CHEMICAL ENGINEERING TRANSACTIONS
VOL. 32, 2013
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet
Chief Editors: Sauro Pierucci, Jiří J. Klemeš

ISBN 978-88-95608-23-5; **ISSN** 1974-9791
DOI: 10.3303/CET1332151

Potential of Microalgae *Scenedesmus obliquus* Grown in Brewery Wastewater for Biodiesel Production

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This work aims to analyze the possibility of growing microalgae *Scenedesmus obliquus* (*S. obliquus*) in a brewery wastewater as a potential candidate for biodiesel production. For this purpose *S. obliquus* was cultivated in a synthetic brewery wastewater at 12,000 Lux of light intensity, with a 12 h period of daily light and aeration. Under these conditions, results revealed an average lipid content of 27 % of dry-weight (dwt) biomass and average biomass and lipid concentrations of respectively, 0.90 and 0.24 g/L (of dwt biomass). The fatty acid methyl esters (FAME) transesterified from the lipids are mainly composed of saturated esters (56.4 %) among which, palmitate (C16:0) is the most significant with a relative percentage of 47.8 % (wt). With regard to the unsaturated esters, the percentage of 10.6 % (wt) obtained for linolenate (C18:3) is below the maximum limit imposed by the EN 14214:2003 standard for this ester in biodiesel. The average molecular mass of these lipids and FAME are respectively 845.2 and 283.1 g/mol.

1. Introduction

Microalgae are increasingly seen as an alternative to the traditional biodiesel feedstocks such as edible vegetable oils, animal fats (Mata et al., 2010a, 2011a) and other residual products like spent coffee grounds (Caetano et al., 2012), which have a limited supply and may associate economic, social and environmental impacts (Mata et al., 2011b). They can grow and be harvested almost continuously, reducing the seasonal problems of raw materials supply for the biofuels industry. Also, their potential high biomass and lipid productivities (Mata et al., 2010b), and the possibility of using nutrients from waste streams (e.g. wastewaters and/or CO_2 flue gas emissions) can help reduce the environmental impacts and costs of cultivating them for biofuels applications (Mata et al., 2012a).
S. obliquus is a freshwater microalga that can grow in industrial wastewaters of different origins showing good adaptation ability (Hodaifa et al., 2008, 2009) and it is a very versatile microalga as raw material for biofuels production (Miranda et al., 2012). It is also considered one of the best candidates for biodiesel production among several microalgae species (Gouveia and Oliveira, 2009), with a lipid content ranging between 18.8 and 29.3 % dwt for a nutrient-replete medium and up to 42 % dwt for a nutrient-deficient medium (Ruiz et al., 2013). Moreover, the optimum temperature range for the *S. obliquus* growth is relatively wider, since its growth rates change little between 14 and 30 °C (Xu et al., 2012). This finding is especially relevant for the outdoor cultivation of this microalga, as temperature is one of the major environmental factors limiting the microalgae productivity (Martinez et al., 1999; Voltolina et al., 2005), which normally varies among hot or cold weather and day or night-time temperatures.
Another important finding is the possibility of doubling the biomass productivity by operating the cultivation and harvesting in continuous mode instead of batch as described by different authors (McGinn et al., 2012;

Please cite this article as: Mata T., Melo A., Meireles S., Mendes A., Martins A., Caetano N.S., 2013, Potential of microalgae scenedesmus obliquus grown in brewery wastewater for biodiesel production, Chemical Engineering Transactions, 32, 901-906 DOI: 10.3303/CET1332151

Ruiz-Marin et al., 2010; Ruiz et al., 2013). This can be explained because biomass is kept in the exponential growth phase due to the continuous addition of new medium, with more nutrients, to the cultivation reactor.
In this work a brewery wastewater was chosen as culture medium since it is generated in large volumes and contains a high environmental load with enough nutrients for this microalga growth (Mata et al., 2012b). Also, this presents several benefits, such as the possibility of combining biodiesel production with the biological removal of contaminants (in particular of N and P) from wastewaters, this way reducing the eutrophication potential and decreasing the costs of biofuels production from the microalgae biomass (Mata et al., 2010b).
Concerning the application of this microalga to wastewaters treatment few studies still exist. An example is the Hodaifa et al. (2008) study that used rinse water from the olive-oil extraction industry for growing microalga *S. obliquus*, showing that although this wastewater is N deficient, the highest percentage of mono and poly-unsaturated fatty acids in the biomass' lipids fraction was reached when 100 % rinse water was used as culture medium. McGinn et al. (2012) also explored the possibility of using microalga *Scenedesmus sp*. for a municipal wastewater treatment in batch and continuous mode. Hence, this study analyzes the potential of microalgae *S. obliquus* cultivated in a synthetic brewery wastewater for biodiesel production.

2. Materials and methods

2.1 Preparation of the synthetic brewery effluent

A culture of *S. obliquus* 276-3d from the German SAG (Sammlung von Algenkulturen Göttingen) collection was used for the purpose of this study, and cultivated in a simulated brewery effluent, which composition is presented in Table 1, as proposed by Tam (2002) and modified in this work. In order to maintain the pH at approximately 6.5 during the batch experiments (and remaining within 5% of the initial value) the synthetic effluent was buffered using salts of sodium phosphate (NaH_2PO_4 and Na_2HPO_4) at 1.65 mM. Ammonium sulfate [$(NH_4)_2SO_4$] was used as an inorganic nitrogen source.

Table 1: Composition of the simulated brewery effluent

Component	Value
Malt extract (g/L)	1.00
Yeast extract (g/L)	0.50
Peptone (g/L)	0.15
Maltose (g/L)	0.86
$(NH_4)_2SO_4$ (g/L)	2.20
Ethanol (mL/L)	2.00
NaH_2PO_4 (g/L)	0.08
Na_2HPO_4 (g/L)	0.14

In order to prevent the precipitation of complexes formed due to the high temperatures attained during autoclaving, some of the culture medium components were autoclaved separately as recommended by Tam (2002). Therefore, the ammonium sulphate, peptone and yeast extract were weighed to a screw cap 1,000 mL Pyrex bottle, filled with 500 mL of distilled water. Maltose, malt extract, and buffer salts were weighed to another screw cap 1,000 mL Pyrex bottle filled with 500 mL of distilled water. Both bottles were then autoclaved at 121 °C for 20 min and after autoclaving the junction of the two components' parts was performed under aseptic conditions (in a laminar flow cabinet). The required volume of ethanol was then measured and added to this mixture.
For preventing contamination by other microorganisms, all the laboratorial material and glassware (test tubes, and glass flasks) were washed with water and detergent, rinsed with distilled water, autoclaved at 121 ºC for 20 minutes and placed in a laminar flow cabinet (CRUMA, 870-FL), under UV radiation for 60 min. Disposable pipettes and sterile loops were used.

2.2 Routine microalgae culturing and acclimatization

Test tubes were inoculated with this microalga in approximately 10 mL of the simulated brewery effluent (to start the cells acclimatization) and closed with cotton plugs (allowing air diffusion). After seven days, the inocula of two test tubes were transferred to 250 mL Erlenmeyer flasks (the culture density in the test tubes was controlled by visual observation) and supplemented with fresh synthetic brewery effluent.

The microalgae cultures were subjected to a light/dark (L/D) photoperiod of 12/12 h, at 30 ± 3 °C of room temperature, and illuminated by fluorescent lamps (36 W, Sylvania Aquastar T8) providing an illuminance of about 4,500 Lux (for test tubes) and 12,000 Lux (for 250 mL Erlenmeyer flasks) measured with a luxmeter (Lutron LX-1102). Air sterilized by filtration (0.2 µm pore diameter) was supplied to the microalgae cultures at an average rate of 2 mL/s by air pumps (Pacific AP6), through the perforated rubber stopper of the 250 mL Erlenmeyer flasks.

2.3 Harvest of the microalgae biomass, lipids extraction and quantification

The method used in this work to harvest the microalgae biomass is the centrifugation in a Super-speed Automatic Centrifuge (SORVALL SS-3) at 3,000 rpm, for 15 min.

For the lipids extraction and quantification it was chosen the Bligh and Dyer (1959) method, modified in this work as follows: (1) First, the biomass sample obtained by centrifugation was weighed in a centrifuge pre-weighed glass tube; (2) Then, the co-solvents were added at ratios of 1.0, 2.0 and 0.8 (v/v) for chloroform (Riedel de Haën, p.a.), methanol (Riedel de Haën, p.a.) and distilled water, respectively. Since it was used a centrifuged biomass (with a known water content) and not lyophilized biomass, these proportions were corrected taking into account a fraction of water in the wet biomass of about 70 % (Figure 1a); (3) The centrifuge tube containing the biomass sample with co-solvents was subjected to ultrasounds for 30 min (in a Baldelin Sonorex TK30 equipment); (4) A second extraction step was then performed by adding the co-solvents at ratios of 2.0, 2.0 and 1.8 (v/v) for chloroform, methanol and distilled water, respectively; (5) The sample was again subjected to ultrasounds for more 30 min (Figure 1b) and then centrifuged at 3,000 rpm, for 15 min (in a ECCO Tvp 25 No. 8601 centrifuge). (6) After centrifugation three layers became visible: an upper layer rich in water and methanol, a middle layer consisting of extracted biomass, and a lower layer rich in lipids and chloroform (Figure 1c). The upper layer rich in water and methanol was discarded and the lower layer rich in lipids and chloroform was recovered to a previously weighed glass tube; (7) The chloroform was evaporated to dryness (in a laboratorial hood at room temperature of about 25 ºC) and the purified lipids extract remained in the glass tube; (8) The tube containing the lipids (pre-weighed when empty) was weighed again in order to determine the microalgae lipid content by gravimetry.

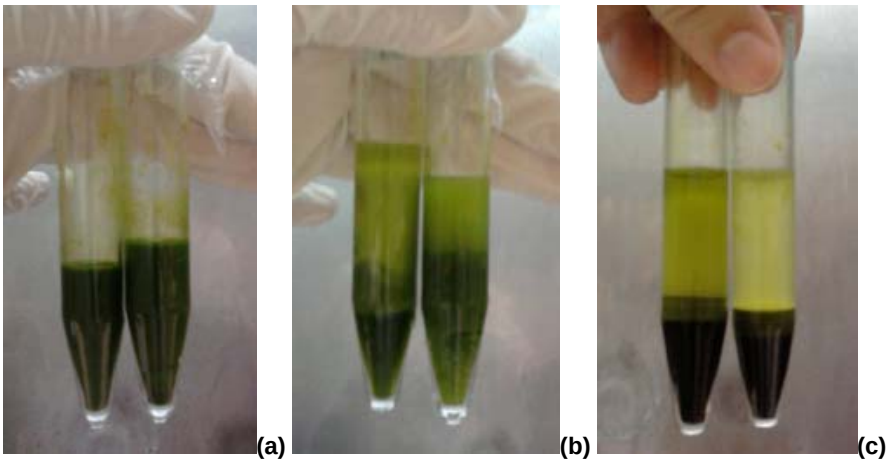

Figure 1: Biomass sample appearance: a) after addition of the first extraction co-solvents, b) after addition of the second extraction co-solvents and subjected to ultrasounds, c) after centrifugation, having three layers visible

2.4 Transesterification of microalgal lipids

In this study, for the transesterification of lipids extracted from the microalgae biomass in order to obtain FAME, it was used the Lepage and Roy (1984) method, with slight modifications as follows: 1) The crude lipids (about 10 mg) was dissolved using 2 mL of a freshly prepared mixture of chloroform-methanol (2:1, v/v) in a 10 mL Pyrex tube with a Teflon-sealed screw cap; 2) 1 mL of methanol as reagent and 0.3 mL of sulfuric acid 95-97 % (Scharlau Chemie, reagent grade) as catalyst were added, for the transesterification of about 10 mg of lipids; 3) After screwing the lid, the tube with the mixture was weighed; 4) The tube was vigorously shaken for 5 min; 5) Finally, reaction of the mixture in the tube at 100 °C, for 10 min, in a digestor (ECO 16 Thermoreactor Velp Scientifica) followed by cooling down the tube to room temperature;

6) Addition of 1 mL of distilled water for phase separation (by adding water two distinct phases are formed, the upper layer rich in water, methanol, glycerol, and sulfuric acid, and the lower layer rich in chloroform and esters); 7) Addition of 1 mL of distilled water for a gentle water washing of esters with chloroform layer (more dense than the water layer) followed by discard of the water rich upper layer (less dense). This step was repeated twice more; 8) The esters rich layer was filtered using a disposable Nylon syringe filter (0.2 μm pore, 13 mm diameter, Cronus, UK); 9) Evaporation to dryness of chloroform from the esters in a laboratorial hood, at room temperature (of about 25 ºC).

2.5 Gas chromatography analysis of FAME

The FAME obtained from the microalgae lipids were analyzed by gas chromatography (GC) according to the EN 14103:2010 standard, using as internal standard methyl heptadecanoate (99.5 % purity, Fluka) with a concentration of 10.256 mg/mL. This analysis was performed using a gas chromatograph (DANI GC 1000 DPC) equipped with a TRB-WAX (Capillary Column, Teknokroma) for FAME´s (30 m, 0.32 mm internal diameter, and 0.25 μm film thickness). The injector temperature was set to 250 ºC, while the flame ionization detector (FID) temperature was set to 250 ºC and the oven temperature to 195 ºC. The carrier gas used was Helium, at a flow rate of 1 mL/min. Injection was made in a split mode, using a split ratio of 1:80, and the volume injected was 0.1 μL.

3. Results and discussion

3.1 Microalgae growth in the synthetic brewery effluent

For the cultivation of microalgae *S. obliquus* in a synthetic brewery wastewater several laboratorial experiments were conducted (Mata et al., 2012b) in order to evaluate the best operating conditions of light exposure, light intensity and culture aeration, regarding to the biomass growth rate, reduction of chemical oxygen demand (COD), and to the total nitrogen (TN) and total carbon (TC) removal. Table 2 summarizes the best results achieved in the course of that study.

Table 2: Summary of the best results obtained for S. obliquus cultivated in a synthetic brewery effluent at different operating conditions (Mata et al., 2012b)

Light intensity (Lux)	Photoperiod	Aeration	Maximum biomass (dry weight)		Maximum reduction COD		Maximum reduction N		Maximum reduction C	
			g/L	day	%	day	%	day	%	day
4,500	12	Yes	0.80	9	33.9	14	15.2	13	38.3	9
4,500	12	No	0.60	5	30.2	12	16.2	12	31.8	12
4,500	24	Yes	0.75	7	66.8	11	11.0	11	63.8	9
4,500	24	No	0.70	6	26.1	13	24.4	13	19.5	13
12,000	12	Yes	0.90	9	57.5	14	20.8	14	56.9	13
12,000	12	No	0.55	11	26.1	14	17.8	14	31.8	14
12,000	24	Yes	0.80	4	40.0	11	11.6	11	53.5	11
12,000	24	No	0.35	11	13.3	13	23.5	13	23.8	13

Following the results presented in Table 2, *S. obliquus* was cultivated in the synthetic brewery effluent at 12,000 Lux of light intensity, for a 12 h period of daily light, and the cultures were subjected to aeration.

3.2 Lipid content and biomass concentration

Table 3 shows the average lipid content and concentration and the average biomass concentration of microalgae *S. obliquus*. Experiments were performed in triplicate and data are expressed as mean ± SD (standard deviation).

Table 3: Average lipid content and concentration and the averaged biomass concentration of S. obliquus cultivated in the brewery effluent

Parameter	Value
Lipid content (dwt % biomass)	27.0 ± 1.00
Biomass concentration (g dwt/L)	0.90 ± 0.05
Lipid concentration (g dwt /L)	0.24 ± 0.02

The average lipid content of microalgae *S. obliquus* is 27.0 % of dry weight biomass, which is within the ranges (11-55%) reported for *S. obliquus* (Ho et al., 2010; Mata et al., 2010b). Also, the average biomass concentration (0.90 g of dry weight biomass per liter) obtained in this study is the same value found by Gouveia and Oliveira (2009) although other authors reported much higher values for the *S. obliquus* maximum biomass concentration (1.84 ± 0.30 g/L, Abou-Shanab et al., 2011, and 3.51 g/L, Ho et al., 2010) while using different cultivation conditions.

3.3 GC analysis of FAME

The lipids from microalgae *S. obliquus* were primarily transesterified and the major FAME composition were determined by GC analysis. The FAME composition was calculated as percentage of the total esters present in the sample, determined from the peak areas, as shown in Table 4.

Table 4: Fatty acid composition of S. obliquus determined as FAME

FAME composition	(wt %)
Myristate (C14:0)	n.d.
Palmitate (C16:0)	47.78
Stearate (C18:0)	8.60
Oleate (C18:1)	21.59
Linoleate (C18:2)	11.42
Linolenate (C18:3)	10.61
Arachidate (C20:0)	n.d.
Total	100.00

n.d. - not detected

The results obtained in this work show that FAME obtained from the lipids of *S. obliquus* are mainly composed of saturated esters (56.4 %), among which palmitate (C16:0) is the most significant with a relative percentage of 47.8 % (wt).
With regard to the unsaturated esters, special attention should be given to linolenate (C18:3) because the EN 14214:2003 standard for the biodiesel quality, establishes the maximum limit of 12 % (wt/wt) for this ester, which is verified in this case as the percentage obtained for linolenate was 10.6 % (wt).
The average molecular mass of lipids and FAME determined for *S. obliquus* were respectively, 845.2 and 283.1 g/mol.

4. Conclusions

Biodiesel from *S. obliquus* is technically feasible, where the oil can be used alone or associated with other oils in order to meet the biodiesel quality requirements. However, in order to be economically competitive with fossil diesel, microalgal cultivation and harvesting, oil extraction and biodiesel production must be optimized. Also, the biorefinery concept should be considered, as the downstream processing of microalgae biomass may result not just on lipids, suitable for biodiesel production, but also on a wide variety of other chemicals that can be used to produce other types of biofuels or for the pharmaceutical or nutraceutical industries. Moreover, the combination of this microalgae growth with the brewery effluent treatment proved to be possible, thereby enabling the reduction in the cost of acquisition of nutrients for microalgae cultivation and, on the other hand opening the possibility for a new type of brewery effluent treatment. Thus, an average lipid concentration of 0.24 g/L (of dwt biomass) was obtained from the *S. obliquus* cultivated in the simulated brewery effluent and the biodiesel could be produced with the oil extracted from *S. obliquus*, containing 56.4 % of saturated esters and less than 12 % of the linolenate (unsaturated) ester.

Acknowledgments

Authors would like to acknowledge financial support from UNICER, project PP-IJUP2011-UNICER-107, as well as to the Centre of Innovation in Engineering and Industrial Technology (CIETI) of ISEP for its support in FAME determination.

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