CO₂-supplemented brewery wastewater treatment by microalgae and biomass upgrading for bioenergy production

Alice Maria Garcia Ferreira
Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal
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Abstract: The ability of microalgae to grow in nutrient-rich environments and to accumulate nutrients from the wastewater, make them attractive for sustainable and low cost wastewater treatment coupled with the production of potentially valuable biomass, which can be used for the production of bioenergy, food, feed, fertilizers, among others. The first objective of this work was to treat the brewery wastewater from Sociedade Central de Cervejas e Bebidas, S.A., using bubble-column PBRs, with 10% (v/v) CO₂ supplementation. The PBRs were inoculated with the microalga Scenedesmus obliquus and several residence times (HRT) were tested: 2.1±0.5, 3.5±0.4, 5.3±0.3, 6.5±0.4, 8.7±0.2 and 10.4±0.2 days. The experiments achieved a maximum volumetric productivity of 0.2 gAFDW L⁻¹ d⁻¹ for a dilution rate of 0.29 d⁻¹, which corresponds to a HRT of 3.5 days. The maximum removal rates attained were: 93 and 89% for ammonia and total nitrogen, respectively; 41% for phosphorus; and 62% for COD. Except for 0.48 d⁻¹, all the treated waters are in accordance with the Portuguese environmental legislation (Decree-Law 236/98). Considering the volumetric productivity, treatment efficiency and lower residence time, 0.29 d⁻¹ represents the optimal dilution rate. The potential of S. obliquus biomass produced in different wastewaters (poultry, swine, cattle, domestic, brewery and dairy) was evaluated for the production of bioH₂ through dark fermentation with Enterobacter aerogenes, and bio-oil through pyrolysis. The results for bioH₂ were 390 mL H₂/gVS, with a purity (H₂/CO₂) of 7, and for bio-oil production was 83%, both for S. obliquus grown in swine waste.

1 INTRODUCTION

1.1 Overview of the brewing industry

The brewing industry holds a strategic economic position with an annual world production up to 2 billion hectoliters in 2014 (Inc., 2016). The brewing process generates large amounts of wastewater and solid wastes. It is widely estimated that for 1 liter of beer, about 3-10 liters of waste effluent is generated. This waste must be disposed off or treated accordingly to discharge regulations set by government entities, in order to protect both human and animal lifes, and the environment (Olajire, 2012; Simate et al., 2011).

1.2 Wastewater management

1.2.1 Conventional wastewater treatment

The first treatment steps consist mainly on physical unit operations, to remove coarse solid matter, but not dissolved pollutants (Simate et al., 2011). In chemical methods, different chemicals can be added to the brewery wastewater to alter its chemistry (e.g. pH adjustment and/or coagulation/floculation). Biological methods are based on the activity of a wide range of microorganisms, which convert the biodegradable organic pollutants present in the wastewaters. In fact, since brewery effluents have both chemical (high organic content) and microbial contaminants they are generally treated by biological methods. However, while these technologies are available to remove nutrients, they consume significant amounts of energy and chemicals, which makes them costly processes for industries and municipalities (Christenson and Sims, 2011).

1.2.2 Microalgae-based wastewater treatment

Microalgae-based treatment seems to be quite promising for combining the biomass growth with the biological removal of the wastewater contaminants in a less expensive and ecologically safer way with the added benefits of resource recovery and recycling (Christenson and Sims, 2011). The treatment of industrial effluents occurs via photosynthesis and
through nutrients recycling with N and P assimilation into the algal-bacterial biomass (Razzak et al., 2013).

The use of microalgae for wastewater treatment presents major advantages since it rests on the principles of natural ecosystems, with the O\textsubscript{2} needed for the bacteria being provided through microalgae photosynthesis, minimizing the hazardous solid sludge formation and greenhouse gas emissions, coupled to the production of useful algal biomass-energy with recycling of the nutrients present in the wastewater (Gouveia et al., 2016). However, some progress has to be made since algal systems still have some important drawbacks: (1) generation times are long (hours to days); (2) harvesting is difficult and costly; (3) light is required; and (4) the biomass concentration is rather low (de la Noé et al., 1992).

Regarding the potential of using microalgae for brewery wastewaters treatment there are already some studies. These works (Darpito et al., 2015; Mata et al., 2013; Kong et al., 2012; Raposo et al., 2010) proved that, under certain conditions, microalgae can successfully incorporate the nutrients present in brewery wastewater for their growth. Additionally, the resulting microalgae biomass can be further upgraded for several purposes such as the production of bioenergy, food, feed, fertilizers, among others.

Scenedesmus obliquus is a versatile organism that can be easily cultivated since it presents a rapid growth and a great adaptation to the environmental conditions (Batista et al., 2015; Mata et al., 2012; Hodaifa et al., 2008).

1.3 Biofuels production

Nowadays, fossil fuels are cheaper and readily available. However, due to their increasing prices and negative long-term impact on the environment, there is the need to find alternatives to them. Of the many options available, microalgae are seen as one of the best potential feedstocks for producing sustainable fuels for transportation, since they can grow and be harvested almost continuously, reducing the seasonal problems of raw materials supply for the biofuels industry. In addition, they have high biomass and lipid productivities, and can use nutrients from waste streams (e.g. wastewaters and/or CO\textsubscript{2} flue gas emissions) which helps reducing their environmental impacts and production costs to a point competitive for fossil fuels (Mata et al., 2014; Mata et al., 2013).

1.3.1 Thermochemical conversion (Pyrolysis)

Pyrolysis consists in the thermal decomposition of materials at medium to high temperatures (350-700 °C), in the absence of oxygen to convert them into different valuable products: bio-char, bio-oil and syngas (Razzak et al., 2013; Silva et al., 2016). After conversion, there’s only minor residues left such as ash for disposal. Major research on pyrolysis products is focused on lignocellulosic biomass. However, microalgae are pointed out as a promising feedstock for pyrolysis processes (Ferreira et al., 2014).

The thermogravimetric analysis (TGA) is the usual technique to first characterize the microalgae behaviour in pyrolysis atmosphere. This technique generates a TG curve which represents the evolution of the mass (weight loss) as a function of the temperature.

1.3.2 Biochemical conversion (Biohydrogen production)

Hydrogen (H\textsubscript{2}) has the highest energy content per unit weight of any known fuel (142 kJ/kg) and can be transported for domestic/industrial consumption through conventional means, being safer to handle than domestic natural gas (Brennan and Owende, 2010). Biological hydrogen (bioH\textsubscript{2}) can be produced by fermentative processes such as dark fermentation. This consists in the conversion of sugars into H\textsubscript{2}, CO\textsubscript{2} and organic acids by microorganisms, through the acidogenic pathway. Microalgae are attractive feedstocks for dark fermentation, since they are able to absorb solar energy and CO\textsubscript{2} and convert it into chemicals, which can be stored as carbon compounds, such as starch, a fermentable substrate for bioH\textsubscript{2} production (Ortigueira et al., 2015).

2 MATERIALS AND METHODS

2.1 Brewery wastewater treatment

2.1.1 Feed effluent

The brewery wastewater was collected from the brewery SCC at Vialonga, after secondary treatment by anaerobic digestion. The effluent was stored in a refrigeration chamber at 10 °C. Due to large amounts of sludge present in the effluent, it was left to settle for 24 hours at room temperature and only the supernatant was used as the algal culture.

2.1.2 Microalgae

The microalga used was Scenedesmus obliquus (ACOI 204/07) from the Coimbra University Algotec, Portugal. *S. obliquus* was maintained on the appropriate culture medium (Bristol), at room
temperature, under orbital shaking (150 rpm) and continuous artificial light of 72 W (4 lamp \( \times 18 \) W/865). Bristol medium contains NaNO\(_3\) (0.250 g/L), KH\(_2\)PO\(_4\) (0.175 g/L), K\(_2\)HPO\(_4\) (0.075 g/L), MgSO\(_4\).7H\(_2\)O (0.075 g/L), Fe-EDTA (0.060 g/L), CaCl\(_2\).2H\(_2\)O (0.033 g/L), NaCl (0.025 g/L) and Chu’s trace elements solution (1 mL/L) (Vonshak, 1986).

2.1.3 Photobioreactor system

The continuous photobioreactor system was composed by five PBRs (14 cm in diameter and 40 cm in height) operating in parallel, that were continuously fed with brewery wastewater from a 20 L polycarbonate carboy containing the feed effluent, through a series of silicone rubber tubes attached to glass tubes at both ends, one immersed in the effluent in the carboy and the other end at each PBR. A working volume of 5 L was maintained by overflow at a fixed level and the outlet streams were collected in plastic containers, through silicone rubber tubing. The air was mixed with CO\(_2\), purified from the brewery, through a "Y" connection, reaching the culture through an aquarium Elite air diffuser (Hagen), placed at the centre bottom of each PBR, at a flow rate of 0.1 vvm (measured with an American Meter Company flow meter). The culture was continuously illuminated by 3 fluorescent lamps (Philips 36 W) assembled at one lateral side of the PBR, with an average light intensity of 3.2 klux (measured with a Phywe Lux-meter). Figure 1 represents a scheme of the system described above.

![Figure 1: Schematic representation of the PBR system for the continuous assays. Adapted from (Marchão, 2016).](image)

2.1.4 Photobioreactor operation

First, each PBR was inoculated with 120 mL of microalgal culture, diluted with 2 L of brewery effluent and operated in fed-batch mode for biomass acclimatization. The PBRs were agitated by filtered compressed air and maintained at room temperature and under artificial light. The PBR volume was gradually increased until a working volume of 5 L. Once the culture started to stabilize, the operational mode was changed from fed-batch to continuous, by feeding fresh brewery wastewater to the culture and the filtered compressed air was replaced by air enriched in CO\(_2\) (10% (v/v)). Air agitation was adjusted manually. In order to test six different hydraulic retention times (HRT) values (2.08, 3.51, 5.27, 6.50, 8.74 and 10.4 days), different feeding rates were imposed manually in each PBR, using Hoffman tubing clamps. Samples were collected from the PBRs 2 times a day (except on weekends) for pH (InoLab WTW) and OD measure (Hitachi U-2000 spectrophotometer) to evaluate the microalgae growth. The pH of the cultures was maintained between 7 and 8 by adjusting the feeding of CO\(_2\). The continuous assays ended when the algal growth reached the stationary phase. The biomass and the effluent were then collected for further analysis.

2.1.5 Biomass and supernatant recovery

At the end of the assay, the collected culture volume was left to settle for 48 hour at room temperature for a primary concentration of the biomass through decantation. The supernatant was collected and filtered through 47 mm Whatman 45 \( \mu \)m filters. The concentrated biomass was recovered by centrifugation at 10 000 rpm during 10 min at 15 °C (Heraeus multiuge 3SR+ centrifuge, Thermo Scientific) and freeze-dried (Heto Power Dry LL3000, Thermo Scientific).

2.1.6 Brewery wastewater and supernatants characterization

The effluent from SCC was first characterized in terms of pH, COD, nitrogen (ammonia and Kjeldahl nitrogen) and phosphorus, before and after being settled for 24 hours, respectively, raw and decanted effluent. In order to evaluate the efficiency of the wastewater treatment by microalgae, the same analyses were performed for the different filtered supernatants collected after the cultivation runs.

COD was determined according to the standard method 5220 B (Apha, 1998). A 20 mL sample was mixed with the proper reagents (\( \sim 0.5 \) g of HgSO\(_4\), 30 mL of H\(_2\)SO\(_4\) and 10 mL of K\(_2\)Cr\(_2\)O\(_7\) solution) and left to reflux for 2 h at 150 °C in a Bloc digester 20 P-Selecta. After cooling, the mixture was diluted with distilled water until a volume of 400 mL and titrated...
with standard ferrous ammonium sulphate (0.25 N), using 2-3 drops of ferroin indicator solution.

Ammonia nitrogen was quantified by titration after a preliminary distillation step based on standard methods 4500-NH3 B and C (Apha, 1998). In a Kjeldahl tube, a 150 mL sample buffered at pH 9.5 with 25 mL of borate buffer was distilled in a Buchi Distillation Unit K-350 for 6 min. The distillate was collected in an Erlenmeyer flask containing 50 mL of boric acid indicator solution and was titrated with a stock solution of H2SO4 0.02 N.

The Kjeldahl nitrogen was determined according to the modified Kjeldahl method adapted from the standard method 4500-Norg B (Apha, 1998). In a Kjeldahl tube, 5 mL of sample and 50 mL of digestion solution were mixed together and digested in a Buchi Digestor Unit K-424 for 4–4.5 h. After cooling, it was diluted with 100 mL of distilled water and distilled in the distillation unit for 6 min with 50 mL of reagent sodium hydroxide-sodium thiosulfate.

A commercial kit for phosphorus determination was used with the Phosver 3 (ascorbic acid) method using Powder Pillows (Spectrophotometer HACH DR/2010). This tests provides the following results, in mg/L: PO4-P (phosphate), P-PO4 (phosphorus presented in the phosphate) and P2O5 (phosphorus pentoxide).

In the end, the removal rate for these nutrients were determined according to equation 1, where Ce and Cs are the concentrations of the parameter in the effluent and in the filtered supernatant, respectively.

\[
\text{Removal(\%) = } \frac{C_e - C_s}{C_e} \times 100 \quad (1)
\]

2.1.7 Microalgal characterization

Determination of ash-free dry weight Microalgal growth was evaluated by measuring the ash-free dry weight (AFDW) of the 6 cultures. This was done by filtering 5 mL of microalgal culture through a preweighed GF/C 25 mm Whatman 45 µm filters. These filters were left to dry overnight at 105 °C and then weighted after cooling in a desiccator (woven). The filters were further incinerated in the muffle furnace (550 °C for 1 h) and finally weighted (w_muffle). The AFDW value was calculated through equation 2.

\[
\text{AFDW (gAFDW/L) = } \frac{w_{\text{oven}} - w_{\text{muffle}}}{V_{\text{sample}}} \quad (2)
\]

Protein content The Lowry method (Lowry et al., 1951) was used to measure the protein content of the microalgal biomass through equation 3, where C is the protein concentration (mg L⁻¹), V is the volume (L) of the NaOH solution used to pretreat the biomass, D is the dilution factor and mbiomass is the amount of biomass (mg).

\[
\text{Protein content (\% w/w) = } \frac{C \times V \times D}{m_{\text{biomass}}} \times 100 \quad (3)
\]

Chlorophyll content Microalg culture samples from the PBR (2 mL) were first concentrated by centrifugation for 10 min at 3900 rpm (Sigma 2-6E, Sartorius). Then, 2 mL of acetone (99.5%, Sigma-Aldrich) and glass beads were added to the tube and the extraction of chlorophyll was performed by submitting the mixture to vortex during 2 min followed by 2 min in an ice bath. The mixture was then centrifuged at 3900 rpm for 20 minutes, and the supernatant collected. The extraction procedure was repeated until a colourless supernatant was obtained. All the tubes were covered with aluminum foil to prevent pigment degradation by light exposure. The total volume of the extract phases collected was quantified. Chlorophylls a and b in the extracts were quantified by spectrophotometry (Hitachi U-2000), measuring the absorbance at 630, 647, 664 and 691 nm. The calculations were performed using equations proposed by (Ritchie, 2008). The chlorophyll concentrations in the culture samples (Chl a and Chl b) were then calculated using equation 4.

\[
\text{Chl (mg/L) = } \frac{\text{C} \times V_{\text{extractant}}}{V_{\text{sample}}} \quad (4)
\]

Finally, the chlorophyll contents (Chl a and Chl b) in the algal cells (mg/g) were calculated by dividing the concentration of the chlorophylls (mg/L) by the cell dry weight (g/L) in the culture samples and total chlorophyll content was obtained by summing the values for Chl a and Chl b.

Total sugars content The sugars present in microalgal cells (300 mg of freeze-dried biomass) were first extracted by quantitative acid hydrolysis, a process described by Hoebler et al. (1989).

Following extraction, the total sugars content was determined by the colorimetric method of phenol-sulfuric reagent (DuBois et al., 1956). Using standard glucose solutions, it was possible to build a calibration curve that was used to calculate the total sugar content of the various samples through their absorbance at 490 nm.

Fatty acid content Fatty acid composition of the biomass samples was analysed, in duplicate, by gas
chromatography (GC). For this, the fatty acids were first transesterified by the method of Lepage & Roy (1986) modified. 100 mg of freeze-dried microalgae were added to Pyrex tubes with Teflon-sealed screw caps. Then, 2 mL of a methanol/acytate chloride (95:5 v/v) mixture and 0.2 mL of heptadecanoic acid in petroleum benzìn 60-80 °C (5 mg/mL) as internal standard solution were also added. The mixture was heated at 80 °C for 1 h and was cooled to room temperature before being diluted with 1 mL of water and 2 mL of n-heptane. The tube contents were left to stand until phase separation. The upper layer was recovered, dried over anhydrous Na2SO4 and was held at that value for 16 min. Individual fatty acid contents were calculated as a percentage of the total fatty acids present in the sample, determined from the chromatographic peak areas.

**Determination of biomass productivity** In continuous culture at steady-state (if biomass decay is negligible), μ equals the dilution rate (D) value, and the volumetric biomass productivity (P_X) can be determined by equation 5, where X is the biomass concentration at steady-state.

\[ P_X (gL^{-1}d^{-1}) = D \times X \] (5)

### 2.2 Biohydrogen production from *Scenedesmus obliquus*

#### 2.2.1 Bacteria culture conditions

The fermentative bacteria *Enterobacter aerogenes*, ATCC 13408 Sputum (American Type Culture Collection, Manassas, USA) was used for production of hydrogen by dark fermentation (bioH₂), according to Batista et al. (2015).

#### 2.2.2 Microalgae biomass

The microalga used as substrate - *Scenedesmus obliquus* – was obtained by growing in several different effluents as culture medium, such as poultry, swine, cattle, domestic, brewery and dairy. The cattle wastewater was pre-treated by anaerobic digestion (AD cattle waste). All these microalgae biomass were obtained from collaboration works at LNEG.

**Volatile solids (VS) determination** The biomass VS content was determined by incinerating the biomass sample (w_sample) at 550 °C in a muffle furnace for 1 h (w_muffle). The crucibles were previously weighted after drying in an oven at 105 °C for 24 h (w_oven). Finally, the VS content was calculated by equation 6. The analyses were performed in duplicate.

\[ VS (gyr/L) = \frac{w_{oven} - w_{muffle}}{w_{sample}} \] (6)

#### 2.2.3 Dark fermentation assays

Batch fermentation experiments were performed in 159 mL serum bottles, closed with butyl rubber stoppers and crimped with aluminum seals. Each contained 26 mL of basal fermentation medium (see section 2.2.1) and the microalgal biomass (Headspace volume/liquid phase volume = 5). The bioreactors containing the fermentation medium and the substrate (S. obliquus) were previously submitted to autoclave sterilization at 121 °C for 15 min. Then, they were aseptically purged by bubbling N2 through them, before inoculation with exponentially grown *E. aerogenes* at 1% (v/v). The fermentation was carried out under orbital shaking (150 rpm) for 6 h at 30 °C. The initial concentration of the substrate (microalgal biomass) was 2.5 g/gyr/LFM (Batista et al., 2015). All the experiments were performed in triplicate.

#### 2.2.4 Analysis of the gas phase from the dark fermentation

The content of H2 and CO2 in the fermentation headspace was analyzed by GC, at atmospheric pressure, in a Varian 430-GC equipped with TCD and a fused silica column (Select Permanent Gases/CO2-Molsieve 5A/Borabound Q Tandem #CP 7430). Injector and column were operated at 80 °C and the detector at 120 °C. Helium was the carrier gas. Previously, calibration curves were obtained, in the range of the expected H2 and CO2 concentrations, using standard mixtures, in order to determine the composition of the gaseous phase. Specific hydrogen productions yields were calculated by dividing the total volume of produced hydrogen by the initial amount.
in terms of VS) of *S. obliquus* and by the fermentation medium volume in the bioreactor, at 6 h of the process.

2.3  Bio-oil production from *Scenedesmus obliquus*

2.3.1 Microalgae biomass

The microalgae biomass was the same used for dark fermentation assays (see section 2.2.2).

2.3.2 TGA analysis

The microalgae biomass was characterized by thermogravimetric analysis (TGA). This technique was performed in a TGA apparatus (NETZSCH STA 409 PC/PG), simulating pyrolysis conditions. The samples were heated from 30 to 1100 °C at a heating rate of 25 °C/min in a nitrogen (99.996%) atmosphere.

2.3.3 Pyrolysis operation

The pyrolysis experiments were performed in a quartz fixed bed reactor (16 mm in internal diameter and 150 mm in length) in nitrogen atmosphere. The quartz reactor was filled with carborundum and it was externally heated using a Termolab circular electric furnace equipped with PID controller to ensure complete pyrolysis. Figure 2 shows a schematic diagram of the pyrolysis apparatus used.

![Schematic diagram of the fixed bed pyrolysis apparatus](Image)

The pyrolysis test was started by placing about 2.5 g of microalgae into the quartz reactor. Nitrogen was used as the carrier gas. The pyrolysis was set to 475 °C and N₂ flow to 200 mL/min. After the 15 min of the pyrolysis process, the reactor was removed of the oven and the bio-char was removed. The bio-oil was recovered by washing the reactor with acetone followed by rotating evaporation of acetone under a reduced pressure. The yields of pyrolysis products (bio-char, bio-oil and bio-gas) were quantified.

3  RESULTS AND DISCUSSION

3.1  Brewery wastewater treatment by *Scenedesmus obliquus*

3.1.1 Microalgae growth evaluation

The volumetric productivity for all the PBRs were calculated based on their AFDW at the end of the trials, and these values are shown in Table 1.

<table>
<thead>
<tr>
<th>HRT (d⁻¹)</th>
<th>D (d⁻¹)</th>
<th>AFDW (g/L)</th>
<th>PX (mgAFDW L⁻¹ d⁻¹)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>0.48</td>
<td>0.36±0.00</td>
<td>173±20</td>
<td>7.09</td>
</tr>
<tr>
<td>3.5</td>
<td>0.29</td>
<td>0.76±0.02</td>
<td>217±6</td>
<td>8.22</td>
</tr>
<tr>
<td>5.3</td>
<td>0.19</td>
<td>0.88±0.14</td>
<td>167±27</td>
<td>7.93</td>
</tr>
<tr>
<td>6.5</td>
<td>0.15</td>
<td>0.58±0.08</td>
<td>83.9±11.6</td>
<td>7.17</td>
</tr>
<tr>
<td>8.7</td>
<td>0.11</td>
<td>0.95±0.07</td>
<td>109±8</td>
<td>6.85</td>
</tr>
<tr>
<td>10.4</td>
<td>0.10</td>
<td>0.81±0.05</td>
<td>77.9±4.8</td>
<td>7.08</td>
</tr>
</tbody>
</table>

The steady-state times for all the cultures are in accordance to the literature, which states that a microalgal culture reaches its steady-state after 3-5 residence times (Teixeira et al., 2007). At the end of the trial, the biomass concentration and volumetric productivity were calculated, and they are represented as a function of the dilution rate in figure 3.

According to figure 3, AFDW is very dependent on the dilution rate. For higher dilution rates, the AFDW presents a drastic reduction (from 0.88 to 0.51 gAFDW L⁻¹). The highest biomass concentration was obtained for a dilution rate of 0.29 d⁻¹ (0.95±0.07 gAFDW L⁻¹). Regarding biomass productivity, it is possible to observe an increase until a maximum at D = 0.29 d⁻¹ (217.09±5.71 mgAFDW L⁻¹ d⁻¹), which is the optimal dilution rate, and then it decreases.

Consulting the literature, the results obtained, although lower, are comparable to volumetric productivities achieved by McGinn et al. (2012) when cultivating *Scenedesmus* in secondary municipal wastewater in continuous mode at a dilution rate of 0.75 d⁻¹ (234 and 267 mg L⁻¹ d⁻¹). Also, the present results are comparable to the ones obtained by Marchão (2016).

3.1.2 Brewery wastewater treatment evaluation

The biological treatment performance by *Scenedesmus* was evaluated by measuring the
nutrient contents and organic load present in the brewery effluent at the end of the trials. The results are shown in Table 2. Maximum removal efficiencies were also calculated and are presented in Table 3.

The results presented in Table 2 show that, excluding $D = 0.48 \text{ d}^{-1}$, all the other treated waters can be discharged into natural water bodies with no harmful consequences for the environment, since they meet the legal discharge requirements in the Portuguese legislation (Decree-Law 236/98).

Since an efficient wastewater treatment comprises both high nutrient removal efficiency and optimal biomass production, $D = 0.29 \text{ d}^{-1}$ represents the better choice, since it allows the highest removal efficiencies for the wastewater treatment (90.5 and 84.6% for ammonia and TKN nitrogen, respectively; 61.9% for COD) at the highest volumetric productivity ($217.09 \pm 5.71 \text{ mgAFDW L}^{-1} \text{ d}^{-1}$). Additionally, 3.5 days represents the lowest HRT which results in removal of both ammonia and TKN nitrogen (71.4 to 92.9% and 73.1 to 88.5%, respectively), but P had the low removal rates. However, the treatment was effective since the final values are below the legal limits.

The present results for N removal are comparable to the reported values in the literature, but the P removal efficiencies are significantly lower. Mata et al. (2012) obtained removal efficiencies around 11-24.4% and 54-66% for N and P, respectively, while Raposo et al. (2010) and Darpito et al. (2015) both achieved removal efficiencies above 85% for N and up to 90% for P, in batch brewery wastewater treatment using *Scenedesmus* and *Chlorella*. For COD removal, they are comparable to the ones achieved by Mata et al. (2012) (13.3 to 66.8%). Comparing the results with the ones presented by Marchão (2016), we see that in general the present work was able to achieve higher removal efficiencies for N and P, but not for COD.

### 3.1.3 Biomass characterization

The results for the biomass characterization are shown in Table 4.

<table>
<thead>
<tr>
<th>$D$ (d$^{-1}$)</th>
<th>Chl a+b (mg/L)</th>
<th>Crude protein (%)</th>
<th>Total sugars (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.48</td>
<td>31.13±2.80</td>
<td>39.3±0.6</td>
<td>23.42±0.06</td>
</tr>
<tr>
<td>0.29</td>
<td>36.65±1.23</td>
<td>34.2±2.0</td>
<td>24.00±3.21</td>
</tr>
<tr>
<td>0.19</td>
<td>28.80±3.38</td>
<td>40.4±4.5</td>
<td>20.06±0.79</td>
</tr>
<tr>
<td>0.15</td>
<td>40.00±1.95</td>
<td>37.7±1.8</td>
<td>23.05±0.70</td>
</tr>
<tr>
<td>0.11</td>
<td>26.99±0.69</td>
<td>37.6±0.7</td>
<td>26.13±1.31</td>
</tr>
<tr>
<td>0.10</td>
<td>36.03±0.99</td>
<td>38.9±2.5</td>
<td>23.19±0.28</td>
</tr>
</tbody>
</table>

### Table 3: Nutrient maximum removal efficiencies.

<table>
<thead>
<tr>
<th>$D$ (d$^{-1}$)</th>
<th>N-NH$_3$ (%)</th>
<th>TKN (%)</th>
<th>P (%)</th>
<th>COD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.48</td>
<td>71.4</td>
<td>73.1</td>
<td>6.1</td>
<td>55.8</td>
</tr>
<tr>
<td>0.29</td>
<td>90.5</td>
<td>88.5</td>
<td>22.4</td>
<td>61.9</td>
</tr>
<tr>
<td>0.19</td>
<td>85.7</td>
<td>84.6</td>
<td>22.4</td>
<td>60.9</td>
</tr>
<tr>
<td>0.15</td>
<td>81.0</td>
<td>80.8</td>
<td>18.4</td>
<td>58.1</td>
</tr>
<tr>
<td>0.11</td>
<td>90.5</td>
<td>80.8</td>
<td>36.7</td>
<td>55.8</td>
</tr>
<tr>
<td>0.10</td>
<td>92.9</td>
<td>88.5</td>
<td>40.8</td>
<td>50.0</td>
</tr>
</tbody>
</table>

The dilution rate equal zero corresponds to the initial effluent.

Figure 3: Biomass concentration (AFDW) and volumetric productivity ($P_X$) of the different *S. obliquus* cultures at the steady-state as a function of dilution rate in the 6 PBR.
The protein contents are slightly higher than the reported values found in the literature, such as 20.4% for Sc grown in Bristol medium (Batista et al., 2014) and 32.7% for Sc used for domestic wastewater treatment (Gouveia et al., 2016). This can be explained by the higher amount of nitrogen present in the brewery wastewater, when compared to Bristol and domestic wastewater.

Regarding total sugars content, the values obtained are above 20%, which are lower than 30.7 and 31.8% obtained for S. obliquus grown, respectively, in Bristol medium (Batista et al., 2014; Miranda et al., 2012), but higher than 11.7% for S. obliquus used for domestic wastewater treatment (Batista et al., 2015). This means that S. obliquus grown in brewery wastewater has a good potential as a fermentative substrate for the production of biohydrogen.

Relatively to chlorophyll contents, the majority of the present values are much higher than the ones reported by Marchão (2016), using S. obliquus for brewery wastewater treatment, with no CO₂ addition.

The Chla/AFDW ratio is a clear indicator of the physiological state of algal cells. A value lower than 1% means that the population is at risk of crashing, either due to predators or the lack of nutrients (Veloso et al., 1991). Figure 4 shows that all the ratios are significantly higher than 1%, which suggests that there is no risk for the algal cells. These high ratios can be explained by the inclusion of CO₂, which has a positive effect on the stability of the microalgae culture.

### 3.2 Biohydrogen production from Scenedesmus obliquus

The H₂ produced was evaluated through parameters like production yield and purity of the bioH₂ produced, which are presented in figure 5.

Most attained values for the different microalgae were comparable to the ones reported by Batista et al. (2014) when fresh synthetic culture medium was used for S. obliquus growth (57.6 mL H₂/gVS) and by Batista et al. (2015) with domestic wastewater as growth medium (56.8 mL H₂/gVS). The highest H₂ volumetric production were 390 and 378 mL H₂/gVS and were attained by S. obliquus cultivated in swine and poultry waste, respectively. It should also be noted that both values are contained in a considerably superior range of values, when compared to the other Sc in study, which can be easily seen in figure 5. These results can be explained not only by their higher VS content (above 90%), but also by the accumulation of organic load from the wastewater they were grown within. All the bioH₂ produced was very rich in H₂, with all H₂/CO₂ ratios higher than 1.

In conclusion, the results presented undoubtedly demonstrated the potential of the S. obliquus biomass as feedstock for hydrogen production by dark fermentation, even if it is grown in industrial effluents, which is particularly valuable from the economic point of view.

### 3.3 Bio-oil production from Scenedesmus obliquus

Figure 6 presents the yields obtained for each pyrolysis product (bio-oil, bio-char and bio-gas). It is possible to conclude that the microalga grown in swine wastewater has a greater potential for bio-oil production with a yield of 82.8%, followed by the S. obliquus grown in brewery wastewater (64.1%). However, all the other microalgae studied presented bio-oil yields lower than the S. obliquus grown in synthetic medium. On the other hand, the microalga
cultivated in poultry waste achieved the highest yield of bio-gas production (46.5%). The bio-char yields do not present significant changes, ranging from 18.1 to 37.2%. The yields obtained for the *S. obliquus* grown in swine wastewater were significantly higher when compared to the others, with bio-oil and bio-char production yields already making 100%. This may be due to the composition of this biomass that enhances the bio-oil production, namely the protein and carbohydrates content, which according to TGA and FTIR data (data not shown), are very high for this microalga. No reference to this was found in literature, however. Other explanation for this result may also be the low amounts of microalga available (less than 2 g), which are susceptible to a higher error.

![Figure 6: Production yields of bio-oil, biochar and bio-gas from pyrolysis of *S. obliquus* biomass grown in different wastewaters. The values correspondents to Bristol were obtained from Silva et al. (2016).](image)

### 4 CONCLUSIONS

The integrated microalgae-based approach studied in this work allowed the combination of the microalgae growth with the brewery and CO$_2$ effluents treatment, enabling the reduction in the cost of acquisition of nutrients and the use of potable water for microalgal cultivation. Moreover it enabled the recovery and valorization of the biomass produced towards biofuel production.

The supplementation of the culture medium with CO$_2$ showed benefits for the microalgal growth and the brewery wastewater treatment, since it prevented nutrient losses through pH mediated processes (phosphate precipitation and ammonia volatilisation), ensuring that most nutrients are available for microalgal assimilation.

Regarding the bioH$_2$ production by dark fermentation process, the hydrogen yields attained for *S. obliquus* grown in wastewater media were higher than the ones using synthetic medium with lower costs, energy input, and GHG emissions and minimal impact on freshwater supplies. Moreover, the biogas produced achieved high purity levels, which is crucial for reducing costs associated with purification. For the pyrolysis experiments, high yields of bio-oil were achieved for all the microalga in study.

### REFERENCES


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