Environmental Research 164 (2018) 32–38

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Combining biotechnology with circular bioeconomy: From poultry, swine, cattle, brewery, dairy and urban wastewaters to biohydrogen

Alice Ferreira[a], Paula Marques[a], Belina Ribeiro[a], Paula Assemany[b], Henrique Vieira de Mendonça[c], Ana Barata[a], Ana Cristina Oliveira[a], Alberto Reis[a], Helena M. Pinheiro[d], Luisa Gouveia[a,*]

[a] LNEG, National Laboratory of Energy and Geology I.P. /Bioenergy Unit, Estrada do Paço do Lumiar 22, 1649-038 Lisbon, Portugal
[b] Federal University of Viçosa/Civil Department, Avenida PH Rolfs s/n, 36570-900 Viçosa, MG, Brazil
[c] Federal University of Juiz de Fora (UFJF), Institute of Biological Sciences, Campus São Pedro, 36036-900, Juiz de Fora, MG, Brazil
[d] iBB, Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Departamento de Bioengenharia, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

ARTICLE INFO

Keywords:
Wastewater bioremediation
Scenedesmus obliquus
Enterobacter aerogenes
Dark Fermentation
Biohydrogen

ABSTRACT

The ability of microalgae to grow in nutrient-rich environments and to accumulate nutrients from wastewaters (WW) makes them attractive for the sustainable and low-cost treatment of WW. The valuable biomass produced can be further used for the generation of bioenergy, animal feed, fertilizers, and biopolymers, among others. In this study, *Scenedesmus obliquus* was able to remove nutrients from different wastewaters (poultry, swine and cattle breeding, brewery and dairy industries, and urban) with removal ranges of 95–100% for nitrogen, 63–99% for phosphorus and 48–70% for chemical oxygen demand. The biomass productivity using wastewaters was higher (except for poultry) than in synthetic medium (Bristol), the highest value being obtained in brewery wastewater (1025 mg/(L.day) of freeze-dried biomass). The produced biomass contained 31–53% of proteins, 12–36% of sugars and 8–23% of lipids, regardless of the type of wastewater.

The potential of the produced *Scenedesmus obliquus* biomass for the generation of $BioH_2$ through batch dark fermentation processes with *Enterobacter aerogenes* was evaluated. The obtained yields ranged, in mL H_2/g Volatile Solids (VS), from 50.1 for biomass from anaerobically digested cattle WW to 390 for swine WW, whereas the yield with biomass cultivated in Bristol medium was 57.6 mL H_2/g_{VS}.

1. Introduction

Wastewater management is an increasing concern worldwide due to the growing population and industrialization. The uncontrolled discharge of domestic and industrial wastewaters into the environment results in serious pollution problems, making wastewater treatment a mandatory process (Posadas et al., 2015).

Many industries produce a huge amount of wastewater in their processes, namely the food processing industry. In particular, the effluents derived from the dairy and brewery sectors are rich in organic compounds, such as proteins, as well as in phosphates, ammonia and/or nitrate (Raposo et al., 2010). Slaughterhouses generate large volumes of wastewaters, which are characterized by high organic (e.g. residual blood, skin, fat, manure, etc.) and nutrient loads. These industries are intense water consumption activities, generating large volumes of wastewaters (Table 1), containing high loads of organics and nutrients that need to be removed before they can be discharged into the environment. Due to their characteristics, these industrial wastes are a suitable cultivation medium for mixotrophic microalgae, with carbon/nitrogen (C/N) and nitrogen/phosphorus (N/P) ratios favorable for microalgae growth (Maroneze et al., 2014).

Microalgal-bacterial processes have been widely studied for nutrients removal and constitute a cost-effective and sustainable alternative to conventional wastewater treatment technologies due to their potential for cost-free oxygenation and efficient nutrient removal (Su et al., 2012, 2011). Microalgae provide, through photosynthesis, the O_2 needed by bacteria for the oxidation of organic matter and NH_4^+. They also consume NH_4^+ in a must faster process than oxidation by bacteria. Simultaneously, the nutrients are assimilated into the algal-bacterial biomass, as a result of their combined auto- and heterotrophic growth.

Abbreviations: $bioH_2$, Biohydrogen; COD, Chemical Oxygen Demand; FAME, Fatty Acids Methyl Ester; FM, Fermentation Medium; GC, Gas Chromatography; HRT, Hydraulic Retention Time; TKN, Total Kjehldahl Nitrogen; VS, Volatile Solids; WW, Wastewater

* Corresponding author.
E-mail address: luisa.gouveia@lneg.pt (L. Gouveia).

https://doi.org/10.1016/j.envres.2018.02.007
Received 22 December 2017; Received in revised form 6 February 2018; Accepted 7 February 2018
Available online 22 February 2018
0013-9351/

Table 1
Wastewater produced by economic sector worldwide, and associated nutrient (nitrogen and phosphorus) and chemical oxygen demand (COD) loads ("Eurostat, 2013–2015," n.d.).

Economic sector		Wastewater flowrate (million m^3/y)	COD (ton/y)	Nitrogen (ton/y)	Phosphorus (ton/y)
Urban		20,400	8,200,000	816,000	200,000
Dairy		538	8,100,000	322,000	–
Brewery		1 040[a]	4,160,000[b]	54,500[b]	31,200[b]
Slaughter-house	**Swine**	35	268,000	22,000	–
	Poultry	21	161,000	13,000	–
	Cattle	12	92 000	7 300	–

[a] Estimated considering a global beer production (in 2015) of 1.93 billion hL (Statista, 2017) and a wastewater/beer production ratio of 5.39 (Brewers Assiociation, 2013).
[b] Estimated considering the general composition of brewery wastewater reported by Simate et al. (2011).

This valuable biomass can be further used as a biofertilizer and/or as a feedstock for biofuel production (Batista et al., 2015; Posadas et al., 2015), such as biohydrogen.

Hydrogen (H_2) is a naturally occurring molecule that is a clean and efficient energy carrier. It has the highest energy content per unit weight of any known fuel (142 kJ/kg) and can be transported for urban or industrial consumption through conventional means, being safer to handle than urban natural gas. Moreover, H_2 is the only carbon-free fuel, its oxidation process producing only water (Das and Veziroglu, 2008).

Presently, nearly all the hydrogen produced worldwide is generated by processes which use fossil fuels (natural gas, heavy oils, naphtha, coal) and only a small fraction is based on water and biomass as raw materials (Acar and Dincer, 2014). However, there is an increased interest in biohydrogen (bioH_2) production because the traditional H_2 production processes are still polluting and expensive (Batista et al., 2014). Moreover, bioH_2 production processes are catalyzed by microorganisms in an aqueous environment at near ambient temperature and atmospheric pressure (Das and Veziroglu, 2008). Another interesting process is microbial electrolysis which is an electricity-mediated bioelectrochemical technology originally developed for H_2 production from waste streams (Zhang and Angelidaki, 2014).

BioH_2 can be produced by fermentative processes such as dark fermentation, which consists in the conversion of sugars into H_2, CO_2 and organic acids by microorganisms, through the acidogenic pathway. In theory, any sugar-containing biomass can be used as feedstock, and the appeal of the process rises if the chosen biomass is readily available at lower costs. In recent years, bioH_2 production through dark fermentation has received increased attention due to its many advantages, such as the high hydrogen production rates, the potential to convert biomass or bio-wastes into hydrogen, and the feasibility of process design and control (Batista et al., 2014).

Microalgae are attractive feedstocks for dark fermentation since they are able to uptake solar energy and CO_2 and convert them into storage chemicals such as starch, a fermentable substrate for bioH_2 production (Ortigueira et al., 2015). Their cell walls are rich in cellulose, but normally lack hemicellulose and lignin, which facilitates the breakdown of the biomass into fermentable sugars, allowing milder pretreatment processes (Batista et al., 2014). In addition, there is the possibility of extracting high-value co-products from the biomass prior to fermentation (e.g. pigments, amino acids or proteins), helping with the economic feasibility of this type of biofuel production (Batista et al., 2015). This process is even more attractive when the microalgae biomass can be produced using wastewaters, constituting a potentially win-win process integrating effluent treatment and energy production.

BioH_2 can be produced by spontaneous microbial consortia, where the feedstocks are unsterilized waste materials, or using more controlled fermentation conditions with specific and highly H_2-producing strains. Good H_2 producers include mesophiles, such as several species of *Clostridium* and *Enterobacter*, and thermophiles like *Thermotoga neapolitana*. These anaerobic bacteria have been described as a good H_2 producers in fermentations with biomass feedstocks derived from cyanobacteria such as *Anabaena* sp. (Ferreira et al., 2012), and microalgae such as *Chlamydomonas reinhardtii* (Kim et al., 2006; Nguyen et al., 2010), *Nannochloropsis* sp. (Efremenko et al., 2012; Nobre et al., 2013), *Chlorella vulgaris* (Liu et al., 2012), and *Scenedesmus obliquus* grown in Bristol medium (Batista et al., 2018, 2014) and urban wastewater (Batista et al., 2015).

The main purpose of this work was to evaluate the pollutant removal efficiency of the microalga *Scenedesmus obliquus* grown in different wastewaters (urban, dairy and brewery industries, cattle, swine and poultry breeding). The composition of the biomass produced was determined and its potential for bioenergy production, such as biohydrogen, was assessed. The valorization of biomass produced in wastewaters into biohydrogen represents a win-win approach and has not been deeply studied. The values of biohydrogen production from biomass cultivated in wastewaters were compared with those using the synthetic medium (Bristol).

2. Material and methods

2.1. Wastewater treatment

2.1.1. Wastewater sources and characterization

In this work, the microalga *Scenedesmus obliquus* was used to treat several different effluents, serving as culture media, namely poultry, swine, cattle, brewery, dairy, and urban wastewaters, in independent runs (Table 1).

All the wastewaters (WW) were used directly as collected from their industrial sources, with two exceptions. The cattle wastewater was pretreated by anaerobic digestion, using a hybrid reactor under mesophilic temperature conditions (37 °C ± 1 °C) and a hydraulic retention time (HRT) of 6 days (Mendonça et al., 2017) and the dairy wastewater was pre-treated in a bench-scale aerobic reactor (activated sludge) with a HRT of 1 day. It should also be noted that the collected brewery wastewater had been pre-treated in a full-scale anaerobic reactor BIOPAQ®IC, at the industrial wastewater treatment plant.

All the wastewaters were characterized in terms of their total nitrogen, ammonia nitrogen, phosphorus and COD contents, at least in duplicate. This characterization was done according to standard methods (APHA, 2012). Ammonia nitrogen was quantified by titration after a preliminary distillation step based on standard methods 4500-NH_3 B and C. The total Kjeldahl nitrogen (TKN) determination was carried out by a modified method adapted from the standard method 4500-N_{org} B. Phosphorus was determined using a commercial kit with the Phosver 3 (ascorbic acid) method using Powder Pillows (Spectophotometer Hach DR/2010). The COD determination was done with test kits from Hach Lange, Hach 5121258–51 (0–150 mgO_2/L as COD), through closed reflux digestion with spectrophotometric measurement. The characterization results for all the wastewaters are shown in Table 2.

2.1.2. Microalga cultivation and biomass characterization

The microalga used was *Scenedesmus obliquus* (ACOI 204/07) from the ACOI Coimbra University Collection of Algae, Portugal.

The photobioreactors used were of the bubble column (0.8 L and 150 L), flat panel (2.6 L) and high rate algal pond HRAP (area = 2.4 m^2; depth = 11 cm; working volume = 365 L; horizontal velocity = 0.11 cm/s promoted by paddle-wheels) types (Table 3).

The microalgae culture conditions used in the various experiments are presented in Table 3. The urban and brewery WW experiments were run outdoors, at the LNEG Alfragide (38°43′54.3″N 9°12′41.3″W) and Lumiar (38 46' 25" N 9 10' 36" W) *campi*, respectively (Lisbon, Portugal). The urban WW experiment was run in October and the brewery

Table 2
Average composition of the different wastewaters used as culture media for *Scenedesmus obliquus* cultivation. Average values are given, with standard deviations from at least two replicate determinations.

Wastewater	N-NH_3 (mg N/L)	TKN (mg N/L)	PO_4^{3-} (mg P/L)	P-PO_4^{3-} (mg P/L)	COD (g O_2/L)	References
Poultry	122.7 ± 1.9	–	–	27.9 ± 1.6	3.7 ± 0.7	Barata (2016)
Swine	2472.4 ± 1.9	3171 ± 208	–	6.9 ± 0.6	14.2 ± 1.2	Barata (2016)
Cattle	498 ± 1.2	618 ± 0.9	23.5 ± 0.01	7.8 ± 0.01	2.9 ± 0.02	Mendonça (2017)
Brewery	4.11 ± 2.1	28.0	20.0 ± 0.16	6.5 ± 0.18	0.2 ± 0.0	Unpublished data
Dairy	204 ± 0.1	312 ± 0.03	18 ± 0.2	6 ± 0.2	3.0 ± 0.4	Mendonça (2017)
Urban	175 ± 0.9	–	14.3 ± 0.11	4.6 ± 0.12	0.08 ± 1.5	Gouveia et al. (2016)

WW experiment in January-February. The insolation values were around 225 h (October) and 210–220 h (January and February) (IPMA, 2017).

All the WW were used for culture without further dilution or additives, except for the swine WW, which was diluted with tap water to a final content of 5% (v/v) (e.g., 25187 ± 25 mg NH_3/L).

Microalgae growth was monitored by measuring the optical density of culture samples, at 540 nm (Rocha et al., 2003), against distilled water, using a Hitachi U-2000 spectrophotometer. In addition the biomass dry weight (DW) was also determined.

At the end of the assay, the biomass was harvested by gravity settling, followed by centrifugation of the sediment at 10,000 rpm (11,300*g*) for 10 min at 15 °C, and freeze-dried (Heto Power Dry LL3000, Thermo Scientific) before biochemical composition evaluation. All subsequent biomass concentration, productivity and biochemical analysis results are given on the basis of the dry weight thus obtained, unless otherwise specified.

The biochemical composition of the microalga biomass (predominantly composed of *Scenedesmus obliquus*) was determined according to the A.O.A.C (2006), and all analyses were done in duplicate.

Total lipid content was evaluated by extraction with n-hexane in a Soxhlet apparatus, for 6 h. Total sugar content was measured through the phenol–sulphuric method (DuBois et al., 1956), on samples previously submitted to quantitative acid hydrolysis and filtered (Hoebler et al., 1989). Protein content was determined by a modified Kjeldahl method (APHA, 2012) using 6.25 as the conversion factor from total nitrogen to crude protein (Jones, 1941).

Fatty acid content 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 and Roy (1986) with modifications. The resulting fatty acid methyl esters (FAME) were analysed in a CP-3800 GC (Varian, USA) equipped with a 30-m SUPELCOWAX 10 capillary column (film 0.32 μm) with helium as carrier gas at a constant flow rate of 3.5 mL/min. The injector and detector (flame ionization) temperatures were 250 and 280 °C, respectively. The split ratio was 1:50 for the first 5 min and 1:10 for the remaining time. The column temperature programme started at 200 °C for 8 min, increased up to 240 °C at a rate of 4 °C/min, and was held at that value for 16 min.

Moisture was determined by drying the biomass in an oven at 105 °C until constant weight. Total ash was determined by incineration at 550 °C in a muffle furnace for 1 h. The volatile solids (VS) content was then calculated by the difference between the dry weight and total ash.

2.2. Biohydrogen production from the microalga biomass

2.2.1. Fermentative bacterium culture conditions

A strain of the bacteria *Enterobacter aerogenes*, ATCC 13408 Sputum (American Type Culture Collection, Manassas, USA), was used for the production of hydrogen by dark fermentation (bioH_2).

The bacterial culture was kept at 4 °C in solid CASO Agar (from MERCK: 15 g/L peptone from casein, 5 g/L peptone from soymeal, 5 g/L sodium chloride and 15 g/L agar–agar) and grown in a synthetic growth medium (20 g/L peptone, CULTIMED, solution with 5 g/L NaCl). The bacterial biomass was harvested at the exponential growth phase. The fermentation medium (FM) for the bioH_2 production assays (basal fermentation medium) contained K_2HPO_4 (7.0 g/L), KH_2PO_4 (5.5 g/L), tryptone (5.0 g/L, Bacto™), yeast extract (5.0 g/L, Bacto™), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4{\cdot}7H_2O$ (0.25 g/L), $Na_2MoO_4{\cdot}2H_2O$ (0.12 g/L), $CaCl_2{\cdot}2H_2O$ (21 mg/L), nicotinic acid (20 mg/L), $NiCl_2$ (20 mg/L), Na_2SeO_3 (0.172 mg/L), with a pH of around 6.8. All media were autoclaved before use (Batista et al., 2015).

2.2.2. Substrate

The biomass (predominantly composed of *Scenedesmus obliquus*) used in this work as substrate for bioH_2 production was obtained after the bioremediation of the different wastewaters (urban, dairy and brewery industries, cattle, swine and poultry breeding), as described in Section 2.1.2.

2.2.3. Dark fermentation assays

Batch fermentation assays were performed in 159 mL serum bottles closed with butyl rubber stoppers and crimped aluminum seals. Each contained 26 mL of FM and the microalga biomass substrate (headspace volume / liquid phase volume = 5) (Batista et al., 2018). The bottles containing the fermentation medium and the substrate (*S. obliquus* biomass) were previously submitted to autoclave sterilization at 121 °C

Table 3
Operational conditions used for the cultivation of Scenedesmus obliquus in different culture media (see references for further details).

Culture medium	Inlet gaseous mixture and Agitation	Light source and intensity (μE/(m^2·s))	Temperature (°C)	Reactor type and volume (L)	Operating strategy and cultivation time (days)	References
Poultry WW	Air diffuser	Artificial (20.2)	25	Bubble column (0.8)	Batch (29)	Barata (2016)
Swine WW	Air + 5% CO_2 diffuser	Artificial (32.4)	25	Bubble column (0.8)	Batch (21)	Barata (2016)
Cattle WW	Air diffuser	Artificial (60.7)	18–21	Flat Panel Airlift (2.6)	Batch (12)	Mendonça (2017)
Brewery WW	Paddle wheels	Sunlight	12–13	High Rate Algal Pond (300)	Batch (12)	Unpublished data
Dairy WW	Air diffuser	Artificial (31.1)	19–22	Flat Panel Airlift (2.6)	Batch (12)	Mendonça (2017)
Urban WW	Air diffuser	Sunlight	18	Bubble column (150)	Fed-Batch (13)	Gouveia et al. (2016)
Bristol	Paddle wheels	Sunlight	20	High Rate Algal Pond (300)	Batch (12)	Batista et al. (2018, 2014)

for 15 min (that works as a thermo-hydrolytic step). The sterilized bottles were then aseptically purged by bubbling N_2 through them before inoculation with the exponentially growing *E. aerogenes* culture 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 (microalga biomass, defined on the basis of their content of volatile solids) was 2.5 g_{VS}/L_{FM} (Batista et al., 2018, 2015). All the experiments were performed in triplicate and the results are expressed as average ± standard deviation. Control fermentation assays, without microalga biomass, were also prepared for comparison.

2.2.4. Analysis of the gas phase (headspace) composition

The gaseous phase samples were collected directly from the headspace of the serum bottles by using a gas-tight syringe. The content of H_2 and CO_2 in the fermentation headspace was analysed by gas chromatography, at atmospheric pressure, in a Varian 430-GC equipped with a thermal conductivity detector (TCD) and a fused silica column (Select Permanent Gases/CO_2-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. Calibration curves were previously obtained, in the range of the expected H_2 and CO_2 concentrations, using standard mixtures, in order to quantify the composition of the gas phase. Volumetric and specific hydrogen productions yields, in mL H_2/L_{FM} and mL H_2/g_{VS}, respectively, were calculated by dividing the total volume of produced hydrogen by the added weight (in terms of VS) of *S. obliquus* and by the fermentation medium volume in the bioreactor bottle, after 6 h of incubation. The gas volumes are expressed at NTP - normal temperature and pressure - conditions (1 atm, 20 °C).

3. Results and discussion

3.1. Pollutant removal by Scenedesmus obliquus in different wastewaters

Fig. 1 shows the maximum removal yields of the N, P and COD loads for each wastewater used as culture medium for microalga growth.

From Fig. 1, it is evident that *S. obliquus* performed very efficiently in the removal of nitrogen from all the wastewaters tested, with removal efficiencies above 95%. The high removal levels of this pollutant, as compared with the other measured parameters, can be explained by the volatilization of ammonia during elevated pH periods (induced by the photosynthetic activity, consuming dissolved CO_2) together with the assimilation of nitrogen by microalgae. The removal of phosphate was also very interesting with values ranging from 63% to 99%. The lower P removal achieved for brewery WW may be due to nitrogen limitation to the culture growth, since the N/P ratio was very low (N/P = 1.4:1) when compared to the other tested WW. Nonetheless, these removal yields can be seen as promising, considering that most of the wastewater treatment plants show low efficiency in removing

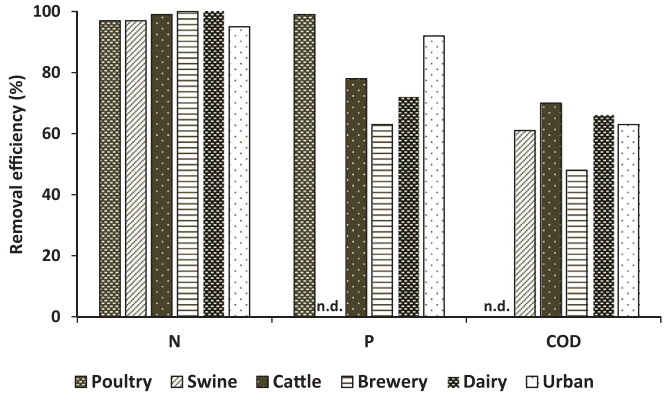

Fig. 1. Maximum removal efficiency of pollution loads (N, P and COD) from different wastewaters used as culture media for *Scenedesmus obliquus*. n.d. – not determined.

phosphorus (Mendonça et al., 2012). The COD removal range (48–70%) can be justified by two factors: (1) there was assimilation of organic carbon through mixotrophic processes, which are common for this species, and (2) the oxidation of organic matter by bacteria, using the oxygen supply provided by the algae photosynthetic activity. The lowest removal efficiency obtained for brewery WW (48%) may be due to the fact that some of the organic matter present in this WW (such as di-o-polysaccharides) cannot be assimilated by the biomass (Lutzu et al., 2015).

It should be noticed that these comparisons have associated errors as the experimental conditions were different (reactor configuration, light type and intensity and temperature).

3.2. Scenedesmus obliquus biomass characterization

The biochemical composition of the microalgae biomass grown in the different wastewaters is presented in Table 4.

Although the same species was used in all cultivations, Table 4 shows clear variations in the biochemical composition of the produced biomass, which indicates that, the culture medium and/or culture parameters/conditions have a significant influence. The highest biomass productivity was registered for *S. obliquus* grown in brewery wastewater. For the other wastewaters, values are lower but within the same order of magnitude. In general, the productivities of the biomass grown in the WW are substantially higher than that obtained in synthetic medium (130 mg/(L.d)), with poultry WW being the exception. This lower productivity may result from the minor light intensity supplied to this PBR (20.2 μE/(m^2·s)). Nonetheless, these results show a clear advantage for microalgae-based WW treatment, since it translates in cost savings in terms of nutrient and water consumption for microalgae cultivation.

For the studied wastewaters, there was a general predominance of protein over sugars and lipids, in the composition of the biomass. The higher protein concentrations, when compared to the microalga grown in synthetic medium (Bristol), were probably due to the abundant availability of ammonia nitrogen in the wastewaters used as cultivation media (Table 2). The cattle WW presented high values of ammonia nitrogen (498 mg N-NH_3/L) and resulted in a biomass with 42% of protein, followed by the dairy WW (204 mg N-NH_3/L) with 53% of protein in the biomass (the highest protein content in all runs). This latter value can be explained not only from the amount of ammonia nitrogen present in the respective WW, but also due to the higher light intensity applied during the cultivation stage (60.7 μE/(m^2·s)), when compared to the run with dairy WW (31.1 μE/(m^2·s)), performed in similar conditions.

Regarding the sugar content, for most of the tested wastewaters the biomass presented lower values than for Bristol medium, except for the poultry WW. For the biomass grown in brewery WW, a sugar content similar to that of Bristol medium was achieved (30.2%), possibly reflecting the high amount of sugars present in the beer production process WW. The sugar contents here determined suggested that the biomass grown in poultry, brewery and swine WW would have a higher potential as substrates for hydrogen production, which was confirmed by the dark fermentation assay results (see below).

Considering the total lipid content, the values registered for poultry, swine, cattle, brewery and dairy WW are very similar to that obtained with Bristol medium, being lower for the urban WW. Overall, taking into account the high nutrient availability in the WW culture media, the values attained for lipid content can be considered satisfactory, since most are similar or higher than that measured for the synthetic medium. The higher lipid contents obtained for swine and poultry WWs could be justified by the presence of CO_2 and the lower light intensity applied, respectively, since both factors (CO_2 and light) were shown to have a greater impact on lipid content for *S. obliquus* (Álvarez-díaz et al., 2015).

In order to quantify the initial concentration of substrate (*S. obliquus*

Table 4
Biochemical composition of the biomass obtained with the different wastewaters. Average values are given, with standard deviations from at least two replicate determinations.

Wastewater	Protein (%)	Total lipids (%)	Total Sugars (%)	Biomass productivity (mg/(L.d))	References
Poultry	n.d.	19.8 ± 0.3	36.2 ± 0.2	100	Barata (2016)
Swine	n.d.	23.3 ± 0.2	23.6 ± 5.2	300	Barata (2016)
Cattle	42 ± 0.02	18.0 ± 0.2	22 ± 0.01	358	Mendonça et al. (2017)
Brewery	31.4 ± 0.04	17.9 ± 0.6	30.2 ± 0.5	1025	Assemany (2017)
Dairy	53 ± 0.1	18. 0 ± 0.9	14 ± 0.6	183	Mendonça (2017)
Urban	32.7 ± 0.1	8.1 ± 0.1	11.7 ± 3.7	440	Gouveia et al. (2016)
Bristol	20.4 ± 0.02	17.1 ± 0.2	30.7 ± 0.8	130	Batista et al. (2018, 2014)

n.d. – not determined.

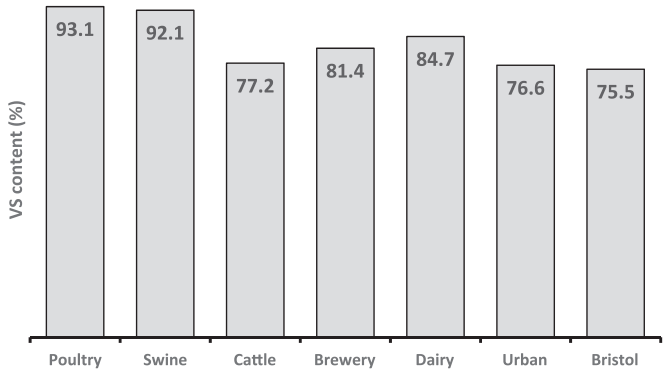

Fig. 2. Total VS content (% on a dry basis) for each *Scenedesmus obliquus* biomass (freeze-dried) cultivated in the different wastewaters and the synthetic medium. The values for urban wastewater and Bristol were obtained from Batista et al., (2015, 2014), respectively.

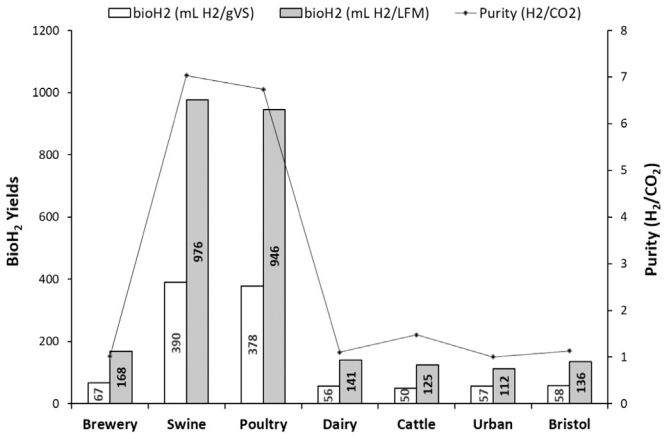

Fig. 3. Hydrogen production yield and gas purity values obtained from the fermentation of dried *Scenedesmus obliquus* biomass, grown in different wastewaters and synthetic medium, by *Enterobacter aerogenes*. The values for urban wastewater and synthetic medium (Bristol) are from Batista et al., (2015, 2014), respectively.

biomass) in the bioH_2 production runs, the organic content of the microalga was determined, in terms of its VS value (A.O.A.C, 2006). The values determined are present in Fig. 2.

Fig. 2 shows that the VS values of *S. obliquus* grown in synthetic medium (Bristol medium, 75.5%) (Batista et al., 2014) and in urban wastewater (76.6%) (Batista et al., 2015) are similar and much lower than those obtained in the other WW. The latter results (77.2–93.1%) may suggest that all the obtained *S. obliquus* biomasses have potential as feedstock for hydrogen production by dark fermentation, justifying the objectives of this work.

3.3. *Scenedesmus obliquus as feedstock for biohydrogen production by Enterobacter aerogenes*

The initial substrate concentration in all fermentations, was 2.5 g_{VS}/L_{FM} aiming to compare the WW biomass results with those observed for the microalga grown in synthetic medium (Bristol). For the dark fermentation assay, the total VS values for each biomass were also used to calculate the specific bioH_2 yield. The hydrogen yield (mL H_2/g_{VS} and mL H_2/L_{FM}) and purity (H_2/CO_2 v/v) values were thus evaluated and compared with those obtained for the microalga grown in synthetic medium (Batista et al., 2014). These results are shown in Fig. 3.

The best results were clearly obtained with the *S. obliquus* grown in swine and poultry WW, with considerably higher values for both hydrogen yields (around 7-fold) and gas purity (around 6-fold) (Fig. 3), in relation to synthetic medium grown biomass. This may be the result of the high sugar content present in the biomass (36.2% for swine and 23.6% for poultry WWs, respectively).

Despite this fact, it is important to highlight that, in this work, the microalgal biomass used as substrate was grown in effluents of complex and different composition. Therefore, the initial concentration of the substrate was established in terms of its VS content (see Fig. 2) and not in terms of its content in sugars. So, other factors may have influenced the process such as the chemical composition of the growth wastewater (Table 2). Furthermore, since the microalgae were not washed before is used as feedstock, consideration should be given to the presence of compounds adsorbed to the surface of the microalga, which could be more available to be metabolised by the bacteria, enhancing the results obtained for poultry and swine WWs. This assumption may explain that although the relationship between bioH_2 production and the feedstock sugar content would lead us to predict a higher production of hydrogen for brewery WW (highest total sugars content), the values did not reach those obtained with swine and poultry.

All the H_2 production yields were higher than the control (synthetic Bristol medium) (except for cattle and urban WW), the swine and poultry WW values being 7-fold and 6.8-fold higher, respectively.

An important point to highlight is the high purity of the produced biogas (H_2/CO_2volume ratio), 7.0 and 6.7, for swine and poultry WW, respectively (Fig. 3), which is crucial to facilitate the hydrogen purification process.

These purity results were much higher than those obtained by other authors when using, as substrate, *S. obliquus* grown in Bristol medium (between 1.13 and 1.67) (Batista et al., 2014) and the leftover biomass of *Nannochloropsis* sp., after the extraction of oils and pigments (between 1.00 and 1.40) (Nobre et al., 2013). A similar value of biogas purity (5.5) was reached by Ortigueira et al. (2015) using a strict anaerobic bacteria (*C. butyricum* DSM 10702) as fermentative microorganism.

Apart from the very high (about seven fold higher) production yields and biogas purity, achieved for microalgae grown in the swine and poultry WW, the results registered for the *S. obliquus* grown in the other effluents (50.0–56.8 mLH_2/gVS and 1.0–1.6, respectively) were of the same order of magnitude of those reported by Batista et al. (2014) (57.6 mL H_2/gVS; and 1.13), when synthetic Bristol was used for microalga growth (Fig. 3). The production yield achieved for the *S. obliquus* biomass produced from the brewery WW was slightly higher (67.1 mL H_2/gVS) (Ferreira et al., 2017).

Moreover, it should be noted that all the values presented in this study were more promising than those achieved by other authors, when used *S. obliquus* biomass after alkaline and/or acid pretreatments (specific production yields between 16.99 and 45.54 mLH_2/gVS) (Yang et al., 2011, 2010) or a wet algal culture (mixed consortia predominantly composed of *Scenedesmus* and *Chlorella* species), also submitted to autoclave pretreatment (29.5 mLH_2/gVS) (Kumar et al., 2016).

Finally, it is important to highlight the fact that in all runs, the *S. obliquus* biomass substrate was sterilized together with the fermentation medium, in order to take advantage of the autoclaving conditions (121 °C, 1.4 bar, for 15 min) which act as a thermal treatment, promoting the breakage of microalga cells and the release of sugars and other compounds (e.g. storage polysaccharides) with potential as feedstock to produce $bioH_2$. Thus, in this work the thermal treatment of the substrate was associated to the fermentation medium sterilization step (essential to a fermentative process with pure bacteria), which is an advantage from the point of view of energy efficiency (Batista et al., 2014).

In conclusion, the results presented in this study undoubtedly demonstrated the potential of the *S. obliquus* biomass as feedstock for hydrogen production by dark fermentation, particularly if it is grown in wastewaters and represents an obvious environmental and economic advantages. This integrated process could be widespread as an example of a real circular bioeconomy (Lakaniemi et al., 2013).

4. Conclusions

The microalgae-based wastewater treatment approach studied in this work resulted in the production of valuable biomass at a potentially lower cost and energy demand, with good potential for hydrogen generation.

Scenedesmus obliquus was able to efficiently remove most of the pollution load present in the various wastewaters (poultry, swine and cattle breeding, brewery and dairy industries, and urban). For nitrogen, the removal yield values were above 95%, with virtually complete removal for brewery and dairy wastewaters. For phosphorus, the removal efficiencies were 63–99% and for COD, 48–70%. All the microalga biomasses produced had a high protein content (31–53%), with also 12–36% of sugars and 8–23% of lipids.

Regarding $bioH_2$ production from algal biomass by the dark fermentation process, the hydrogen yields attained for *S. obliquus* grown in wastewater media, namely 390 and 378 mL H_2/g_{VS}, respectively, for biomass grown in swine and poultry WW, were considerably higher than those resulting from biomass grown using synthetic medium. These higher yields are potentially associated to lower costs on chemicals end energy input, lower CO_2 emissions and minimal impact on freshwater supplies. Moreover, the $bioH_2$ produced achieved high purity levels (the H_2/CO_2 volume ratio was up to 7 for biomass grown on swine WW), which is crucial for reducing costs associated to its purification.

In conclusion, this microalgae-based system opens up the possibility of wastewater treatment with reduced costs and environmental impacts, improving the sustainability and profitability of the process, and could be one of the best strategies for the future of bioenergy production and circular bioeconomy.

Acknowledgments

The study was supported by the Project ERANETLAC/0001/2014 (ELAC2014/BEE0357) GREENBIOREFINERY-Processing of brewery wastes with microalgae for producing valuable compounds, COST Action 1408 EUALGAE- European network for algal-bioproducts, project “WW-SIP — From Urban Wastewater Treatment Plant to Self-Sustainable Integrated Platform for Wastewater Refinement” (LIFE10 ENV/IT/000308). Henrique Mendonça thanks the Coordination for the Improvement of Higher Level or Education Personnel (CAPES) for financial support (process nW: 99999.010229/2014-00).

Paula Assemany thanks her sandwich PhD scholarship (CNPq 234510/2014-5) funded by the Brazilian National Council for Scientific and Technological Development (CNPq). H. M. Pinheiro acknowledges the funding received by iBB-Institute for Bioengineering and Biosciences, from Fundação para a Ciência e a Tecnologia, Portugal (UID/BIO/04565/2013), and from Programa Operacional Regional de Lisboa 2020 (Project No. 007317). The authors would like to thank Sociedade Central Cervejas e Bebidas (SCC), Portugal, to the access of brewery effluent, AVIBOM (poultry effluents) and Graça Gomes (LNEG) and Natércia Santos (LNEG) for microalgae culture maintenance and laboratory assistance.

References

A.O.A.C, 2006. Official Methods of Analysis, 18th ed. Association of the Official Analytical Chemists (A.O.A.C.), Gaithersburg MD, USA.

Acar, C., Dincer, I., 2014. Comparative assessment of hydrogen production methods from renewable and non-renewable sources. Int. J. Hydrog. Energy 39, 1–12. http://dx.doi.org/10.1016/j.ijhydene.2013.10.060.

Álvarez-díaz, P.D., Ruiz, J., Arbib, Z., Barragán, J., Garrido-pérez, M.C., Perales, J.A., 2015. Technology Wastewater Treatment and Biodiesel Production by Scenedesmus Obliquus in a Two-stage Cultivation Process, 181, pp. 90–96. doi:10.1016/j.biortech.2015.01.018.

APHA (American Public Health Association), 2012. Standard methods for the examination for water and wastewater, 22nd ed. Washington, DC.

Assemany, P.P., 2017. Energetic valorization by anaerobic digestion of algal biomass cultivated in wastewater. Universidade Federal de Viçosa.

Barata, A.F., 2016. Microalgae: Sustainable Economical and Environmental Production. Faculdade de Ciências, University of Lisbon.

Batista, A.P., Ambrosano, L., Graça, S., Sousa, C., Marques, P.A.S.S., Ribeiro, B., Botrel, E.P., Neto, P.C., Gouveia, L., 2015. Combining urban wastewater treatment with biohydrogen production – an integrated microalgae-based approach. Bioresour. Technol. 184, 230–235. http://dx.doi.org/10.1016/j.biortech.2014.10.064.

Batista, A.P., Gouveia, L., Marques, P.A.S.S., 2018. Fermentative hydrogen production from microalgal biomass by a single strain of bacterium Enterobacter aerogenes e Effect of operational conditions and fermentation kinetics. Renew. Energy 119, 203–209. http://dx.doi.org/10.1016/j.renene.2017.12.017.

Batista, A.P., Moura, P., Marques, P.A.S.S., Ortigueira, J., Alves, L., Gouveia, L., 2014. Scenedesmus obliquus as feedstock for biohydrogen production by Enterobacter aerogenes and Clostridium butyricum. Fuel 117, 537–543. http://dx.doi.org/10.1016/j.fuel.2013.09.077.

Brewers Assiociation, 2013. Wastewater Management Guidance Manual (WWW Document). ⟨https://www.brewersassociation.org⟩ (Accessed 18.12.17).

Das, D., Veziroglu, T.N., 2008. Advances in biological hydrogen production processes. Int. J. Hydrog. Energy 33, 6046–6057. http://dx.doi.org/10.1016/j.ijhydene.2008.07.098.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356. http://dx.doi.org/10.1021/ac60111a017.

Efremenko, E.N., Nikolskaya, A.B., Lyagin, I.V., Senko, O.V., Makhlis, T.A., Stepanov, N.A., Maslova, O.V., Mamedova, F., Varfolomeev, S.D., 2012. Bioresource Technology Production of biofuels from pretreated microalgae biomass by anaerobic fermentation with immobilized Clostridium acetobutylicum cells. Bioresour. Technol. 114, 342–348. http://dx.doi.org/10.1016/j.biortech.2012.03.049.

Eurostat 2013–2015[WWW Document], n.d. URL ⟨http://ec.europa.eu/eurostat⟩.

Ferreira, A., Ribeiro, B., Marques, P.A.S.S., Ferreira, A.F., Dias, A.P., Pinheiro, H.M., Reis, A., Gouveia, L., 2017. Scenedesmus obliquus mediated brewery wastewater remediation and CO_2 bio fi xation for green energy purposes. J. Clean. Prod. 165, 1316–1327. http://dx.doi.org/10.1016/j.jclepro.2017.07.232.

Ferreira, A.F., Marques, A.C., Batista, A.P.B., Marques, P.A.S.S., Gouveia, L., Silva, C.M., 2012. Biological hydrogen production by Anabaena sp. – yield, energy and CO_2 analysis including fermentative biomass recovery. Int. J. Hydrog. Energy 37, 179–190. http://dx.doi.org/10.1016/j.ijhydene.2011.09.056.

Gouveia, L., Graça, S., Sousa, C., Ambrosano, L., Ribeiro, B., Botrel, E.P., Neto, P.C., Ferreira, A.F., Silva, C.M., 2016. Microalgae biomass production using wastewater: treatment and costs: scale-up considerations. Algal Res. 16, 167–176. http://dx.doi.org/10.1016/j.algal.2016.03.010.

Hoebler, C., Barry, J.L., David, A., Delort-Laval, J., 1989. Rapid acid hydrolysis of plant cell wall polysaccharides and simplified quantitative determination of their neutral monosaccharides by gas-liquid. J. Agric. Food Chem. 37, 360–367. http://dx.doi.org/10.1021/jf00086a020.

IPMA, 2017. Instituto Português do Mar e da Atmosfera [WWW Document]. URL ⟨https://www.ipma.pt/pt/otempo/prev.descritiva/inde2017⟩ (Accessed 1 January 2017).

Jones, D.B., 1941. Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins. Circular.

Kim, M., Baek, J., Yun, Y., Jun, S., Park, S., Kim, S., 2006. Hydrogen Production from Chlamydomonas Reinhardtii Biomass Using a Two-step Conversion Process : Anaerobic Conversion and Photosynthetic Fermentation, 31, pp. 812–816. doi:10.

1016/j.ijhydene.2005.06.009.
Kumar, G., Sivagurunathan, P., Bao, N., Thi, D., Zhen, G., Kobayashi, T., 2016. Evaluation of different pretreatments on organic matter solubilization and hydrogen fermentation of mixed microalgae consortia. Int. J. Hydrog. Energy 41, 21628–21640. http://dx.doi.org/10.1016/j.ijhydene.2016.05.195.
Lakaniemi, A.-M., Tuovinen, O.H., Puhakka, J.A., 2013. Anaerobic conversion of microalgal biomass to sustainable energy carriers – a review. Bioresour. Technol. 135, 222–231. http://dx.doi.org/10.1016/j.biortech.2012.08.096.
Lepage, G., Roy, C.C., 1986. Direct transesterification of all classes of lipids in a one-step reaction. J. Lipid Res. 27, 114–120.
Liu, C., Chang, C., Cheng, C., Lee, D., 2012. Fermentative hydrogen production by Clostridium butyricum CGS5 using carbohydrate-rich microalgal biomass as feedstock. Int. J. Hydrog. Energy 37, 15458–15464. http://dx.doi.org/10.1016/j.ijhydene.2012.04.076.
Lutzu, G.A., Zhang, W., Liu, T., 2015. Feasibility of using brewery wastewater for biodiesel production and nutrient removal by Scenedesmus dimorphus Feasibility of using brewery wastewater for biodiesel production and nutrient removal by Scenedesmus dimorphus. Environ. Technol. 37, 1–54. http://dx.doi.org/10.1080/09593330.2015.1121292.
Maroneze, M.M., Barin, J.S., Menezes, C.R., De, Queiroz, M.I., Queiroz, L., Jacob-lopes, E., 2014. Treatment of cattle-slaughterhouse wastewater and the reuse of sludge for biodiesel production by microalgal heterotrophic bioreactors. Sci. Agric. 4, 521–524.
Mendonça, H.V., 2017. Treatment and Valorization of Cattle Wastewater by Biological Processes. Federal University of Juiz de Fora (UFJF).
Mendonça, H.V., Ribeiro, C.B.D.M., Borges, A.C., Bastos, R.R., 2012. Remoção de nitrogênio e fósforo de águas residuárias de laticínios por sistemas alagados construídos operando em bateladas. Ambient. e Água - An Interdiscip. J. Appl. Sci. 7, 75–87. http://dx.doi.org/10.4136/ambi-agua.805.
Mendonça, H.V. de, Ometto, J.P.H.B., Otenio, M.H., Reis, A.J.D. dos, Marques, I.P.R., 2017. Bioenergy recovery from cattle wastewater in an UASB-AF hybrid reactor. Water Sci. Technol. 76, 2268–2279. http://dx.doi.org/10.2166/wst.2017.325.
Nguyen, T.A.D., Kim, K.R., Nguyen, M.T., Kim, M.S., Kim, D., Sim, S.J., 2010. Enhancement of fermentative hydrogen production from green algal biomass of Thermotoga neapolitana by various pretreatment methods. Int. J. Hydrog. Energy 35, 13035–13040. http://dx.doi.org/10.1016/j.ijhydene.2010.04.062.
Nobre, B.P., Villalobos, F., Barragán, B.E., Oliveira, A.C., Batista, A.P., Marques, P.A.S.S., Mendes, R.L., Sovová, H., Palavra, A.F., Gouveia, L., 2013. A biorefinery from Nannochloropsis sp. microalga – extraction of oils and pigments. Production of biohydrogen from the leftover biomass. Bioresour. Technol. 135, 128–136. http://dx.doi.org/10.1016/j.biortech.2012.11.084.
Ortigueira, J., Alves, L., Gouveia, L., Moura, P., 2015. Third generation biohydrogen production by Clostridium butyricum and adapted mixed cultures from Scenedesmus obliquus microalga biomass. Fuel 153, 128–134. http://dx.doi.org/10.1016/j.fuel.2015.02.093.
Posadas, E., Morales, M. del M., Gomez, C., Acién, F.G., Muñoz, R., 2015. Influence of pH and CO_2 source on the performance of microalgae-based secondary domestic wastewater treatment in outdoors pilot raceways. Chem. Eng.
Raposo, M., Oliveira, S., Castro, P., 2010. On the utilization of microalgae for brewery effluent treatment and possible applications of the produced biomass. J. Inst. Brew. 116, 285–292.
Simate, G.S., Cluett, J., Iyuke, S.E., Musapatika, E.T., Ndlovu, S., Walubita, L.F., Alvarez, A.E., 2011. The treatment of brewery wastewater for reuse: state of the art. Desalination 273, 235–247.
Statista, 2017. Beer Production Worldwide from 1998 to 2015 (in billion hectoliters) (WWW Document). 〈https://www.statista.com/statistics/270275/worldwide-beer-production/〉 (accessed 30.05.17).
Su, Y., Mennerich, A., Urban, B., 2012. Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: influence of algae and sludge inoculation ratios. Bioresour. Technol. 105, 67–73. http://dx.doi.org/10.1016/j.biortech.2011.11.113.
Su, Y., Mennerich, A., Urban, B., 2011. Municipal wastewater treatment and biomass accumulation with a wastewater-born and settleable algal-bacterial culture. Water Res. 45, 3351–3358. http://dx.doi.org/10.1016/j.watres.2011.03.046.
Yang, Z., Guo, R., Xu, X., Fan, X., Li, X., 2010. Enhanced hydrogen production from lipid-extracted microalgal biomass residues through pretreatment. Int. J. Hydrog. Energy 35, 9618–9623. http://dx.doi.org/10.1016/j.apenergy.2010.09.009.
Yang, Z., Guo, R., Xu, X., Fan, X., Luo, S., 2011. Fermentative hydrogen production from lipid-extracted microalgal biomass residues. Appl. Energy 88, 3468–3472. http://dx.doi.org/10.1016/j.apenergy.2010.09.009.
Zhang, Y., Angelidaki, I., 2014. Microbial electrolysis cells turning to be versatile technology: recent advances and future challenges. Water Res. 56, 11–25. http://dx.doi.org/10.1016/j.watres.2014.02.031.