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# Bioresource Technology

journal homepage: [www.elsevier.com/locate/biortech](http://www.elsevier.com/locate/biortech)

## A biorefinery from *Nannochloropsis* sp. microalga – Extraction of oils and pigments. Production of biohydrogen from the leftover biomass

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### HIGHLIGHTS

- ▶ *Nannochloropsis* sp. was studied as a biorefinery context.
- ▶ Production of fatty acids for biodiesel, high added-value compounds and biohydrogen.
- ▶ Extracts fractionation by SFE (oils for biodiesel and pigments for food).
- ▶ Fermentation of biomass leftover by *E. aerogenes* yielded maximum 60.6 mL H<sub>2</sub>/g<sub>dry biomass</sub> alga.
- ▶ Best SFE conditions extracted 45 g<sub>lipids</sub>/100 g<sub>dry biomass</sub> (40 °C, 300 bar, CO<sub>2</sub> + ethanol).

### ARTICLE INFO

Article history:  
Available online xxx

Keywords:  
*Nannochloropsis*  
Oils  
Carotenoids  
Biohydrogen  
Biorefinery

### ABSTRACT

The microalga *Nannochloropsis* sp. was used in this study, in a biorefinery context, as biomass feedstock for the production of fatty acids for biodiesel, biohydrogen and high added-value compounds. The microalgal biomass, which has a high lipid and pigment content (mainly carotenoids), was submitted to supercritical CO<sub>2</sub> extraction. The temperature, pressure and solvent flow-rate were evaluated to check their effect on the extraction yield. The best operational conditions to extract 33 g<sub>lipids</sub>/100 g<sub>dry biomass</sub> were found to be at 40 °C, 300 bar and a CO<sub>2</sub> flow-rate of 0.62 g/min. The effect of adding a co-solvent (ethanol) was also studied. When supercritical CO<sub>2</sub> doped with 20% (w/w) ethanol was used, it was possible to extract 45 g<sub>lipids</sub>/100 g<sub>dry biomass</sub> of lipids and recover 70% of the pigments. Furthermore, the remaining biomass after extraction was effectively used as feedstock to produce biohydrogen through dark fermentation by *Enterobacter aerogenes* resulting in a hydrogen production yield of 60.6 mL/g<sub>dry biomass</sub>.

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### 1. Introduction

Renewable carbon-neutral liquid biofuels are needed to replace petroleum-derived transport fuels in the near future as they contribute to global warming and are of a limited availability. A promising alternative is microalgae, because of their much higher photosynthetic efficiency, areal productivity and oil content and also that they do not compete with food cultures, arable land, and potable water, and they have the possibility of being harvested on a daily basis (Gouveia and Oliveira, 2009; Gouveia, 2011). However, the current implementation of microalga-based systems has been economically constrained and still has some technological

drawbacks. The main constraints include the limited amount of biomass which can be obtained with currently available photobioreactors, the low biomass productivity, the low harvesting efficiency and the relatively low microalga intrinsic lipid content (Amaro et al., 2011).

In terms of microalgal lipids, the total amount and the type of fatty acids are specific for each species of microalgae and this is linked to environmental factors (e.g. light intensity, pH, salinity, temperature, concentration of nitrogen, and other nutrients; Gouveia and Oliveira, 2009; Gouveia et al., 2009). The lipid content of microalgae is approximately 20–50% of its dry weight with a possibility to attain up to 80% (Spolaore et al., 2006).

In addition to the energy content of the microalgal biomass, these microorganisms have the capacity to synthesize bioactive molecules, such as carotenoids, antioxidants, anti-inflammatory

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and other valuable organic compounds, which can be used in food, feed, cosmetics, biomaterials, nanostructures and the pharmaceutical industry. In particular, the carotenoids such as astaxanthin, canthaxanthin and zeaxanthin have been regarded as free-radical scavengers and cancer preventives and are believed to play an important role in the protection against a large number of chronic and acute health conditions (e.g. Stahl and Sies, 2005).

All these applications are in addition to the original energy purposes. The biorefinery approach consists in the production of a wide range of biofuels and chemicals. This could be done through the use of various technologies in a cost-effective and environmentally sustainable way. The concept is not new, however, it contributes to making biofuel production economically viable (Gouveia, 2011).

The extraction of lipids and carotenoids from microalgae is usually carried out using classical extraction methods, such as using organic solvents and Soxhlet extractions (e.g. Gouveia and Oliveira, 2009; Bai et al., 2011). However, the selectivity is low and a large amount of toxic solvent is needed. In recent years, supercritical fluid extraction (SFE) has become an important alternative to the conventional separation methods. CO<sub>2</sub> is the most used supercritical solvent because the compounds can be obtained without contamination by toxic organic solvents and thermal degradation. The product is solvent free and compatible with the use of the label “natural” (Bruno et al., 1993). On the other hand, supercritical CO<sub>2</sub> has a high diffusivity, a low viscosity, and a low surface tension which improves the mass transfer inside the matrix. Many applications involving supercritical fluid extraction of lipids and bioactive compounds (e.g. carotenoids, fatty acids) from microalgae have been studied (Andrich et al., 2005; Macías-Sánchez et al., 2005; Nobre et al., 2006; Gouveia et al., 2007; Liao et al., 2010). The use of a polar co-solvent like ethanol can improve CO<sub>2</sub> solvent properties for more polar compounds, increasing the extraction rate, as well as the recovery yield. This is particularly interesting when applying supercritical CO<sub>2</sub> to the bioactive compounds extracted from microalgae (Nobre et al., 2006). Furthermore, other authors (Andrich et al., 2005; Macías-Sánchez et al., 2005; Liao et al., 2010) have used SFE with success to obtain lipids and carotenoids from the microalga *Nannochloropsis*, but none of these works have studied the fractionation of the extracts in order to separate lipids from carotenoids.

After the extraction of oils and pigments from the microalga biomass, the remaining biomass could be used in a fermentation process as a substrate to produce hydrogen. The conversion of lipid-extracted microalgal biomass residues into hydrogen plays a dual role in renewable energy production and sustainable development of the microalgal biodiesel industry (Yang et al., 2011). Dark and photo-fermentations are considered the more advantageous biological processes, due to the possibility of using several different feedstocks as a substrate, namely organic wastes and microalgal biomass (Ferreira et al., 2011, 2012; Yang et al., 2011) coupled with the wastewater treatment and CO<sub>2</sub> mitigation to obtain clean energy (Lindblad et al., 2002; Hallenbeck, 2009).

*Nannochloropsis* sp., a marine microalga, commonly used in aquaculture, has recently become more widely recognized as a potential source of lipids for biodiesel production (Gouveia and Oliveira, 2009; Moazami et al., 2012). It has also been considered as a potential source of carotenoids (Lubián et al., 2000), such as astaxanthin, canthaxanthin and zeaxanthin.

Several studies have been done with the microalga *Nannochloropsis*. Examples can be shown from past work in the eighties, where the effect of environmental factors on growth rate, cell-lipid content and productivities in laboratory and outdoor cultures were studied (Boussiba et al., 1987). Recently, the production metrics were studied in a scalable outdoor photobioreactor for commercial applications (Quinn et al., 2012), in terms of the biomass and lipid

productivities, as well as evaluating the process of mixing the culture and its influence on the energy consumption. The supercritical fluid extraction of the bioactive lipids (Andrich et al., 2005), the factors and strategies that affect fatty acid accumulation, such as light intensity and N starvation (van Vooren et al., 2012) and the net energy analysis of the production of biodiesel and biogas from *Nannochloropsis* (Razon and Tan, 2011) are only some of the many examples of the studies done with this microalga.

The aim of this work was to use the biomass of the *Nannochloropsis* sp. and apply the biorefinery approach for the production of oil (for biodiesel production), high added-value compounds (carotenoids) and biohydrogen. This work deals with the culture of the microalga, the supercritical fluid extraction of the oil and high added-value compounds (carotenoids), and the production of biohydrogen using the remaining biomass. Furthermore, for comparison the supercritical fluid extraction process with conventional methods, the oil and carotenoids from the microalga were also obtained by Soxhlet and Bligh & Dyer, acetone and ethyl acetate extractions, respectively.

## 2. Methods

### 2.1. Microalga

*Nannochloropsis* sp. (NANNO-2 from SERI algotec) was grown in modified GPM medium (0.200 g/L KNO<sub>3</sub>, 0.038 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.034 g/L H<sub>3</sub>BO<sub>3</sub>, 0.030 g/L Na<sub>2</sub>EDTA, 4.30 mg/L MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.45 mg/L FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.30 mg/L ZnCl<sub>2</sub>, 0.13 mg/L CoCl<sub>2</sub>·6H<sub>2</sub>O) in 75% filtered seawater (GF/C filter 1.2 µm pore) and 25% de-ionized water.

The microalga was cultivated in polyethylene bags (PBR) of 10 L capacity, agitated by bubbling air (1 vvm/mL L<sup>-1</sup> min<sup>-1</sup>), at a constant temperature of 25 ± 1 °C and under 25.7 µmol/µEinstein light intensity (measured at the surface of the PBR with Phywe Lux-Meter) by fluorescence lamps (Philips TL-D 36 W/54-765).

Each culture was grown for 40 days, in order to guarantee high lipid accumulation during the stationary phase (20 days). The growth of the cultures was measured through O.D.<sub>540 nm</sub> in a cuvette's path length of 1 cm (Hitachi U-2000) and dry weight concentration (GF/C filter 1.2 µm pore).

The harvesting of the biomass was carried out by stopping agitation, followed by centrifugation at 10,000 rpm for 10 min (Beckman Avanti J-251) and dried in an oven at 70 °C.

### 2.2. Extraction

Previously to extractions, the microalga *Nannochloropsis* sp. was ground using a ball mill Retsch® model MM400. 0.5 g of dry biomass was milled for 3.5 min, using 8 balls (10 mm Ø) and speed of 25 s<sup>-1</sup>. This procedure allowed to homogenize the samples, as well as increasing the contact area of the microalgae with the extraction solvent.

#### 2.2.1. Total lipid content of biomass

Total lipid content in the microalga biomass was assessed by two different methods: Soxhlet extraction and Bligh and Dyer (1959). The Soxhlet extraction was carried out using 1 g of microalgae for 6 h. The amount of total lipids was determined gravimetrically. Two solvents were tested: hexane and ethanol.

In the Bligh and Dyer extraction (Bligh and Dyer, 1959) 150 mg of ground microalga biomass was added to a mixture of methanol, chloroform and water (10:5:4, v/v/v) and stirred for 24 h. Afterward, the sample was centrifuged, removing the liquid and stored in a separating funnel. This process was repeated two more times using a stirring time of 2 h. Chloroform and water were added to

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