Brewery wastewater treatment by microalgae using closed bubble-column photobioreactor

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Abstract

Microalgae seem to be an alternative to the traditional biodiesel feedstocks. Their potential high biomass and lipid productivities and the possibility of using nutrients from waste streams (e.g. liquid wastewaters and/or CO₂ flue gas emissions) can help reduce the environmental impacts and costs of cultivation. Moreover, microalgae are a feedstock for production of feeds, fertilizers, other fuels and chemicals.

The main objective of this work was to evaluate the growth and the brewery wastewater treatment by Scenedesmus obliquus, in bubble-column type photobioreactors, under batch (5 L capacity) and continuous mode (6 L capacity). Seven residence times (τ) were tested: 1.72, 2.26, 3.85, 5.58, 7.55, 9.46 and 11.80 days. Regarding the trial in batch mode, it was obtained an ash-free dry weight (AFDW) of 0.93 ± 0.01 g/L and a maximum and average biomass productivity of 156.80 ± 4.65 mg/L.day and 87.11 ± 3.37 mg/L.day, respectively. Concerning the continuous mode, the highest AFDW was obtained for the dilution rate (D) of 0.08 day⁻¹ (τ = 11.80 days): 0.95 ± 0.04 g/L. The optimal dilution rate for biomass productivity was 0.26 day⁻¹ (τ = 3.85 days), reaching 224.30 ± 22.99 mg/L.day. Batch culture and continuous culture at D = 0.26 day⁻¹ presented the best wastewater treatment efficiency. Batch production attained a 75% removal for total Kjeldahl nitrogen, 43% for phosphorous and 70% for chemical oxygen demand (COD). On the other hand, at D = 0.26 day⁻¹, slightly higher removal rates for COD and N were reached (74 and 76%, respectively), but the removal of P was not so efficient (23% removal). Except for the dilution rate of 0.58 day⁻¹ (τ = 1.72 days), the treated water had values that are in accordance with Portuguese environmental legislation (Decree-Law 236/98) for discharge in receiving water bodies. S. obliquus biomass presented a maximum protein content of 50.09% for D = 0.26 day⁻¹ and a minimum of 21.15% for the culture in batch mode. At that dilution rate of D = 0.26 day⁻¹ the biomass productivity, removal of nutrient and biomass protein content were maximum, and these results are in accordance to the highest percentage of viable cell (92.33 ± 0.53 %).

Key words: Photobioreactor, brewery wastewater treatment, biomass growth, Scenedesmus obliquus.

1. Introduction

Microalgae are an extremely heterogeneous group of mainly photoautotrophic organisms, that can be eukaryotic or prokaryotic (Mata et al., 2010). Microalgae can assume different types of nutritional modes (e.g. autotrophic, heterotrophic, mixotrophic). Some algae strains can combine autotrophic photosynthesis and heterotrophic assimilation of organic compounds in a mixotrophic process (Brennan & Owende 2010). Regarding the production systems, closed photobioreactors (PBRs) have attracted much interest because they allow a better control of the cultivation conditions than open systems. With closed photobioreactors, higher biomass productivities are obtained and contamination can be more easily prevented (Ugwu et al., 2008). Vertical column PBRs stands out because they offer the most efficient mixing with low shear stress and the highest volumetric mass transfer rates. They are low cost, compact and easy to operate. On the other hand, one of the major limitation is small illumination surface area (Ugwu et al., 2008).

PBRs can be operated in batch, semicontinuous/febtach and continuous mode. Continuous reactions offer increased opportunities for system investigation and analysis (Mata et al. 2010). While batch studies are critical for determining basic growth phases and characteristics of the algal species, steady-state studies are necessary to better understand and measure the specific growth parameters (Tang et al., 2012).
There are several factors influencing algal growth: abiotic factors such as light, temperature, nutrient concentration, O₂, CO₂, pH, salinity, and toxic chemicals; biotic factors which concerns pathogenicity and competition by other algae and operational factors such as shear produced by mixing, and dilution rate (Mata et al., 2010). The potential threat of global climate change has increased, mainly because of greenhouse gas emissions from fossil fuel usage for transport, electricity and thermal energy generation. Biofuels are seen as real alternatives to replace fossil fuels. (Brennan & Owende, 2010). The main advantages of using microalgae for biofuels are: a rapid growth potential combined with high oil content, oil productivity of microalgae cultures exceeds the yield of the best oilseed crops, they need less water than terrestrial crops and the cultivation can be performed in non-arable land, not competing with production of food (Brennan & Owende, 2010). Microalgae can be used for the production of liquid biofuels (bioethanol, biodiesel, biobutanol and bio-oil) or gaseous biofuels (biomethane, biohydrogen, syngas) (Beer et al. 2009).

Furthermore, microalgae have the potential for co-production of non-energy products like carbohydrates, proteins, and high value bioproducts, such as pigments, vitamins and polyunsaturated fatty acids (PUFAs) (Trivedi et al. 2015). The economic viability of algae-based biofuels is still unfeasible. However, microalgae-based biorefineries could play a major role in turning economics and create many opportunities in the global microalgae business. Only the co-production of high added value products and environmental benefits, such as water bioremediation and CO₂ mitigation, could eventually compensate the high production costs of mass microalgae cultivation and support a microalgae-based bioeconomy (Gouveia 2014).

Many species of microalgae are able to effectively grow in wastewater conditions through their ability to utilize organic carbon and inorganic N and P in the wastewater, as well as heavy metals (Brennan & Owende, 2010). Some advantages of employing microalgae in treatment of wastewater are: cost effectiveness; low energy requirement; efficient recycling of nutrients, as the produced biomass is useful for several applications and reduction in sludge formation (Al-rajhia et al., 2012; Pittman et al., 2011):

Algal harvesting consists of biomass recovery from the culture medium and may contribute to 20–30% of the total biomass production cost. (Brennan & Owende 2010). In the first place it is performed a bulk harvesting employing processes as flocculation, flotation or gravity sedimentation. After that, the obtained slurry is thickened with more energy intensive techniques (Brennan & Owende 2010). Gravity sedimentation is the most common harvesting technique for algae biomass with low value and in wastewater treatment because of the large volumes treated (Brennan & Owende, 2010). On the other hand, for high-value products it is often recommended to use continuously operating centrifuges (Mata et al., 2010).

In the brewing industry, a large amount and variety of wastes are produced. Brewery effluents are generally treated by biological methods. Biological treatment compared to physicochemical or chemical methods, has three advantages: the treatment technology is mature, presents high efficiency in COD and BOD removal (80 to 90%) and requires low investment cost (Simate et al. 2011). Therefore, after the brewery wastewater has undergone physical and chemical pretreatments, the wastewater can suffer biological treatment (Simate et al., 2011). There are two processes: anaerobic (without oxygen) and aerobic (with air/oxygen supply). Anaerobic pre-treatment followed by aerobic post-treatment will result in a positive energy balance, reduced sludge production and space saving (Driessen & Vereijken 2003). Regarding the potential of using microalgae for brewery wastewaters biological treatment few studies still exist. Some works (Raposo et al. 2010; Kong et al. 2012; Mata et al. 2013; Farooq et al. 2013; Mata et al. 2014; Darpito et al. 2015) proved that under certain conditions microalgae succeeds to grow in the brewery wastewater by using their easy biodegradable contaminants as nutrients. Besides removing the organic compounds in the wastewater, the final microalgae biomass obtained can be used as animal feed, as fertilizer, for the extraction of valuable compounds and even for biodiesel production.

Because of an ease cultivation, rapid growth and adaptation to the environmental conditions, *Scenedesmus obliquus* is a versatile organism for the use in domestic and industrial wastewater treatment (Hodaiha et al. 2008; Mata et al. 2012; Batista et al. 2015).

The main objective of this work was to evaluate the growth and the wastewater treatment by *S. obliquus* using simple bubble-column type PBRs, which were operated in batch and continuous regimes.

2. Materials and methods

2.1 Effluent

The brewery effluent for the growth of the microalg is collected from the anaerobic digester BIOPAQ®IC from the Vialonga Factory of SCC - Sociedade Central de Cervejas e Bebidas, S.A. The effluent was stored in a refrigerating chamber at 4°C until needed for use as the algal cultivation
medium, after adjustment to room temperature. At that time the effluent was characterized. For the batch production, the effluent was used without any supplementation or pre-treatment. The effluent was let to settle 24 h in the dark at room temperature and the upper layer was then separated for characterization and use as the algal culture medium for the production of _S. obliquus_ in continuous mode. This step was necessary to allow settling of the suspended materials, that could origin clogging problems in the feeding tubes.

2.2 Microalgae

In this study it was used _Scenedesmus obliquus_ (ACOI 204/07, Coimbra University Culture Collection, Portugal). In a previous study, that microalga, _Chlorella vulgaris_, _Chlorella protothecoides_, _Neochloris oleoabundans_ and a previously isolated consortium from an urban effluent (Gouveia et al. 2016) were tested separately for growth in the referred effluent. _S. obliquus_ showed better results concerning the adaptation to the effluent and presented higher biomass concentration.

_S. obliquus_ was maintained on standard liquid media (Bristol), in an incubator (New Brunswick Scientific Co. Inc EDISON NJ USA) at 25 °C, under an agitation speed of 120 rpm and continuous illumination delivered by 4 fluorescent lamps (Philips 18 W), providing an average light intensity of 1.1 klux (measured with a Phywe Lux-meter).

2.3. Photobioreactors

It was used a 5 L Polyethylene terephthalate (PET) bottle as a bubble-column photobioreactor (PBR) to grow _S. obliquus_ in batch mode, indoors, in the laboratory. The PBR has a diameter of 14 cm and 34 cm in height.

6 L PET bottles with 14 cm diameter and 40 cm in height were used as bubble-column PBRs to grow _S. obliquus_ in a continuous mode in the laboratory. In order to perform a parallel test of six different residence times (τ), was designed a continuous photobioreactor system that consisted in six PBRs (one for each residence time), placed in parallel, associated to a 20 L polycarbonate carboy containing the effluent, as depicted in the figure 1. The effluent was fed to each one of the PBRs by a silicone rubber tube attached to a glass tube immersed in the effluent on the carboy. The outlet stream (overflow) was collected in plastic containers by silicone rubber tubing fixed in an opening in each of the PBR.

2.4 Photobioreactor operation

For batch production of _S. obliquus_ in the brewery effluent, the inoculum was a microalgal culture grown in a 250 mL Erlenmeyer on Bristol medium, until it reached an optical density of OD$_{540}$ ~ 1.2. The PBR was inoculated with 160 mL of inoculum culture and the working volume was completed till 4 L (dilution factor=1/25) with effluent. The essay was conducted until the stationary phase. A parallel PBR was operated as a control, in the same conditions, but only with effluent (without inoculation).

For continuous mode, the inoculum was a microalgal culture grown in batch mode in a PBR, until it reached an OD$_{540}$ ~ 3.5. To start-up, the PBRs were inoculated with 550 mL of inoculum culture each and 4 L effluent and were operated in batch mode. Once the culture started to stabilize, the operational feeding mode was switched from batch to continuous, feeding effluent to each photobioreactor. The carboy was covered with aluminum foil to prevent the light from reaching the effluent, in order to avoid the growth of photosynthetic organisms. The working volume of 5 L was maintained by overflow of the culture once the liquid reached the desired level leading to the withdrawal of culture by the silicone rubber tubing. Microalgae growth and wastewater treatment were tested as a function of residence time/dilution rate. Six τ were tested in parallel: 2.26, 3.85, 5.58, 7.55, 9.46 and 11.80 days. Another residence time was tested (1.72 days), in order to draw a conclusion about the dilution rates that the microalgae can withstand before occurring washout. The steady state was considered established when three consecutive OD readings provided the same value (usually after 3-5 turnovers (residence times)) (Teixeira et al. 2007).

The different feeding rates were controlled manually using Hoffman tubing clamps. In order to minimize fluctuations in the flow rates, the liquid level on the carboy was maintained, replacing the effluent every day with fresh effluent.

Residence times were calculated from the equation (1).

\[ \tau = \frac{V}{\bar{F}} \]  

(1)

where V is the photobioreactor volume (L) and \( \bar{F} \) (L/day) is the mean value of flow rate.

An aquarium Elite air diffuser (Hagen) was used for air supply and agitation. The culture was illuminated continuously with an average light intensity of 3.2 klux (measured with a Phywe Lux-meter), supplied by 3 fluorescent lamps (Philips 36 W) assembled on one lateral side of the PBR.

For both modes, samples were collected 2-3 times a day (except weekends) for OD readings from the PBR to evaluate the microalgal growth. From 1.5 – 2.5 L of culture the microalga biomass was harvested for characterization and evaluate the efficiency of the wastewater treatment.
The 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 borax 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 by a modified Kjeldahl method adapted from the standard method 4500-N₅O B (APHA 1998). In a Kjeldahl tube, 5 mL of sample and 50 mL of digestion reagent (per liter: 134 g K₂SO₄ + 650 mL H₂O + 200 mL H₂SO₄ + 2 g HgO/25 mL H₂SO₄ 6N) were added. The mixture was 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 (per liter: 500 g NaOH + 25 g Na₂S₂O₃.5H₂O). The distillate was collected in an Erlenmeyer containing 50 mL of boric acid indicator solution and was titrated with a stock solution of H₂SO₄ 0.02 N.

All of these analyses were performed in duplicate.

A commercial kit for phosphorous determination has been used (also called orthophosphate) Phosver 3 (ascorbic acid) method using Powder Pillows (Cat. 212599) in a HACH DR/2010 spectrophotometer. This test gives the results in mg/L of PO₄³⁻ (phosphate), P₂O₅ (phosphorus pentoxide) and P–PO₄³⁻ being the phosphorus presented in the phosphate.

2.6.2 Microalgae growth

In batch mode, the biomass concentration was measured by optical density readings at 540 nm (OD₅₄₀) in duplicate, against water and by determination of ash-free dry weight (AFDW). To construct the growth curve, it was plotted the ln (OD₅₄₀ of the sample – OD₅₄₀ of control) versus time of incubation. That subtraction of the OD readings was intended to avoid the disadvantage of the coloration that the wastewater contributes to the suspension and to take in account only the contribution of the biomass. AFDW was determined by filtering the samples through a Whatman GF/C 25 mm filter of 1.2 μm pore size, drying overnight at 105°C and then for 1h at 550°C.

In continuous mode, the growth of the microalga was

<table>
<thead>
<tr>
<th></th>
<th>N-NH₃ (mg N/L)</th>
<th>Kjeldahl N (mg N/L)</th>
<th>PO₄³⁻ (mg/L)</th>
<th>P–PO₄³⁻ (mg P/L)</th>
<th>P₂O₅ (mg/L)</th>
<th>COD (mg O₂/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>15.40 ± 0.13</td>
<td>22.40 ± 0.00</td>
<td>20.00</td>
<td>6.50</td>
<td>15.00</td>
<td>354.17 ± 17.68</td>
</tr>
<tr>
<td>Continuous</td>
<td>21.65 ± 0.00</td>
<td>23.80 ± 1.98</td>
<td>26.80</td>
<td>8.70</td>
<td>20.10</td>
<td>241.67 ± 11.79</td>
</tr>
</tbody>
</table>

Results are the average of the replications.
monitored by reading the OD₅₄₀, in duplicate, against water. It was also determined the AFDW, in the day of the inoculation and then when the culture was stabilized (according to the OD₅₄₀ readings).

The pH was also measured using a laboratory pH meter (InoLab WTW).

For the batch culture, the specific growth rate (µ, day⁻¹) of was calculated from the growth curve of the microalgae. First, it was determined the exponential phase of growth which is a linear phase and then µ is determined as the slope of that straight line, according to the equation (2):

\[
\ln N = \mu t + \ln N_0
\]  

(2)

where \(N\) is the biomass at time \(t\) at the exponential phase of growth and \(N_0\) the biomass at the beginning of the exponential phase.

The duplication time (\(t_d\), day) was calculated from the equation (3):

\[
t_d = \ln 2 / \mu
\]  

(3)

The maximum volumetric biomass productivity (\(P_{X,max}\), g/L day) was calculated from the equation (4) where \(X_t\) was the final biomass concentration (g/L) at the time \(t\) (day) and \(X_0\) the initial biomass concentration (g/L) at \(t_0\) (day):

\[
P_{X,max} = (X_t - X_0) / (t - t_0)
\]  

(4)

In continuous culture at steady state, \(\mu\) equals the dilution rate (D, day⁻¹), that is defined by equation (1).

\[
D = \frac{F}{V} = \frac{1}{\tau}
\]  

(1)

Volumetric biomass productivity (\(P_x\), g/L day) can be determined by:

\[
P_x = D \times X
\]  

(2)

where \(X\) is the biomass concentration in the steady state.

2.6.3 Characterization of the wastewaters and supernatant after biomass recovery

Both effluents used in batch and continuous systems were characterized. In order to evaluate the efficiency of the wastewater treatment, all the analysis performed for the effluent (pH, COD, nitrogen and phosphorous) were done for the filtered supernatant, using the same methods. COD, nitrogen and phosphorous removal were calculated according to the equation (7):

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

(7)

where \(C_e\) is the concentration of the measured parameter in the effluent and \(C_s\) is the concentration in the filtered supernatant.

2.6.4 Cell viability and esterase activity

Multi-parameter flow cytometry (FC) was used to assess the microagal cell viability and enzymatic activity of the steady-state cultures using a FACSCalibur flow cytometer (Becton-Dickinson Instruments, Belgium). Cell suspension and buffer volumes were adjusted to 300-400 events s⁻¹ and the final volume was 500 μL.

Cell viability was determined by detecting membrane permeabilization with Propidium iodide (PI). Samples were diluted with phosphate buffer solution (PBS, pH 7.0) and stained with 4 μL of PI stock solution (Invitrogen, Carlsbad, USA). The viability of microalgal cells was expressed as the percentage of viable cells (non-stained cells) in the total amount of cells analysed.

Enzymatic activity was measured by detection of esterase activity with cFDA. Samples were diluted with MCI buffer, stained with 5 μL of cFDA and incubated in darkness for 40 minutes prior to analysis. The enzymatic activity of microalgal cells was expressed as the percentage of active cells (stained cells) in the total amount of cells analysed.

All FC analyses were performed in duplicate.

2.6.5 Quantification of chlorophyll content

Microalg culture samples (2 mL) were first concentrated by centrifugation for 10 min at 3900 rpm (Sigma 2-6E, Sartorius). 2 mL of acetone (99.5%, Sigma-Aldrich) and glass beads were added to the tube and extraction was performed by submitting the mixture to vortex during 2 min followed to 2 min in an ice bath. This procedure was repeated three times, then the mixture was centrifuged at 3900 rpm for 20 minutes, and the supernatant collected. The extraction was carried out until a colourless supernatant was obtained. All the tubes were covered in aluminium foil to prevent pigment degradation derived by light. The total liquid phase collected was quantified.

Chlorophylls a and b in the extractant (\(C_a\) and \(C_b\)) were quantified by spectrophotometry (Hitachi U-2000) and using the equations proposed by Ritchie (2008):

\[
\text{Chl} (mg/L) = C (mg/L) \times \text{extractant volume (ml)}/ \text{sample volume (ml)}
\]  

(10)

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) and total chlorophyll content was obtained by summing Chl a and Chl b.

The quantification was made in duplicate, for all the samples.

2.6.6 Fatty acid composition

Fatty acid composition of microalgal biomass samples was analysed, in duplicate, by Gas Chromatography (GC). Before GC analysis, the fatty acids were transesterified by the method of Lepage & Roy (1986) with modifications.

In Pyrex tubes with Teflon liner screw caps, to a 100 mg of freeze-dried microalgae were added 2 mL of a methanol/acetyl chloride (95:5 v/v) mixture and 0.2 mL of heptadecanoic acid in petroleum benzine 60-80°C (5 mg/mL) as internal standard solution. The mixture was heated in 80°C for 1 h and was let to cool to room temperature before being diluted with 1 mL of water and 2 mL of n-heptane. The tubes contentents were let to stay till separation of the phases. The upper layer was recovered, dried over anhydrous Na2SO4, filtered, in glass Pasteur pipettes, and collected in vials, thus obtaining the fatty acid methyl ester (FAMEs) ready for injection or to be stored at -18°C prior to use.

FAMEs were analysed in a CP-3800 GC (Varian, USA) equipped with 30 m SUPELCOWAX 10 capillary column (film 0.32 μm) with Helium as carrier gas at a constant rate of 3.5 mL/min. Injector and detector (flame ionization) temperatures were 250 °C and 280 °C respectively. The split ratio was 1:50 for 5 min and 1:10 for the remaining time. The column temperature programme started at 200 °C for 8 min, increased at 4 °C/min until 240 °C and held for 16 min. Fatty acid composition was calculated as percentage of the total fatty acids present in the sample, determined from the peak areas.

2.6.7 Quantification of biomass protein content

The Kjeldahl nitrogen present in the final microalgae biomass was determined by the modified Kjeldahl method previously described, using 200 mg of freeze-dried microalga. Protein content was calculated by multiplying Kjeldahl nitrogen by the conversion factor of 5.95 (López et al., 2010).

3. Results and discussion

3.1 Wastewater characterization

The wastewater composition used in the batch and continuous studies in PBRs can be seen in Table 1. The two wastewaters were from different campaigns to collect the effluent from the brewery. For the continuous mode, it can be noted a lower organic load (COD) than in the effluent used for batch mode. This is due to the settling of the effluent, in which most of the suspended solids settled and were not included in the recovered upper layer.

3.2 Growth evaluation of *Scenedesmus obliquus* in photobioreactors in batch mode (5 L) and continuous mode (6 L)

The trial in batch mode lasted 12 days (286 hours) and ended when the algal growth reached the stationary phase (Figure 2). The microalga reached its exponential growth after 45.8 h (in the second day of cultivation), with a maximum growth rate of 0.046 h⁻¹ (1.10 day⁻¹) and a doubling time of 15.13 h. The obtained growth rate was almost 10 times higher than the value found by Raposo et al. (2010) for *C. vulgaris* grown in industrial brewery effluent in 15 L plastic bags. On the other hand, McGinn et al. (2012) achieved a growth rate of 1.4 day⁻¹ for Sc using 300L internally illuminated Brite-Box photobioreactors with secondary municipal wastewater effluent, which is not a much higher value comparing to this study.

![Figure 2. Growth curve and pH behaviour of *S. obliquus* in brewery effluent in a 5 L PBR, in batch mode. A linear regression was applied to the exponential phase, to calculate the growth rate.](image)

The time that the microalgae cultures using the continuous mode took to achieve its steady state was in accordance to the literature (after 3-5 residence times). Regarding the parallel testing, the maximum cultivation time was obtained for τ = 11.80 (35 days) and the minimum was for τ = 2.26 (10 days). For τ = 1.72, the test only lasted 3 days. This trial was stopped in that day, due to a malfunction of the aeration that caused the culture to settle in the bottom of the PBR.

All the results regarding the growth of *S. obliquus* in the wastewater using PBRs are depicted in Table 2.
Table 2. Summary of growth related results obtained (biomass concentration, biomass productivity and pH) for S. obliquus grown in PBRs using brewery wastewater, under batch (5 L capacity) and continuous modes (6 L capacity).

<table>
<thead>
<tr>
<th>τ (day)</th>
<th>D (day⁻¹)</th>
<th>AFDW (g/L)</th>
<th>Pₓ (mg/L day)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.72</td>
<td>0.58</td>
<td>0.33 ± 0.00</td>
<td>189.45 ± 0.00</td>
<td>9.18</td>
</tr>
<tr>
<td>2.26</td>
<td>0.44</td>
<td>0.31 ± 0.02</td>
<td>138.39 ± 7.83</td>
<td>9.25</td>
</tr>
<tr>
<td>3.85</td>
<td>0.26</td>
<td>0.86 ± 0.09</td>
<td>224.30 ± 22.99</td>
<td>9.53</td>
</tr>
<tr>
<td>5.58</td>
<td>0.18</td>
<td>0.79 ± 0.05</td>
<td>141.23 ± 9.51</td>
<td>9.45</td>
</tr>
<tr>
<td>7.55</td>
<td>0.13</td>
<td>0.91 ± 0.23</td>
<td>120.91 ± 30.45</td>
<td>9.39</td>
</tr>
<tr>
<td>9.46</td>
<td>0.11</td>
<td>0.81 ± 0.02</td>
<td>85.88 ± 1.87</td>
<td>9.49</td>
</tr>
<tr>
<td>11.80</td>
<td>0.08</td>
<td>0.95 ± 0.04</td>
<td>80.51 ± 3.00</td>
<td>9.42</td>
</tr>
<tr>
<td>Batch mode</td>
<td></td>
<td>0.93 ± 0.01</td>
<td>87.11 ± 3.37</td>
<td>9.53</td>
</tr>
</tbody>
</table>

Results are the average of the replications.

Still regarding the trial in batch mode, it was obtained an ash-free dry weight (AFDW) of 0.93 ± 0.01 g/L and a maximum biomass productivity of 156.80 ± 4.65 mg/L.day. A very similar maximum biomass (0.90g/L) was found by Mata et al. (2012) for S. obliquus using synthetic brewery effluent. Furthermore, Kong et al. (2012) achieved a significantly smaller biomass production, using Botryococcus brauni in this type of wastewater. However, better results regarding biomass concentration and productivity were achieved by Darpito et al. (2015), for the cultivation of C. protothecoides in anaerobically treated brewery wastewater.

3.2.1 Effect of dilution rate on biomass concentration and productivity

The biomass (AFDW) concentration and productivity as a function of dilution rate are shown in Figure 3.

AFDW drastically reduces from 0.86 ± 0.09 to 0.31 ± 0.02 as dilution increases from 0.26 to 0.44 day⁻¹. This decrease in biomass concentration is predicted in the literature (Teixeira et al., 2007). The highest biomass concentration was obtained for D = 0.08 day⁻¹ (0.95 ± 0.04 g/L). Increasing biomass productivity was found with increasing dilution rate from 0.08 to 0.26 day⁻¹ and then the productivity declined from 0.26 to 0.44 day⁻¹. Therefore, the optimal dilution rate was 0.26 day⁻¹, reaching a biomass productivity of 224.30 ± 22.99 mg/L.day. This trend in the effect of D on biomass productivity was also presented by Tang et al. (2012), as a decline of biomass productivity was obtained for dilution rates lower and higher than the optimal value.

After the test of six dilution rates, a smaller residence time of 1.72 days (D = 0.59 day⁻¹) was tested to verify if in this conditions the microalga culture would washout. The results showed that the washout did not occur, and the obtained biomass concentration was 0.33 ± 0.00 g AFDW/L. This value is slightly higher than the result for the highest dilution in the parallel testing, 0.44 day⁻¹. Moreover, the biomass productivity was surprisingly higher than the expected, not following the trend observed in Figure 3. At the third day of operation, it was noted the settling of the biomass at the bottom of the PBR, which interfered with the mixture and led to accumulation of biomass.

The maximum biomass productivity obtained in the continuous mode was lower, but close to the values found by McGinn et al. (2012): 234 and 267 mg/L.day, using S. obliquus grown in secondary municipal wastewater effluent in two chemostats at a dilution rate of 0.75 day⁻¹.

3.3 Wastewater treatment evaluation

The results of the nutrient characterization after treatment and maximal removal rates are depicted in Tables 3 and 4, respectively. The results regarding ammoniacal and total Kjeldahl nitrogen, phosphorous, COD and pH were compared to emission value limits (EVLs) of the Portuguese legislation (Decree-Law no. 236/98), to assess the possibility of discharging the treated effluent in water flow. The Kjeldahl nitrogen was considered to be the total nitrogen present in the treated effluent.

All the conditions, except D = 0.58 day⁻¹, presented results that met the requirements in the legislation, proving that S. obliquus using this PBR system is sufficiently efficient to treat brewery wastewater.
Batch culture and continuous culture at $D = 0.26 \text{ day}^{-1}$ presented the best wastewater treatment. The batch production of *S. obliquus* attained a 75% removal for total Kjeldahl nitrogen, 43% for phosphorous and 70% for COD. On the other hand, at $D = 0.26 \text{ day}^{-1}$, slightly higher removal rates for COD and N were reached (74 and 76%, respectively), but the removal of P was not so efficient (23% removal).

Being the objective the wastewater treatment together with the biomass production, one can select the culture at $D = 0.26 \text{ day}^{-1}$ ($\tau = 3.85$ days) to be the most favourable condition, since the maximum productivity was obtained (224.30 mg/L day), the total nitrogen and COD removals were the highest and phosphorous removal was the third best.

Not considering the culture at the highest dilution rate of $D = 0.58 \text{ day}^{-1}$, removal of N ranged from 65 to 76%, which is much higher than the results obtained by Mata et al. (2010): 11 – 24%, but lower than those reported by Raposo et al. (2010) and Darpito et al. (2015), where a removal above 85% was achieved. All the previous results regarded brewery wastewater treatment in batch mode. One has to consider that the microalgae did not consume the entire N. Ammonia is easily removed by out-gassing to the atmosphere, due to the high pH, which shifts the equilibrium in favour of NH$_3$ (Martinez et al., 2000). In fact, a near complete removal of ammonia was reached for batch mode and the lowest dilution rates.

Phosphorous removal rates were always lower than those observed for N removal, ranging from 9 to 43% (excluding the result for $D = 0.58$). These results are lower than those attained by Raposo et al. (2010): 54 – 66% and Darpito et al. (2015): more than 90%. For cultures grown under continuous mode, the removal of P was significantly lower than in batch mode.

In continuous mode, McGinn et al. (2012) achieved a better wastewater treatment by *S. obliquus* using urban wastewater, with near complete N and P removal in a very short retention time (1.33 day).

Concerning COD removal, removal rates from 55 to 74% were obtained, which are in accordance with the results by Mata et al. (2010), wherein a maximum of 66.8% removal was achieved.

### Table 3. Characterization of the filtered supernatant, after biomass recovery, for all trials of *S. obliquus* growth in batch mode in 5 L PBR and in 6 L PBRs continuous system, using brewery wastewater.

<table>
<thead>
<tr>
<th>D (day$^{-1}$)</th>
<th>N-NH$_3$ (mg N/L)</th>
<th>Kjeldahl N (mg N/L)</th>
<th>PO$_4$$^{3-}$ (mg P/L)</th>
<th>P-P0$_4$$^{3-}$ (mg P/L)</th>
<th>P$_2$O$_5$ (mg O$_2$L)</th>
<th>COD (mg O$_2$L)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>0.56</td>
<td>5.60</td>
<td>23.20</td>
<td>7.60</td>
<td>17.40</td>
<td>81.20 ± 6.04</td>
<td>9.40</td>
</tr>
<tr>
<td>0.11</td>
<td>0.65 ± 0.13</td>
<td>5.60</td>
<td>24.30</td>
<td>7.90</td>
<td>18.20</td>
<td>108.33 ± 11.79</td>
<td>9.52</td>
</tr>
<tr>
<td>0.13</td>
<td>0.84 ± 0.13</td>
<td>5.60</td>
<td>19.60</td>
<td>6.40</td>
<td>14.70</td>
<td>81.20 ± 6.04</td>
<td>9.51</td>
</tr>
<tr>
<td>0.18</td>
<td>2.05 ± 0.26</td>
<td>9.80</td>
<td>22.10</td>
<td>7.20</td>
<td>16.50</td>
<td>81.20 ± 6.04</td>
<td>9.45</td>
</tr>
<tr>
<td>0.26</td>
<td>2.89 ± 0.66</td>
<td>5.60</td>
<td>20.50</td>
<td>6.70</td>
<td>15.30</td>
<td>62.50 ± 5.89</td>
<td>9.52</td>
</tr>
<tr>
<td>0.44</td>
<td>7.19 ± 0.13</td>
<td>8.40</td>
<td>23.00</td>
<td>7.50</td>
<td>17.20</td>
<td>104.17 ± 17.68</td>
<td>9.27</td>
</tr>
<tr>
<td>0.58</td>
<td>12.97 ± 1.72</td>
<td>23.80</td>
<td>26.80</td>
<td>8.70</td>
<td>20.00</td>
<td>128.21 ± 0.00</td>
<td>9.25</td>
</tr>
</tbody>
</table>

**Batch mode**

<table>
<thead>
<tr>
<th></th>
<th>N-NH$_3$ (mg N/L)</th>
<th>Kjeldahl N (mg N/L)</th>
<th>PO$_4$$^{3-}$ (mg P/L)</th>
<th>P-P0$_4$$^{3-}$ (mg P/L)</th>
<th>P$_2$O$_5$ (mg O$_2$L)</th>
<th>COD (mg O$_2$L)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.19 ± 0.00</td>
<td>5.60</td>
<td>11.20</td>
<td>3.70</td>
<td>8.40</td>
<td>107.41 ± 5.24</td>
<td>9.32</td>
<td></td>
</tr>
</tbody>
</table>

**Legislation**

(1) Legislation — Decree-Law no. 236/98 (PT)
Results are the average of the replications.

### Table 4. Nutrient removal rates of *S. obliquus* cultures grown in brewery wastewater, using PBRs operated in batch and continuous mode.

<table>
<thead>
<tr>
<th>D (day$^{-1}$)</th>
<th>N-NH$_3$</th>
<th>Kjeldahl N</th>
<th>P</th>
<th>COD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>97</td>
<td>76</td>
<td>13</td>
<td>66</td>
</tr>
<tr>
<td>0.11</td>
<td>97</td>
<td>76</td>
<td>9</td>
<td>55</td>
</tr>
<tr>
<td>0.13</td>
<td>96</td>
<td>76</td>
<td>26</td>
<td>66</td>
</tr>
<tr>
<td>0.18</td>
<td>91</td>
<td>59</td>
<td>17</td>
<td>66</td>
</tr>
<tr>
<td>0.26</td>
<td>87</td>
<td>76</td>
<td>23</td>
<td>74</td>
</tr>
<tr>
<td>0.44</td>
<td>67</td>
<td>65</td>
<td>14</td>
<td>57</td>
</tr>
<tr>
<td>0.58</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>47</td>
</tr>
</tbody>
</table>

**Batch mode**

<table>
<thead>
<tr>
<th></th>
<th>N-NH$_3$</th>
<th>Kjeldahl N</th>
<th>P</th>
<th>COD</th>
</tr>
</thead>
<tbody>
<tr>
<td>99</td>
<td>75</td>
<td>43</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

Results are the average of the replications.

### 3.4 Physiological state of the microalgal cells

Flow Cytometry was used to assess the viability and enzymatic activity (of esterase) of the microalga *S. obliquus* for all conditions tested. All the results are summarized in Table 5 and the effect of the dilution rate in these parameters can be observed in Figure 4.

Regarding cell viability, the batch culture mode showed the best result (95.13% of the cells present intact membrane). For the six tested dilution rates, the range of values obtained was narrow, reaching from 85.49 to 92.33% of cells with membrane intact.
In which concerns esterase activity, the maximum percentage of active cells was obtained for \( D = 0.13 \text{ day}^{-1} \), followed by the result for batch mode: 75.72 and 69.12 % of cells with esterase activity, respectively.

Table 5. FC analyses to assess the viability and enzymatic activity of \textit{Scenedesmus obliquus} grown in PBRs operated in batch and continuous mode with brewery wastewater.

<table>
<thead>
<tr>
<th>D (day(^{-1}))</th>
<th>% Viable cells</th>
<th>% Enzymatically active cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>88.24 ± 0.15</td>
<td>52.43 ± 0.98</td>
</tr>
<tr>
<td>0.11</td>
<td>85.77 ± 0.02</td>
<td>55.79 ± 3.99</td>
</tr>
<tr>
<td>0.13</td>
<td>85.49</td>
<td>75.72</td>
</tr>
<tr>
<td>0.18</td>
<td>90.95</td>
<td>65.39</td>
</tr>
<tr>
<td>0.26</td>
<td>92.33 ± 0.53</td>
<td>49.47 ± 3.82</td>
</tr>
<tr>
<td>0.44</td>
<td>86.04 ± 0.88</td>
<td>42.40 ± 4.65</td>
</tr>
<tr>
<td>0.58</td>
<td>82.80 ± 0.18</td>
<td>83.42 ± 5.45</td>
</tr>
</tbody>
</table>

Batch mode: 95.13 ± 0.82 69.12 ± 0.35

Results are the average of the replications.

Increasing percentage of cell viability was found with increasing dilution rate from 0.13 to 0.26 day\(^{-1}\) and then the percentage decreased from 0.26 to 0.58 day\(^{-1}\). The optimal dilution for cell viability was the same as for biomass productivity (0.26 day\(^{-1}\)).

A decline of the percentage of cell activity with increasing dilution from 0.13 to 0.44 day\(^{-1}\) can be observed, where the minimum achieved was 42.40 ± 4.65 %. However, for the two lowest dilution rates, lower percentage of enzymatically active cells (around 50%) was also found. This could be due to the age of cultures, as it took 31 and 35 days to achieve the steady state of the cultures at \( D = 0.11 \) and 0.08 day\(^{-1}\). That values were considerably higher than the ones obtained for the other cultures: 3 to 20 days.

3.5 Biomass composition

The results obtained for the biochemical characterization of \textit{Scenedesmus obliquus} biomass after treating the effluent are presented in Table 6.

The continuous mode proved to be advantageous regarding the protein content of the produced biomass, since the minimum content was obtained for the culture in batch mode (21.15%). The highest protein content (50.09 %) was found for \( D = 0.26 \text{ day}^{-1} \), which is the optimal value for biomass productivity (and cell viability). This result is within the range presented by Becker (2007) for \textit{S. obliquus}. All the protein content values obtained in the continuous mode were higher than the ones reported by Batista \textit{et al.} (2014): 20.4%, using Bristol medium; Gouveia \textit{et al.} (2016): 32.7% using urban wastewater and by Hodaifa \textit{et al.} (2008): 30.8%, using wastewater from olive-oil extraction.

It was achieved Chl contents within the range from 18.52 mg/g for \( D = 0.26 \text{ day}^{-1} \) to 45.71 mg/g for \( D = 0.11 \text{ day}^{-1} \). For the cultures in the PBRs operated in continuous mode, the results were close to each other’s, except for the maximum (45.71 mg/g), which is higher than the expected. However, it is inferior than the maximum Chl content achieved by Raposo \textit{et al.} (2010), with \textit{C. vulgaris} grown in brewery wastewater.

Table 6. \textit{S. obliquus} biomass composition obtained after brewery wastewater treatment.

<table>
<thead>
<tr>
<th>D (day(^{-1}))</th>
<th>Chl a (mg/L)</th>
<th>Chl a+b (mg/g)</th>
<th>Protein content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>16.22</td>
<td>20.53</td>
<td>45.37</td>
</tr>
<tr>
<td>0.11</td>
<td>25.03</td>
<td>45.71</td>
<td>42.40</td>
</tr>
<tr>
<td>0.13</td>
<td>17.42 ± 0.12</td>
<td>26.78 ± 2.62</td>
<td>37.93</td>
</tr>
<tr>
<td>0.18</td>
<td>15.45 ± 1.87</td>
<td>22.34 ± 1.27</td>
<td>45.28</td>
</tr>
<tr>
<td>0.26</td>
<td>12.91 ± 0.15</td>
<td>18.52 ± 0.51</td>
<td>50.09</td>
</tr>
<tr>
<td>0.44</td>
<td>8.64 ± 0.13</td>
<td>38.81 ± 1.17</td>
<td>39.90</td>
</tr>
<tr>
<td>0.58</td>
<td>7.94 ± 0.03</td>
<td>27.85 ± 1.67</td>
<td>37.81</td>
</tr>
</tbody>
</table>

Batch mode: 14.76 ± 0.85 18.94 ± 0.80 21.15

Results are the average of the replications.

4. Conclusions

Using the microalga \textit{Scenedesmus obliquus}, the performance of the PBRs operated in batch and continuous modes was interesting, as the brewery wastewater treatment was efficient, even at low residence times (~2 days). The results were unfavourable only for the highest dilution rate tested. High removal rates were achieved for nitrogen and COD removal (up to 80%, and around 70%, respectively). However, phosphorus removal rates were lower (up to 40%). The resulting treated effluent was ready for discharge in a natural water body, in accordance to the Portuguese environmental legislation (decree-law 236/98). Thus, the brewery wastewater
treatment by *Scenedesmus obliquus* can be done, combined with other treatment technologies.

The continuous cultivation mode proved to be advantageous regarding biomass productivity, and chlorophyll and protein contents in the biomass. Using this feeding mode, at selected dilution rates, the N and COD removal rates were even higher than for the batch operational mode. The value of dilution rate was found to have a strong influence on some performance parameters, mainly on biomass productivity and esterase activity. Taking into account wastewater treatment performance and biomass productivity, quality and cell viability, continuous culture at D = 0.26 day^{-1} is the best option.

Possible guidelines for future work would be: evaluate this PBR system for wastewater treatment by *Scenedesmus obliquus* at larger scale; optimization of conditions to maximize biomass productivity not compromising wastewater treatment efficiency nor the economic viability of the process; study of the influence of CO₂ on the wastewater treatment and microalgal growth and the investigation of the biorefinery concept.

5. References

Al-rajhia, S. et al., 2012. Treatments of Industrials Wastewater by Using Microalgae. , 41.


Simate, G.S. et al., 2011. The treatment of brewery wastewater for

