/s}$	Biomass yield coefficient (mg/mg)

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