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Combining urban wastewater treatment with biohydrogen production – An integrated microalgae-based approach

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HIGHLIGHTS

- Efficient treatment of urban wastewater with microalgae.
- Energetic valorization of microalgae biomass.
- Production of biohydrogen through dark fermentation using *Enterobacter aerogenes*.
- Fermentation kinetics monitored and fitted to a modified Gompertz model.
- Highest bioH_2 production yield (56.8 mL H_2/g_{VS}) obtained for *Scenedesmus obliquus*.

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ABSTRACT

The aim of the present work was the simultaneous treatment of urban wastewater using microalgae and the energetic valorization of the obtained biomass. *Chlorella vulgaris* (*Cv*), *Scenedesmus obliquus* (*Sc*) and a naturally occurring algal *Consortium C* (*ConsC*) were grown in an urban wastewater. The nutrient removals were quite high and the treated water fits the legislation (PT Dec-Lei 236/98) in what concerns the parameters analysed (N, P, COD). After nutrient depletion the microalgae remained two more weeks in the photobioreactor (PBR) under nutritional stress conditions, to induce sugar accumulation (22–43%). The stressed biomass was converted into biohydrogen (bioH_2), a clean energy carrier, through dark fermentation by a strain of the bacteria *Enterobacter aerogenes*. The fermentation kinetics were monitored and fitted to a modified Gompertz model. The highest bioH_2 production yield was obtained for *S. obliquus* (56.8 mL H_2/g_{VS}) which was very similar when using the same algae grown in synthetic media.

1. Introduction

The modern societies have to face big challenges, such as food and energy supply, scarcity and security of water, and environment/climate protection.

The connection between these issues is very strong: (1) the food and energy (for heat, light, power and transportation) demand is estimated to double in the 2008–2035 period; (2) the annual global freshwater demand is expected to grow by about 10–12% per decade. Agriculture is by far the largest consumer of freshwater – about 70% of all freshwater withdrawals go to irrigated agriculture (Oilgae, 2014).

All these challenges and drawbacks of the society so far, must be supported by an (1) agriculture/algaeculture providing food and feed in a sustainable way using as less freshwater as it can be; (2) intensive wastewater treatment and/or desalination of brackish and salt water; (3) production, as much as possible, of bioenergy and biofuels using economically and environmentally more sustainable renewable energy sources. Avoiding the use of fossil fuels can also limit the global warming and climate change.

Microalgae biotechnology can contribute to solve these main key challenges.

The wastewater treatment using microalgae offers some interesting advantages over conventional methods, such as low energy requirement (no need of agitation to oxygenate) (Oswald, 2003), Green House Gas emission reduction (the entire system is carbon negative), reduction of sludge formation (no use of hazardous chemicals), lower costs and all in parallel with the production of useful algal biomass. The biomass produced by nutrient remediation from the wastewater is energy rich and can be further processed to make biofuels or other valuable products such as

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biofertilizers, biopolymers, bioplastics, lubricants, paints, dyes and colorants.

The simultaneous wastewater treatment and production of valuable algal biomass enhances the environmental and economic benefits of the process (e.g., Razzak et al., 2013). According to Brennan and Owende (2010) the combination of these processes will be the most plausible commercial application in the short term, and is probably one of the most sustainable ways to produce bio-energy and bio-products. Wijffels and Barbosa (2010) also highlighted the importance of reusing wastewater with remediation of the nutrients, as the production of biodiesel from microalgae, requires approximately 1.5 L water/kg of biofuel produced in closed systems (being worst for open systems where fresh water needs to be added to compensate the evaporation). The annual water consumption is in the range of 11–13 million L/ha assuming a productivity of 40,000 L biodiesel/ha year (Wijffels and Barbosa, 2010).

The use of microalgae to treat wastewater is not innovative, and has been already reported by several authors (e.g., Min et al., 2011; Abdelaziz et al., 2013). According to Christenson and Sims (2011) for the production of large scale algae-biofuels it is mandatory that the primary goal should be the wastewater treatment, concomitant to the biomass production. In spite of the huge potential of using microalgae biomass after wastewater treatment for biofuel production, the studies published so far just included biodiesel (e.g., Alva et al., 2013; Fathi et al., 2013). The use of wastewater-algae to biocrude, by hydrothermal liquefaction, was showed by Roberts et al. (2013) using a pilot-scale algal cultivation ponds fed with municipal wastewater.

The cultivation of microalgae in wastewater use 90% less freshwater and reduce the N requirement by up to 94% (Rawat et al., 2013). Lam and Lee (2012) also depicted that up to 50% of the energy use and GHG emissions (when cradle to the gate LCA analysis is employed) is due to the chemical fertilization for the microalgae cultivation.

Several biofuels could be obtained from algae biomass, including: biodiesel, biohydrogen, biomethane, bioethanol or other hydrocarbon fuel variants, such as JP-8 fuel, gasoline, etc.

Hydrogen is considered a fuel of the future mainly due to its high conversion efficiency (142 kJ/g), recyclability and non-polluting nature (water as a unique combustion product). Biological hydrogen production (bioH_2) is found as environment friendly and less energy intensive when compared with thermochemical and electrochemical conventional processes (e.g. gasification, water electrolysis).

Microalgae and cyanobacteria can be used for biohydrogen production either by directly using light energy to convert photosynthetically water to H_2 (bio-photolysis) and as a substrate for dark fermentation by anaerobic bacteria (Ferreira et al., 2012). The fermentative processes are usually faster and lead to higher hydrogen yields (Hallenbeck, 2005). Hydrogen production from fermentation of microalgae biomass was recently explored by our research group using *Anabaena* sp. (Ferreira et al., 2012), *Nannochloropsis* sp. (Nobre et al., 2013) and *Scenedesmus obliquus* (Batista et al., 2014; Ferreira et al., 2013) and also by other authors (e.g. Lakaniemi et al., 2011; Yang et al., 2011; Liu et al., 2012). None of these studies used microalgae biomass cultivated in wastewater. Therefore, the main aim and particular innovation of the present study was to energetically valorize an urban wastewater, combining microalgae-based wastewater treatment with the production of a clean energy carrier.

This work is part of a Life project (WW-SIP, LIFE10 ENV/IT/000308) where three microalgae (*Chlorella vulgaris*, *S. obliquus* and *Consortium C* isolated from the wastewater) were grown in a tubular vertical Photobioreactor prototype (150 L), using an urban wastewater. The present study gives an insight into bio-energy potential of this biomass in terms of conversion to biofuels, specifically bioH_2. The three microalgae were submitted to a nutritional stress, to induce sugar accumulation, and were used as feedstock for dark fermentation by a strain of *Enterobacter aerogenes*. The kinetics of fermentative bioH_2 production was monitored, and the maximum volumetric production, specific yields, and production rates were determined.

2. Methods

2.1. Microalgae cultivation

Three microalgae were cultivated outdoor using an urban wastewater from Águas da Figueira (AdF, Figueira da Foz, PT) collected after primary treatment: *C. vulgaris* – INETI 58, LNEG_UB, Portugal (*Cv*); *S. obliquus* – ACOI 204/07, Coimbra University Algotec, Portugal (*Sc*) and a *Consortium C* (*ConsC*) – isolated from the wastewater (including different strains such as *Chlorella*, *Scenedesmus*, *Chaetophora* and *Navicula*).

The photobioreactor (PBR) used was a tubular vertical one of 150 L of capacity. The PBR included 12 poly(methyl methacrylate) tubes with 100 mm diameter and 2000 mm height.

The microalgae were grown in the PBR, under natural light and temperature (exterior) until wastewater was treated (according to PT Decreto-Lei n° 236/98), i.e. until nutrient depletion. Afterwards the microalgae remained two more weeks in the PBR, under nutritional stress conditions, to induce sugar/oil accumulation.

The biomass was collected, dried and its biochemical composition was evaluated.

2.1.1. PBR monitorization

The urban wastewater and the daily samples of supernatant (after removal of the microalgae biomass) collected from the PBR, were evaluated in terms of ammonium, nitrates, phosphorus and Chemical Oxygen Demand (COD).

Ammonium was analysed using a Crison multimeter MM41 with an Ion Selective Electrode NH_4^+. Commercial kits were used for nitrates (Nitrate Cell Test 1.14542 – Test kit Spectroquant Merck), phosphorus (Phosver 3-powder pillows, Cat. 2125-99 Hach) and COD (Cat 21258-51, Hach) determinations using a Spectrophotometer HACH DR/2010.

2.1.2. Microalgae biomass characterization

The biochemical composition of the microalgae biomass was achieved according to the A.O.A.C (2006), and all analyses were done in duplicate. Moisture was determined by drying in an oven at 105 °C until constant weight. Total ash was determined by incineration at 550 °C in a muffle furnace. The volatile solids (VS) content was determined by the difference from the moisture and total ash.

For the biomass characterisation in terms of lipids and pigments the microalgae were ground for 4 min and frequency of 25 s^{-1}, in a ball mill (MM400, Retsch). Total lipid content was evaluated by extraction with *n*-hexane in a Soxhlet, over 6 h. Total pigments were extracted with acetone, with glass beads through mechanical (vortex) and thermal (ice) shocks and quantified by spectrophotometry. Total sugar content was evaluated by the phenol–sulphuric method (Dubois et al., 1956), on filtered samples previously submitted to quantitative acid hydrolysis. This method consists in a two stage hydrolysis: first acid hydrolysis (H_2SO_4 72% w/w, 30 °C, 1 h) attacks the polysaccharide fibres making them soluble and then by autoclave (H_2SO_4 4% w/w, 120 °C, 1 h) hydrolyses the sugar polymers, yielding sugar monosaccharides with minimum degradation. Protein content was estimated by the Kjeldahl method using 6.25 as the conversion factor of total nitrogen to crude protein.

2.2. Biohydrogen production potential from the microalgal biomass

2.2.1. Bacteria culture conditions

E. aerogenes, ATCC 13048 Sputum (American Type Culture Collection, Manassas, USA) fermentative bacteria was used for bioH_2 production. The bacteria culture was kept at 4 °C in solid CASO Agar and grown in a synthetic growth media (20 g/L peptone solution with 5 g/L NaCl). Bacteria were harvested from the exponential growth phase. For the bioH_2 production assays the fermentation media contained K_2HPO_4 (7.0 g/L), KH_2PO_4 (5.5 g/L), tryptone (5 g/L), yeast extract (5 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4{\cdot}7H_2O$ (0.25 g/L), $CaCl_2{\cdot}2H_2O$ (0.021 g/L), $Na_2MoO_4{\cdot}2H_2O$ (0.12 g/L), nicotinic acid (0.02 g/L), Na_2SeO_3 (0.172 mg/L), $NiCl_2$ (0.02 g/L). The initial pH medium was constant, with values around 6.8. Both media were always sterilized before use.

2.2.2. Dark fermentation assays

Batch fermentation experiments were performed in 159 mL glass flasks, containing a phases volumetric ratio (gaseous headspace: liquid fermentation medium) of 5, under orbital shaking (220 rpm). The microalgae biomass (*Cv*, *Sc* and *ConsC*) obtained after wastewater treatment and stressed, were used as a substrate at a concentration of 2.5 g/L.

The bioreactors containing fermentation medium and microalgal biomass were previously sterilized in autoclave at 121 °C for 15 min. The reactors and fermentation medium were purged with bubbling N_2 for 2 min to eliminate O_2, before inoculation with exponentially grown *E. aerogenes* at 10% v/v (corresponding to 0.1 g dry weight biomass/L culture media), and sealed with butyl rubber stoppers. The process was carried out under orbital shaking (220 rpm), at 30 °C, and the kinetics of biohydrogen production was followed over 8 h of fermentation.

2.2.3. Analysis of the gas and liquid phases from the dark fermentation

The concentrations of H_2 and CO_2 were determined by gas chromatography in a Varian 430-GC gas chromatograph with TCD and a fused silica column (select Permanent gases/CO_2-Molsieve 5A/Boraabound Q tandem #CP 7430). The gases injector and column were at 80 °C and the detector at 120 °C. Argon was the carrier gas at 32.4 mL/min flow rate. A gas-tight syringe was used to directly withdraw 0.5 mL of the gaseous headspace which was injected into the gas chromatograph.

The supernatants of the fermentation trials, obtained by centrifugation (15,000 rpm/2 min) and filtration of the liquid phases (0.22 μm), were analyzed by HPLC in terms of, ethanol and organic acids concentration. The analysis was performed in a Merck Hitachi HPLC system (Darmstadt, Germany) equipped with an Aminex HPX-87H column and a refraction index detector. The temperature of the column was set to 50 °C, and the eluent consisted of H_2SO_4 5 mM at 0.5 mL/min flow rate. The volume of the injection was 20 μL.

3. Results and discussion

3.1. Characterization of microalgal biomass cultivated in wastewater

The results of Table 1 show that the treated water obtained using microalgae fits the legislation (PT Dec-Lei 236/98) concerning the parameters analysed (N, P, COD) for *Sc* and *ConsC*.

However, for *Cv*, all the parameters fit the Portuguese legislation, except for P, although 77% of removal was achieved. An explanation for this fact can be related to the high initial level of P in the urban wastewater used for this trial, which was quite high when compared to the other ones. The high value was probably due to an unexpected discharge of pollutants and/or to the seasonality of Figueira da Foz region, where the treatment plant is located.

Concerning ammonium removal, a high percentage was obtained: 98% for *Sc*, 96% for *Cv* and 99.5% for *ConsC*. The high ammonium removal is enhanced by the fact that if ammonium, nitrate or nitrite are present in the medium, microalgae will primarily use ammonium until depletion, and then the remaining forms, nitrite followed by nitrate (Abdelaziz et al., 2013).

High removal rates were also obtained for phosphorus (*Sc*-100%, *ConsC*-100% and *Cv*-77%).

The best removal rate for COD was obtained with *Sc* (54%). The other microalgae have a low removal rate or even increased the organic charge. Several assumptions could be made, i.e. the low biodegradability of carbon or even a different algal–bacterial symbiosis. Nevertheless, COD parameter fits the legislation.

The biochemical composition of the microalgal biomass, obtained before and after nutritional stress, is presented in Tables 2 and 3, respectively.

The microalgae cultured in urban wastewater, harvested before nutritional stress (Table 2) presented, in general, a biochemical composition characterized by high protein content (33–56% w/w) and lower lipid and sugar content. This profile is typical of microalgae grown without nutrient limitation, when protein synthesis and cellular division are not impaired.

It is conceivable that microalgae may have used the organic matter content of the effluent to grow mixotrophically, although it was not possible to measure this. However, regarding the low organic load and high luminosity, this contribution would not have been substantial when compared to autotrophic growth mode.

Table 2
Chemical composition of microalgal biomass cultured in urban wastewater. Average ± standard deviation values, expressed in ash free dry weight (% AFDW).

	Scenedesmus obliquus	*Chlorella vulgaris*	*Consortium C*
Crude protein (%)	32.7 ± 0.1	56.4 ± 0.1	51.6 ± 0.6
Total lipids (%)	8.1 ± 0.1	10.0 ± 0.3	13. 6 ± 3.9
Total sugars (%)	11.7 ± 3.7	27.7 ± 0.7	20. 6 ± 4.0
Total Pigments (%)	1.2 ± 0.1	1.2 ± 0. 1	0.9 ± 0.1

Table 1
Nutrient characterization in PBR, at starting point and after nutritional stress.

	NH_4^+ (mg/L)		NO_3^- (mg/L)		$P\text{-}PO_4^{3-}$ (mg/L)		COD (mg/L)	
	Initial[a]	Final[b]	Initial[a]	Final[b]	Initial[a]	Final[b]	Initial[a]	Final[b]
Scenedesmus obliquus	209	4.3	<2.2	2.9	5.5	0.0	123	57
Chlorella vulgaris	37	1.3	23.3	<2.2	67	15.3	60	82
Consortium C	444	2.0	<2.2	5.3	7.0	0.0	137	132
Legislation[c]		10		50		10		150

[a] Initial – PBR starting point inoculated with urban wastewater and microalgae culture.
[b] Final – PBR after two weeks of nutrient depletion (nutritional stress).
[c] Legislation – PT Decreto-Lei n° 236/98.

Table 3
Chemical composition of microalgal biomass, cultured in urban wastewater, after two weeks of nutrient depletion (nutritional stress). Average ± standard deviation values, expressed in ash free dry weight (% AFDW).

	Scenedesmus obliquus	*Chlorella vulgaris*	*Consortium C*
Crude protein (%)	35.7 ± 0.2	34.0 ± 0.7	45.3 ± 1.2
Total lipids (%)	25.2 ± 1.5	12.8 ± 0.6	12.8 ± 0.9
Total sugars (%)	42.6 ± 8.5	21. 9 ± 1.5	28.6 ± 1.1
Total Pigments (%)	1.0 ± 0.1	0.5 ± 0. 1	0.8 ± 0.1

Moreover, as discussed above, the lipid content of the microalgal biomass obtained was relatively low (8.1–13.6% w/w), contrary to what is usually associated with mixo/heterotrophic growth (e.g. Ren et al., 2014).

As expected, the protein content decreased after nutritional stress (Table 3), since in the absence of a nitrogen source, protein and chlorophyll synthesis is reduced and its content (% dry weight) can decrease almost an order of magnitude (Breuer et al., 2012). Conversely, this depletion leads to a lipid and/or sugar accumulation (e.g. Breuer et al., 2012; Dragone et al., 2011), since carbon is accumulated in the form of triacylglycerols and reserve sugars (e.g. starch) within the cells. This mechanism can be explored in terms of maximizing biofuel production (biodiesel, bioethanol, biohydrogen).

However, the lipid increase was not significant for *Cv* and *ConsC*, with final values of only 13% (w/w) lipids (Table 3).

S. obliquus presented a significant increase both in total lipid and sugar contents after nutritional stress. The lipid content increased from 8% up to 25% (w/w). This alga has proved to be a good source of single cell oil for biodiesel production (by e.g. Mandal and Malick, 2009; Gouveia and Oliveira, 2009), cultivated using synthetic medium. Furthermore, the increase in sugar content upon nutritional stress was more significant for *Sc* attaining quite high values (43% w/w) which is in accordance to Miranda et al. (2012), that reported a maximum sugar content of 45% (w/w) (mainly glucose) in a closed PBR continuously illuminated. Ho et al. (2012) also stated a value of 39% (w/w), which is far higher than the ones reported by Harun et al. (2010) (10–17% w/w).

The sugar content of the *Chlorella* presented in this work is also higher than the usually reported, 21.9% against 12–17% reported by Harun et al. (2010). The isolated alga *ConsC* presented, as well, quite high sugar content (about 29% w/w) after nutritional stress.

The quite high sugar content present in the algae-based wastewater in this study is a positive feature regarding the potential use of the biomass as a fermentative substrate for hydrogen or ethanol production. Miranda et al. (2012) proved its potential for ethanol; Ferreira et al. (2013), Batista et al. (2014) and Yang et al. (2011) are examples of studies that showed the hydrogen production using *Scenedesmus* as a feedstock through dark fermentation.

C. vulgaris was also widely studied for several applications, such as biofuels, in especial for biodiesel, however biohydrogen production using the bacteria *Clostridium butyricum* (Liu et al., 2012) or anaerobic digested sludge (Lakaniemi et al., 2011) was also documented.

3.2. Energetic valorization of microalgal biomass – conversion to biohydrogen

The main innovation of this manuscript is that, to our knowledge, so far, no study was reported for bioH_2 production using, as feedstock, microalgae biomass after remediation of the nutrients present in wastewater.

Hence, dark fermentation experiments were carried out using, as substrate, the three microalgae biomasses studied in this work (*Cv*, *Sc* and *ConsC*) and the bioH_2 production was monitored until stabilization (8 h) (Fig. 1). As can be noted, in all cases a typical cumulative hydrogen production behavior was observed, with an initial lag phase (variable according to the microalga), followed by a rapid increase (exponential) in the bioH_2 production, and finally reaching a stationary phase which occurs around 4–5 h for all algae. This is highly advantageous in terms of the short processing time required and reducing energy expenditure. These results show that the strain of the bacteria *E. aerogenes* (facultative anaerobic) used in this study proved to be efficient in converting different microalgal biomass into hydrogen through a dark fermentation process. In addition, the process was faster when compared to the ones that use other fermentative microorganisms, namely strict anaerobic bacteria, such as *C. butyricum* (Ferreira et al., 2013; Batista et al., 2014).

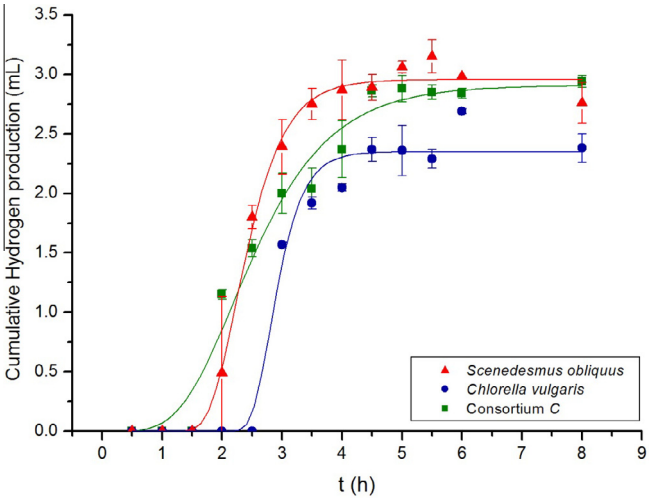

Fig. 1. BioH_2 fermentation kinetics of microalgal biomass by *Enterobacter aerogenes*. The lines correspond to the fitting of the modified Gompertz model.

The experimental data were fitted to a modified Gompertz model (Zwietering et al., 1990) which has been extensively used to describe the fermentative bioH_2 production profile. The equation can be expressed as (1):

$$H(t) = H\exp\left\{-\exp\left[\frac{Rme}{H}(\lambda - t) + 1\right]\right\} \quad (1)$$

where $H(t)$ represents the cumulative volume of hydrogen production (mL), H the maximum hydrogen production potential (mL), R_m the maximum production rate (mL/h), λ the duration of the lag phase (h), t the incubation time (h) and e the exp(1) (2.71828).

A very good fitting of this model (Fig. 1) for all experimental data ($R^2 > 0.978$) was observed, as can be confirmed on Table 4.

The highest hydrogen production potential observed was registered for *Sc* (2.96 mL). However, the *ConsC* presented a very similar value (2.91 mL), which is advantageous considering that these microalgae which appeared naturally in the wastewater are environmentally more adapted and more robust.

Although the use of the *Cv* biomass reached a high bioH_2 production rate (2.9 mL/h, in this case the lag phase was much longer (λ = 2.5 h) and the final cumulative volume of bioH_2 was lower (2.35 mL) than for the other algae. This behavior can be related with the influence of the inoculum–substrate ratio on the lag-phase time (Sun et al., 2011). In fact, the lag phase is defined as the phase which is necessary for adaptation of the fermentative microorganisms to new environment. In this study, the *Cv* fermentation assay was conducted using an inoculum concentration slightly lower than for the other algae (0.5 g/L vs. 0.7 g/L) due to

Table 4
Fitting parameters of the modified Gompertz model to hydrogen production kinetics and calculated specific bioH_2 production obtained from the fermentation of microalgal biomass by *Enterobacter aerogenes*.

	H (mL)	R_m (mL/h)	λ (h)	R^2	Chi^2	mL $H_2/g_{VS\ alga}$
Scenedesmus obliquus	2.96 ± 0.04	2.44 ± 0.24	1.79 ± 0.06	0.9945	0.0113	56.8
Chlorella vulgaris	2.35 ± 0.08	2.87 ± 0.68	2.50 ± 0.11	0.9820	0.0283	40.8
Consortium C	2.91 ± 0.11	1.20 ± 0.17	1.28 ± 0.17	0.9778	0.0365	46.8

a lower pre-inoculum cell growth (the reactor inoculation was always 10% v/v). Considering that the lag phase depends on the metabolic activity of the microorganisms, it is possible that a lower inoculum concentration may have caused a delay on the onset of hydrogen production. This could be particularly important if one considers that the bacterial population inoculum had to adapt to a fresh fermentation media with different composition (including a complex substrate such as microalgal biomass).

In opposition, the *ConsC* began the bioH_2 production earlier (λ = 1.3 h) but at a much slower rate (1.2 mL/h) which can be related to the cellular complexity of the consortium composition. In fact, besides green algae (Chlorophyta) of the genera *Scenedesmus*, *Chlorella* and *Chaetoceros*, one of the algae genus identified in the consortium was the diatom *Navicula*. The Bacillariophyta, or diatoms, are characterized by having cell walls made of silica (frustules) and chrysolaminarin (β-1,3 linked glucan) as storage product, while the Chlorophyta have cellulose cell walls and starch (β-1,4 linked glucan) as storage product (Tomaselli, 2004). Thus, it is possible that the proportion of diatom biomass presented in *ConsC* has as a consequence a higher resistance to cellular disruption and/or solubilization through the process of autoclave sterilization, which can be considered as a thermal pre-treatment. This may have resulted in a lower biodegradability of the substrate (microalgal biomass), contributing to the slower hydrogen production rate observed.

The specific hydrogen production for each algae feedstock is depicted in Table 4. This parameter was calculated by dividing the H (derived from Eq. (1)) by the microalgal biomass volatile solid (VS) content.

In this work, the attained value of 56.8 mL H_2/g_{VS} for *Sc* was of the same order of magnitude than the one reported by Batista et al. (2014) (57.6 mL H_2/g_{VS}) when fresh synthetic culture medium was used for *Sc* growth. This result highly promising and represents obvious environmental and economic advantages.

For *Cv* the maximum specific production achieved (40.8 mL H_2/g_{VS}) was significantly higher than the one stated by Lakaniemi et al. (2011) (10.8 mL H_2/g_{VS}) using anaerobic digested sludge as inoculum, proving that the pure culture of bacteria (facultative anaerobic) used was more efficient for biologically converting this biomass into H_2. Nevertheless, when using *Clostridium* (strictly anaerobic) as fermentative bacteria, a value of 81 mL H_2/g_{dw} was reached by Liu et al. (2012) and 103 mL H_2/g_{VS} by Batista et al. (2014).

Although the hydrogen volume produced by the *Sc* and the *ConsC* is similar (2.96 and 2.91 mL, respectively), the volatile solid content is different (76.6 and 89.5%, respectively) and consequently the specific hydrogen productions are different. In fact, the fermentation of *Sc* generated 56.8 mL H_2/g_{VS} while the *ConsC* yielded a lower value (46.8 mL H_2/g_{VS}). This can be related to the higher sugar content of *Sc* (43% w/w) followed by the *ConsC* (29% w/w) total sugars. This is in agreement with the statement of Hallenbeck (2005) where *E. aerogenes* dark fermentation process is directly proportional to the total sugar content of the feedstock. Moreover, the specific hydrogen production attained with the fermentation of the *ConsC* was higher than the one achieved with *Cv* (40.8 mL/g_{VS}) which is also in agreement with its lower sugar content (29% w/w). However, the most important aspect to note here is to associate the promising results obtained with the *ConsC* fermentation, in terms of biohydrogen production, with the environmental and economic benefits, cited previously.

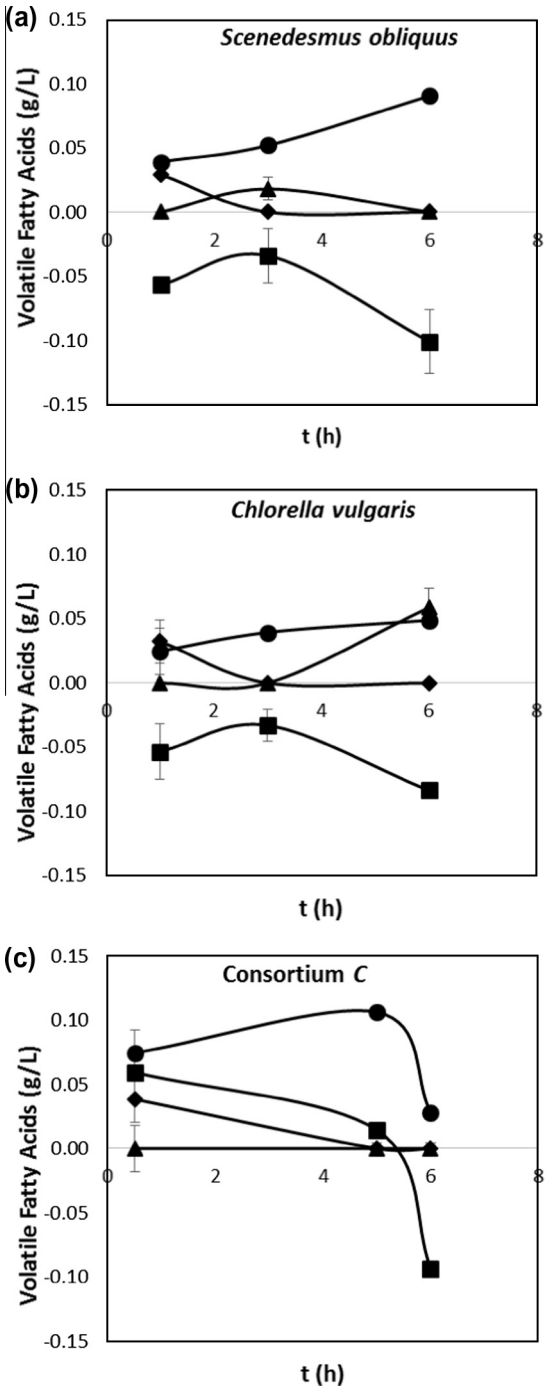

Fig. 2. Production/consumption of succinic (●), lactic (▲), formic (◇) and propionic (■) acid along the fermentation of microalgal biomass by *Enterobacter aerogenes*.

Dark fermentation by enteric bacteria is a mixed process of sugars and/or organic wastes conversion into end-products such as ethanol, volatile fatty acids, carbon dioxide and hydrogen (Hallenbeck, 2005). The relative proportion of these products depends on the nature of the substrate and the operational conditions. Considering this, in the present work, the evolution of the liquid phase composition along fermentation, was followed. Consumption of propionic acid as well as the production of succinic acid by the bacteria *E. aerogenes* was observed for all microalgae feedstock tested (Fig. 2). The production of succinic acid, although in small concentration, has already been reported for some strains of *E. aerogenes* bacteria (Urdanetta et al., 1995; Nobre et al., 2013; Batista et al., 2014). The evolution on formic and lactic acid on fermentation media is less significant. No ethanol production was detected, which can suggest that the metabolism was more diverted towards the formate route (yielding H_2 and CO_2) rather than the acetyl CoA via (yielding ethanol and acetate) (Hallenbeck, 2005). Although, in any case, the final concentration of total volatile fatty acids in the fermentation broth is relatively low (around 0.7 g/L).

4. Conclusions

The integrated microalgae-based approach studied enabled to achieve simultaneous goals: (i) efficient wastewater treatment/nutrient removal; (ii) efficient and economic microalgae production avoiding the use of potable water and synthetic-nutrients; (iii) energetic valorization of microalgal biomass through biofuel production ($bioH_2$). The hydrogen yields attained were comparable to the ones using synthetic medium, beyond lower costs, energy input, and GHG emissions involved in fertilizer production and minimal impact on freshwater supplies. Thus, the possibility of producing biofuel(s) with nutrient remediation from wastewater improves the sustainability and profitability of the whole system and could be one of the best strategies for the bioenergy future.

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