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Fermentative hydrogen production from microalgal biomass by a single strain of bacterium *Enterobacter aerogenes* – Effect of operational conditions and fermentation kinetics

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ABSTRACT

Biohydrogen production through dark fermentation is a promising technology for generating renewable energy, while using microalgal biomass as a third generation feedstock can further increase the sustainability of the process. In the present study, *Scenedesmus obliquus* was used as model microalga substrate for studying the impact of operational parameters in batch dark fermentation trials using a strain of *Enterobacter aerogenes* bacteria.

(i) The initial gas-liquid ratio in the bioreactor (from 1.3 to 8.2) was tested, resulting in higher bioH_2 yields for ratios above 5.

(ii) Different bacterial growth, inoculation procedures and fermentation media were tested in combined experiments. The best conditions were chosen by maximising bioH_2 yield and minimising production time and costs.

(iii) The autoclave sterilization effect on sugar extraction and bioH_2 yield was tested for different microalga concentrations (2.5–50 g/L) with best results attained for 2.5 g/L (81.2% extraction yield, 40.9 mL H_2/g alga).

For the best operational conditions, fermentation kinetics were monitored and adjusted to the Modified Gompertz model, with t_{95} (time required for bioH_2 production to attain 95% of the maximum yield) below 4.5 h. The maximum hydrogen production was higher when using wet algal biomass enabling the energy consuming biomass drying step to be skipped.

1. Introduction

The production of hydrogen from renewable sources is a promising alternative for the future, considering the need for cleaner energy carriers and a reduction in carbon dioxide emissions. Hydrogen is a carbon-free fuel (upon oxidation produces only water) and the most energy-dense fuel per unit mass (142 kJ/g).

The European Strategic Energy Technology Plan [1] has identified hydrogen-based technologies among the technologies needed in Europe to achieve its target for 2020: a 20% reduction in greenhouse gas emissions; a 20% share of renewable energy sources in the energy mix and a 20% reduction in primary energy use. Hydrogen can play an important role in the reduction of local air pollutants, as well as in the decarbonisation of Europe's Transport system.

At present, however, hydrogen is mainly produced from fossil fuels (e.g. natural gas steam reforming, coal gasification) or water (e.g. electrolysis, photolysis) through energy intensive processes. It is therefore essential to develop and optimize more environmentally-friendly, energy-efficient and sustainable hydrogen production processes, namely through biological biomass fermentation.

The production of hydrogen through anaerobic fermentation is a relatively simple process and can use a wide spectrum of substrates, including waste products [2] and microalgae, such as *Scenedesmus obliquus* [3], *Nannochloropsis* sp. [4], *Chlorella vulgaris* [5], *Dunaliella tertiolecta* [5], *Spirogyra* sp [6]. *Enterobacter aerogenes* is known as an efficient biohydrogen producing bacterium, as it is one

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of the most widely studied model strains [7–9]. Moreover, it is a facultative anaerobic bacterium, which is able to grow in the presence of oxygen, and therefore makes the manipulation in bioprocesses much easier compared with the use of strict anaerobes [10]. In this context, *E. aerogenes* has proven to be efficient in the bioconversion of the microalgae *Anabaena* sp. [11], *Nannochloropsis* sp. [4,12], *Scenedesmus obliquus* [3,13] and *Chlorella vulgaris* [13]. *S. obliquus* is a green microalga which is widely used for biofuel production purposes [14–16]. In fact, it can accumulate either oil or sugar and it is used for biodiesel [14,17] or bioethanol production [18,19], showing also its potential as a feedstock for biogas ($bioH_2$ and $bioCH_4$) through anaerobic production processes [3].

Dark fermentation is a complex system where environmental and operational factors such as temperature, pH and H_2 partial pressure, regulate metabolic pathways of hydrogen producing microorganisms [20]. A lower partial pressure in the headspace of the reactors is one of the parameters which facilitate the mass transfer of hydrogen from the liquid to gas phase. Also, besides the type of substrate and its pretreatment, the inoculum sources and enrichments also greatly influence the biohydrogen production. In addition, the bacteria culture seed and inoculation procedure are also very important factors for the startup of the hydrogen production process. Thus, when dealing with complex substrates, such as microalgal biomass, it is crucial to study the effect of these parameters which influence the dark fermentation pathways and consequently the biohydrogen yields [20,21].

The aim of the present work was to explore the operational conditions which were best suited for biohydrogen production using *S. obliquus* microalga as a model substrate. The experiments were conducted in batch mode and the influence of the following parameters on the $bioH_2$ production was tested: (i) Initial gas-liquid volume ratio in the bioreactor; (ii) Bacteria growth and inoculation procedure; (iii) Sterilization. Finally, the fermentation kinetics parameters were also monitored.

2. Material and methods

2.1. Microalga production

Scenedesmus obliquus ACOI 204/07 from Coimbra University Algotec (Portugal) was used in this study. The culture was grown in a Bristol culture medium at pH 7 [22]. Initially, 1 L glass bubble column photobioreactors, with filtered bubbling air, at a constant temperature of 25 ± 1 °C, under low light (150 $\mu E/m^2.s$) were used. Afterwards, *S. obliquus* biomass was produced in two outdoor open raceway ponds (300 L capacity, 2 m^2 each), agitated by paddlewheels at approximately 5 m/min, with natural light (light/dark cycles). For the recovery of the microalgal biomass from the raceway ponds the agitation was stopped, the biomass settled down, most of the liquid phase was poured out and the biomass collected was centrifuged (10000 rpm; 10min) (Avanti J25, Beckman). The biomass was dried in an oven at 80 °C overnight (16 h), and had a proximate composition of: 30.7% total sugars, 17.1% crude fat, 20.4% crude protein and 20.2% total minerals (all % w/w in dry weight) [3]. The algal biomass was used as carbon and energy source in the fermentations for H_2 production. For biohydrogen production kinetics assays (see Section 3.4), wet microalgal biomass, collected after centrifugation, was also tested.

2.2. Fermentative bacterium

Enterobacter aerogenes ATCC 13048 Sputum (American Type Culture Collection, Manassas, USA), was used for the fermentation experiments, being harvested from exponentially grown cultures (Section 2.3.2). The original culture was kept at 4 °C in solid CASO Agar (MERCK: 15 g/L peptone from casein, 5 g/L peptone from soymeal, 5 g/L sodium chloride and 15 g/L agar–agar).

2.3. Biohydrogen production experiments

Batch fermentation assays were performed in 159 mL serum bottles closed with butyl rubber stoppers and crimped with aluminium seals.

Initially, the bioreactors containing both fermentation medium and microalgal biomass (substrate) were sterilized in an autoclave (121 °C, 15 min and 1.4 bar). Afterwards, the sterilized bioreactors were aseptically purged with bubbling N_2 (2 min) to replace O_2 and were inoculated with different initial concentrations of exponentially grown *E. aerogenes*, according to the objectives.

The fermentation was carried out under orbital shaking (220 rpm), for 24 h at 30 °C. Control fermentation tests, without microalgal biomass, were also prepared for all assays. Peptone water (PW) and Complex Fermentation Medium (CFM) (see composition in Section 2.3.2), were used as pre-inoculum and fermentation medium, respectively [except in the cases indicated in Table 1 for the assays on bacteria growth and inoculation procedure (Section 2.3.2)].

2.3.1. Initial gas-liquid volume ratio

The serum bottles (159 mL) were filled with determined volumes of liquid media (containing CFM and 10 g/L microalga) in

Table 1
Experimental conditions and inoculation procedure effect on the bacteria growth and biohydrogen production. Fermentation conditions – [*Sc obliquus*]: 10 g_{Alga}/L_{FM}; Gas-liquid volume ratio: 5; Fermentation time: 24 h.

Pre-Inoculum		Inoculation		Fermentation		
Medium[a]	Conditions	Procedure ($L_{Pre\text{-}inoculum}/L_{FM}$)	Bacterial concentration ($g_{dry\ biomass}/L_{FM}$)	Fermentation medium[a] (FM)	Specific yield (mLH_2/g_{Alga})	Biogas purity (H_2/CO_2)
PW	Aerobic	1% (v/v)	0.01	PW	11.3	1.1
		10% v/v)	0.1	PW	9.6	1.1
		1% (v/v)	0.01	CFM	19.9	1.5
		10% (v/v)	0.1	CFM	20.9	1.1
		Concentration by Centrifugation	1.0	CFM	23.5	1.4
PW	Anaerobic	1% (v/v)	0.003	CFM	15.3	1.2
				BM1	14.2	1.3
RCM	Anaerobic	1% (v/v)	0.01	CFM	17.8	1.1
				BM1	11.8	1.3
RCM	Aerobic	1% (v/v)	0.03	BM1	7.5	1.2

[a] PW: Peptone Water; CFM: Complex Fermentation Medium; RCM: Reinforced Clostridia Medium; BM1: Basal Medium.

order to get gas-liquid ratios of 1.3, 3.6, 5.0, 5.9 and 8.2. All the reactors were inoculated with 1 g/L bacterium.

2.3.2. Bacterial growth and inoculation procedure

E. aerogenes is a facultative anaerobic bacterium, so pre-inoculums can be grown either in aerobiosis (conical flask with cotton stopper) or anaerobiosis (encapsulated serum bottles purged with bubbling N_2).

In this work, different bacterial synthetic growth media and operational conditions were tested to obtain the pre-inoculum (Table 1), namely:

- Peptone Water (PW): 20 g/L peptone, 5 g/L NaCl.
- Complex Fermentation Medium (CFM): 7.0 g/L K_2HPO_4, 5.5 g/L KH_2PO_4, 5 g/L tryptone (pancreatic digest of casein, Bacto™), 5 g/L yeast extract, 1.0 g/L $(NH_4)_2SO_4$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.021 g/L $CaCl_2 \cdot 2H_2O$, 0.12 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.02 g/L nicotinic acid, 0.172 mg/L Na_2SeO_3, 0.02 mg/L $NiCl_2$, with a pH of 6.8 [23].
- Reinforced Clostridial Medium (RCM), Difco™: 10 g/L peptone, 10 g/L beef extract, 3 g/L yeast extract, 5 g/L dextrose, 5 g/L NaCl, 1 g/L starch, 0.5 g/L cysteine, 3 g/L sodium acetate, 0.5 g/L agar. pH 6.8.
- Modified Basal Medium 1 (BM1): 5 g/L trypticase soya broth (TSB) without dextrose, 5 g/L yeast nitrogen base (YNB), 1 g/L NH_4Cl, 0.56 g/L cysteine hydrochloride, 0.4 g/L $K_2HPO_4 \cdot 3H_2O$, 0.1 g/L $MgCl_2 \cdot 6H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5.0 mg/L Na_2.EDTA.$2H_2O$, 1.5 mg/L $CoCl_2 \cdot 6H_2O$, 1.0 mg/L $MnCl_2 \cdot 4H_2O$, 1.0 mg/L resazurin, 1.0 mg/L $FeSO_4 \cdot 7H_2O$, 1.0 mg/L $ZnCl_2$, 0.4 mg/L $AlCl_3 \cdot 6H_2O$, 0.3 mg/L $Na_2WO_4 \cdot 2H_2O$, 0.2 mg/L $CuCl_2 \cdot 2H_2O$, 0.2 mg/L $NiSO_4 \cdot 6H_2O$, 0.1 mg/L H_2SeO_3, 0.1 mg/L H_3BO_3, 0.1 mg/L $Na_2MoO_4 \cdot 2H_2O$. The pH was adjusted to 6.8 with 50 mM phosphate buffer [24].

Different procedures for bacteria inoculation on the autoclaved serum bottles (containing liquid media and the microalga biomass) were also studied:

- Addition of a pre-defined concentration of bacterium - The exponentially grown cells (pre-inoculum) were harvested by centrifugation, washed and re-suspended in sterilized Millipore water according to the regular cell washing procedure. Then a certain volume of cell suspension was added to the serum bottles, in order to have an initial bacterial concentration of 1 $g_{dry\ biomass}/L$.
- Direct addition of a certain volume of exponentially grown cultures (pre-inoculum), corresponding to 1% or 10% (v/v) of the bioreactor liquid phase (ranging from 0.003 to 0.1 $g_{dry\ biomass}/L$).

A substrate concentration of 10 g/L alga and a gas-liquid ratio of 5 were used in these studies.

2.3.3. Effect of sterilization in autoclave

The effect of autoclaving (121 °C, 15min, 1.4 bar), acting as a sterilization process concomitantly with microalga thermal pre-treatment was studied. In this context *S. obliquus* biomass solubilization (i) and bioH_2 production (ii) were analyzed. So,

(i) The sterilized liquid phase was centrifuged and the supernatant collected for analysis (see Section 2.4.2);
(ii) Experiments were conducted using the autoclaved media at a gas-liquid ratio of 5 and inoculating 10% (v/v) of bacterial cells.

For these experiments, different initial microalga concentrations (substrate) were tested (from 2.5 to 50 g/L).

2.3.4. Fermentation kinetics

For the best operational conditions obtained (gas-liquid ratio of 5 and bacterial inoculation of 10% (v/v)) and initial substrate concentration (2.5 g/L), the fermentation kinetics was monitored, in terms of cumulative bioH_2 production.

These experiments were also carried out with wet microalgal biomass, i.e. after centrifugation (69% humidity) avoiding energy-consuming drying steps.

2.4. Analytical methods

2.4.1. Analysis of the gaseous phase

The gaseous phase samples were collected directly from the headspace of the serum bottles by using a gas-tight syringe. The contents of H_2 and CO_2 were analysed through gas chromatography, at atmospheric pressure, in a Varian 430-GC (Middelburg, Netherlands) equipped with thermal conductivity detector (TCD) and a fused silica column (Select Permanent Gases/CO2-Molsieve 5A/Borabound Q Tandem #CP 7430). Injector and column were operated at 80 °C and the detector at 120 °C. Argon was the carrier gas at 32.4 mL/min flow rate.

2.4.2. Analysis of the liquid phase

All liquid samples were centrifuged (15000 rpm/2min) and filtered through cellulose nitrate membranes with 0.22 μm pore diameter, before analysis.

Total sugar content was evaluated by phenol–sulphuric method [25], on samples previously submitted to a two-step quantitative acid hydrolysis (H_2SO_4 72% w/w/30 °C/1 h followed by dilution to 4% w/w H_2SO_4 and autoclave for 1 h).

High Performance Liquid Chromatography (HPLC) was used for analysing liquid media composition in terms of volatile fatty acids, monosaccharides (cellobiose, glucose, arabinose and xylose) and glycerol contents. A Merck Hitachi HPLC system (Darmstadt, Germany) equipped with an Aminex HPX-87H column (BioRad) and a refractive index detector was used. The temperature of the column was 50 °C, and H_2SO_4 5 mM at 0.5 mL/min flow rate was used as eluent.

2.5. Statistical analysis

Independent experiments and analysis were always performed in duplicate, and results are presented as average ± standard deviation. The results were evaluated in terms of ANOVA Post Hoc Comparisons-Scheffé test, at 0.05 probability level, using STATISTICA Software (version 8.0) from StatSoft Inc. (2007).

3. Results and discussion

3.1. Effect of the initial gas-liquid volume ratio

The gas-liquid volume ratio is a relevant operational parameter to be taken into account in dark fermentative hydrogen processes, since it can significantly affect the biogas production yield [20,26,27]. In this study, the influence of different headspace volumes on the fermentation performance was evaluated, corresponding to gas-liquid volume ratios studied were between 1.3 and 8.2 (Fig. 1).

From Fig. 1 it can be observed that an increase in the gas-liquid ratio from 1.3 to 8.2 there is an increase in the specific bioH_2 production yield, even though for values > 5 no significant differences ($p < 0.05$) were observed. Therefore, in subsequent studies it was decided to maintain this ratio at 5, since it allows attaining high production yields while processing larger volumes of fermentation

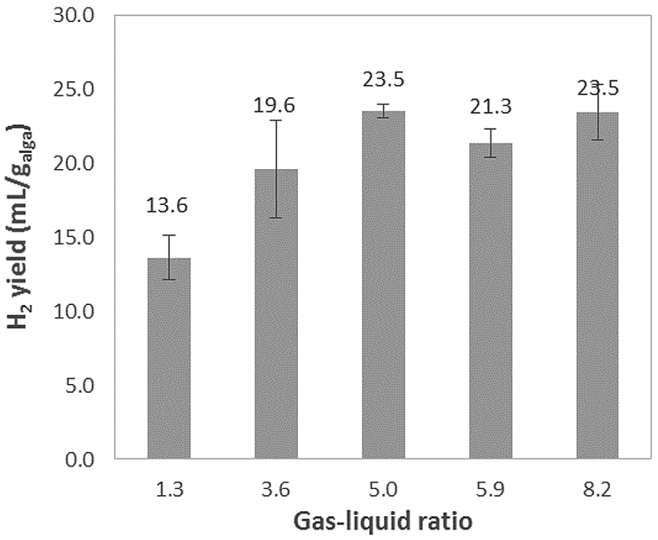

Fig. 1. Effect of initial gas-liquid volume ratio on specific biohydrogen production. Microalgal concentration: 10 g/L; Inoculum concentration: 1 g/L; fermentation time: 24 h.

broth.

Higher yields obtained at larger headspaces may be related to lower partial and total pressure in the serum bottles, which may have affected *E. aerogenes* performance. In fact, during the fermentation process the hydrogenase ([NiFe]-hydrogenase 3, [28]) is involved in reversibly oxidizing and reducing ferredoxin and if the hydrogen concentration in the liquid phase increases, ferredoxin reduction is favored at the expense of oxidation, decreasing the H_2 production [20]. Moreover, lower partial pressure in the headspace of the reactors facilitates the mass transfer of hydrogen from the liquid to the gas phase [20]. Accordingly, partial pressure of H_2 in the reactor headspace will determine the concentration of dissolved hydrogen gas in the liquid phase, which considerably influences hydrogen production performance [21].

However, there has to be a compromise regarding higher gas-liquid ratios would require larger bioreactor volumes and/or lower processing of liquid broth/microalga biomass feedstock. An alternative procedure would be the continuous removal of the produced gases. Oh et al. [27] reported a modified technique to measure the gas production by using a large ratio headspace volume/culture medium. By reducing the partial pressure of the produced gas, the potential for gas product inhibition is reduced, thereby eliminating the need for more expensive gas respirometers. Nasr et al. [29] also evaluated the impact of CO_2 sequestration on H_2 yield, H_2 production rate, metabolic pathways, and microbial community structure in a novel continuous flow biohydrogen production system. The authors observed that the removal of CO_2 from the headspace shifted the H_2 producing pathways forward, increasing H_2 production yield and rate by 22% and 23%, respectively. They associated this behavior with the fact that CO_2 sequestration changed the propionate consumption pathway to be thermodynamically favorable, thus producing more acetate and H_2 [29].

3.2. Effect of bacterial growth and inoculation conditions

Bacterial growth and inoculation conditions can have a major impact on the production yield of a biotechnological process. *E. aerogenes* is a facultative anaerobic bacterium, which means that it can grow (pre-inoculum) either under aerobic or anaerobic conditions using relatively poor media such as peptone water. However, the hydrogen fermentative stage should occur under anaerobic conditions regardless of the operating conditions and physico-chemical parameters.

In this work, experiments were carried out using different pre-inoculum and fermentation media compositions, inoculation procedures and inoculum concentration, in order to study each effect on the bacterial performance and process yields (Table 1).

It can be concluded that when using peptone water as pre-inoculum medium, the *E. aerogenes* grew better under aerobic rather than anaerobic conditions (pre-inoculum with 0.01 vs. 0.003 $g_{dry\ biomass}$/L, respectively), for 1% (v/v) inoculation.

However, using low cost peptone water also as fermentation medium led to significantly ($p < 0.05$) lower bioH_2 yields when compared to CFM, either for 1% (11.3 vs. 19.9 mL H_2/g alga) or 10% (9.6 vs. 20.9 mL H_2/g alga) inoculation.

For all the cases studied, there were no significant differences ($p > 0.05$) when using 1% or 10% (v/v) inoculum (ca. 0.01 to 0.1 $g_{dry\ biomass}$/L) on the resulting hydrogen yields. In comparison, for the "regular procedure" (cell concentration by centrifugation) the specific hydrogen productions were a little bit higher, although not significantly ($p > 0.05$), despite the inoculated bacterial biomass concentration being much higher (1 g/L). So, this procedure was not employed in the subsequent experiments since the results were not outweighing the higher consumption of time and energy.

RCM was also tested as pre-inoculum medium, under aerobic, anaerobic and anoxic conditions. The highest bacterial biomass concentrations was attained under aerobic conditions (0.03 $g_{dry\ biomass}$/L), but H_2 yields was much lower (7.5 mL H_2/g alga) using BM1 as fermentation medium. In fact, this medium proved to be inefficient for *E. aerogenes* bioH_2 production, for all the cases studied (Table 1).

In conclusion, from the results attained it was decided to use peptone water as pre-inoculum medium, under low cost easy-manipulation aerobic conditions, and CFM as fermentative medium. The standard inoculation procedure, involving time-consuming centrifugation and cell-washing steps, which enabled to achieve higher biomass concentrations (inoculum concentrations up to 1 g/L), was found to be unnecessary since the bioH_2 yields were similar to much simpler direct bacterial inoculation procedure. Therefore, in subsequent trials, it was decided to use 10% (v/v) direct bacterial inoculation.

3.3. Effect of sterilization/pretreatment on microalga metabolites solubilization

The sterilization of the fermentation medium containing the substrate (fermentation liquid phase) prior to inoculation is essential when the fermentative microorganism is a pure culture. On the other hand, the application of temperatures above 100 °C for 15–30 min with gradual pressure release, on microalgal biomass, is considered an efficient pretreatment (hydrothermal pretreatment) [30], positively influencing its anaerobic biodegradability [31]. Kumar et al. [32] showed that autoclave pretreatment promoted more release of soluble organics (protein and carbohydrate) than other treatment methods. These authors attributed this ability to the involvement of pressure (1.03 bar) and temperature (121 °C) to break the cell wall and released of extra-cellular compounds.

In this study, *S. obliquus* was not submitted to any pretreatment except the required autoclave sterilization of the fermentation medium (supplemented with the microalgal biomass), which is energetically advantageous. So, the effect of autoclaving (121 °C, 15min, 1.4 bar), acting simultaneously as sterilization method and microalga hydrothermal pretreatment was studied, in order to evaluate its efficiency.

Table 2
Autoclave sterilization (121°C/15min/1.4 bar) effect on the sugar extraction and specific hydrogen production, for different microalgal concentrations. Fermentation conditions - [*Sc obliquus*]: 2.5–50 g_{Alga}/L_{FM}; Inoculum concentration: 0.1 g/L (10%, v/v); Gas-liquid volume ratio: 5; Fermentation time: 24 h.

[*S. obliquus*] (g_{Alga}/L_{FM})	Total sugars		Specific H_2 production	
	Concentration in the FM (g/L)[a]	Extraction Yield[b] (%, w/w)	mL/ g_{Alga}	mL/ $g_{SugarExt}$
2.5	0.58	81.2	40.9	176
5	0.79	55.4	23.6	149
10	1.37	48.0	20.9	152
25	2.13	29.8	10.4	123
50	2.33	16.3	7.7	166

[a] Total sugars concentration after autoclaving fermentation media containing the microalgal biomass for 15 min.
[b] Percentage of microalgal sugars extracted to the liquid phase, after autoclaving, considering an initial 30.7% (w/w) total sugar proportion on Scenedesmus obliquus dried biomass [3].

Regarding the effect of sterilization on *S. obliquus* metabolites release, the sterilized liquid phase was centrifuged and the supernatant collected for analysis. Table 2 shows the total sugars solubilized from the microalga biomass under autoclaving conditions (121 °C, 15min and 1.4 bar).

It was observed that increasing microalga concentration from 2.5 to 50 g_{Alga}/L_{FM}, the total sugars content present in the liquid media also increased from 0.58 to 2.33 g/L. However, considering that *S. obliquus* dried biomass contains 30.7% (w/w) of sugars [3], the sugar extraction yield decreased from 81 to 16% with the increase of microalga concentration.

BioH_2 production trials, using *S. obliquus* as feedstock, were conducted using the autoclaved media along with microalga biomass. When increasing the microalgal concentration, a decrease in the specific H_2 production from 40.9 to 7.7 mL H_2/g_{alga} was observed (Table 2), which could be related to the observed decrease in the sugar extraction yield for higher alga concentrations (Table 2). Taking this in account, the specific H_2 production was also calculated per gram of sugar initially present in the liquid medium. The results had shown a similarity for all *S. obliquus* concentrations, being produced 176 and 166 mL of H_2 per g of microalga for 2.5 and 50 g_{alga}/L_{FM}, respectively, without any influence of alga concentration, revealing the expected link between hydrogen production and total sugar concentration.

This behaviour may also be associated to an increase of the ammonia concentration in the liquid phase with the increase of *S. obliquus* concentration. In fact, since the microalga cell wall has high protein content (±50–60%), its hydrolysis, due to the hydrothermal pretreatment, may result in a high ammonium concentration in the fermentation liquid phase [30], which might be toxic to *E. aerogenes*.

In general, the present results of hydrogen production are higher than the ones obtained by other authors, when using *S. obliquus* biomass after alkaline and/or acid pretreatments [33,34].

In a dark fermentation, H_2 production occurs simultaneously with the formation of metabolic products as ethanol and volatile fatty acids (VFAs). However, the presence of lactate, butyrate, acetate, and/or formate, can disturb the hydrogen production efficiency [35]. In this work, it was observed that negligible concentrations of ethanol (0.08–0.20 g/L), succinic (0.07–0.25 g/L), formic (0.02–0.07 g/L) and acetic acid (0.10–0.29 g/L) were produced (Table 3), which were certainly not enough to affect the activity of the *E. aerogenes* strain used in this study. This is in accordance with the constant pH value of the medium during the fermentation process (6.6–6.8) (results not shown).

In conclusion, the results presented in Tables 2 and 3 indicate that microalgal biomass is a complex substrate and that harsher pretreatments should be investigated to attain higher hydrogen yields. However, this is time consuming, as well as energetically and economically unfavorable. On the other hand, by lowering the alga concentration down to 2.5% a sugar extraction yield above 80% is attained, as well as the maximum specific H_2 production yields. Therefore further kinetic studies (Section 3.4) were conducted at this optimal concentration.

3.4. Biohydrogen production kinetics

For the best operational conditions and initial substrate concentration (2.5 g_{alga}/L_{FM}), the cumulative bioH_2 formation and the liquid phase composition were followed throughout the fermentation process. These experiments were carried out using dry and wet *S. obliquus* biomass, in order to assess the impact and the need of the energy-consuming drying step on biohydrogen production.

Typical cumulative hydrogen production behaviour was observed, with an initial lag phase, followed by a rapid increase (exponential) in the bioH_2 production, and finally reaching a stationary phase (Fig. 2). The experimental data were fitted to the modified Gompertz model [36,37], which is extensively used to describe the fermentative H_2 production:

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

Table 3
Sugars, organic acids and glycerol contents, in the complex fermentation medium (CFM), after thermal treatment (15min, 121 °C), in the presence of *Scenedesmus obliquus* microalga, before (0 h) and after (24 h) dark fermentation process. Inoculum concentration: 0.1 g/L (10%, v/v); Gas-liquid volume ratio: 5.

Alga concentration	2.5 g/L		5 g/L h		10 g/L		25 g/L		50 g/L	
	0 h	24 h	0 h	24	0 h	24 h	0 h	24 h	0 h	24 h
Cellobiose (g/L)	0.07	–	0.08	–	0.10	–	0.13	–	0.16	–
Glucose (g/L)	0.26	–	0.33	–	0.38	–	0.69	–	0.77	–
Xylose (g/L)	0.11	–	0.11	–	0.14	–	0.39	–	0.50	–
Arabinose (g/L)	0.07	–	0.08	–	0.08	–	0.00	–	0.00	–
Succinic acid (g/L)	0.07	0.19	0.08	0.19	0.10	0.25	0.09	0.22	0.06	0.16
Lactic acid (g/L)	0.02	0.00	0.02	0.01	0.02	0.02	0.04	0.04	0.06	0.07
Formic acid (g/L)	–	0.00	–	0.02	–	0.07	–	0.02	–	0.03
Acetic acid (g/L)	0.13	0.22	0.27	0.23	0.07	0.29	0.11	0.23	0.10	0.28
Propionic acid (g/L)	0.44	0.41	0.43	0.42	0.43	0.47	0.47	0.36	0.40	0.24
Isobutyric acid (g/L)	0.02	–	0.03	–	0.01	–	0.03	–	0.02	–
Butyric acid (g/L)	0.01	–		–	0.02	–	–	–		–
Glycerol (g/L)	0.04	–	0.06	–	0.09	–	0.16	–	0.22	–
Ethanol (g/L)	–	0.08	–	0.10	–	0.10	–	0.20	–	0.20

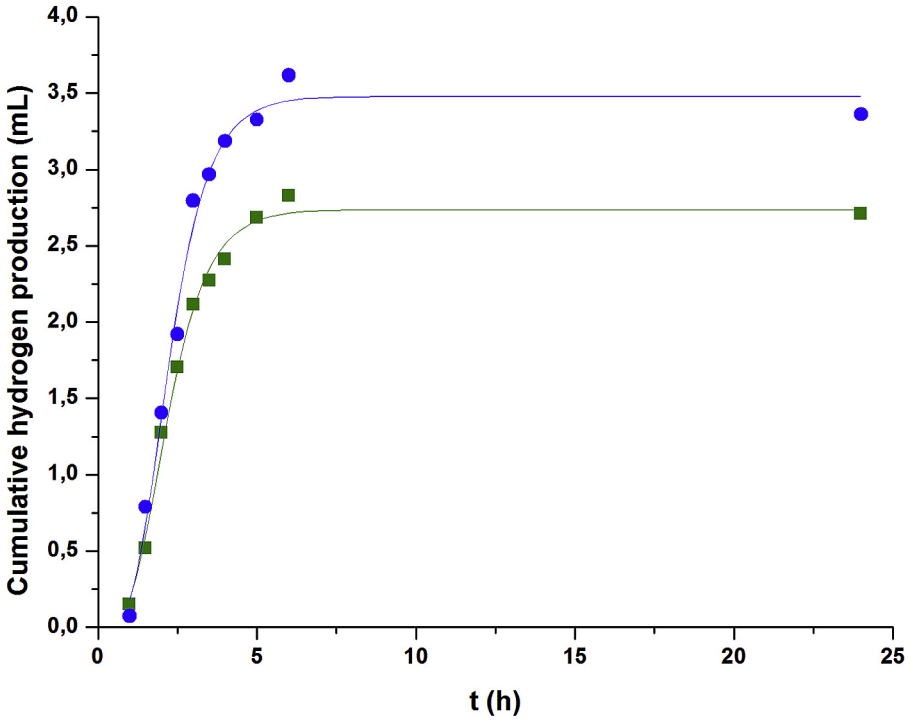

Fig. 2. BioH_2 fermentation kinetics of microalgal biomass by *E. aerogenes*. The lines correspond to the fitting of the modified Gompertz model. Alga concentration: 2.5 g/L - ■ dry ● wet; Inoculum concentration: 0.1 g/L (10%, v/v); Gas-liquid volume ratio: 5.

Table 4
Fitting parameters of the modified Gompertz model to hydrogen production kinetics and calculated specific bioH_2 production obtained by fermentation of microalgal biomass. Microalgal concentration: 2.5 g/L; Inoculum concentration: 0.1 g/L (10%, v/v); Gas-liquid volume ratio: 5.

	H (mL)	R_m (mL/h)	λ (h)	t_{95} (h)	R^2	R^2 adj.	reducedChi^2
S. obliquus - dry	2.74 ± 0.05	1.16 ± 0.08	1.00 ± 0.08	4.44	0.9936	0.9918	0.0073
S. obliquus - wet	3.48 ± 0.08	1.52 ± 0.13	1.08 ± 0.10	4.43	0.9904	0.9877	0.0186

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 Euler number.

As can be seen in Fig. 2 and in Table 4, a very good fitting of this model to the experimental data was verified ($R^2 > 0.99$). The kinetics parameters H, R_m and λ were estimated and summarized in Table 4. The fermentation using wet *S. obliquus* biomass resulted in a higher maximum H_2 production ($H = 3.48$ mL) compared to the dry biomass ($H = 2.74$ mL), which is in agreement with a previous study with *E. aerogenes* by Batista et al. [3]. This is advantageous, considering that the time-consuming and energetically-demanding biomass drying step could be avoided, increasing energetic efficiency and economic performance of this bioprocess.

The fermentation of wet *S. obliquus* biomass has also presented a higher maximum rate of hydrogen production ($R_m = 1.52$ mL/h) than that of dry biomass ($R_m = 1.16$ mL/h), which probably because wet biomass could be more readably available for bacterial degradation. The R_m values obtained in this work were higher than those observed by Kumar et al. [32], using a wet algal culture (mixed consortia predominantly composed of *Scenedesmus* and *Chlorella* species) also submitted to autoclave pretreatment ($R_m = 0.32$ mL/h).

A very small lag phase period ($\lambda = 1.00–1.08$ h), was observed for both wet and dry *S. obliquus* fermentations, indicating that the operating conditions were favorable to the microbial metabolism [37]. This is highly advantageous in terms of the short processing time required and reducing energy expenditure. Batista et al. [13] used microalgae biomass grown in urban wastewaters as substrate for biohydrogen production by *E. aerogenes*, reporting longer lag phases ($\lambda = 1.3–2.5$ h) but higher R_m (1.2–2.9 mL/h) compared to the present study (Table 4), resulting in a similar maximum hydrogen production potential ($H = 2.4–3.0$ mL).

In order to assess the onset of the stationary phase, an additional kinetic parameter derived from the Gompertz function (eq. (1)), t_{95}, was also calculated according to the equation.

$$t_{95} - \lambda = \frac{H}{R_m e}(1 - \ln(-ln0.95)) \quad (2)$$

This parameter is assumed to be the time required for hydrogen production to attain 95% of the maximum yield, which provides a measure of how fast the maximum production is achieved, and can be useful for an appropriate kinetic evaluation, when comparing data from experiments carried out in different conditions [38]. Both wet and dry *S. obliquus* biomass fermentation by *E. aerogenes* presented t_{95} values of 4.4 h (Table 4). This is considerably low when comparing to other hydrogen producing bacteria such as *Clostridium butyricum* for which 48 h fermentation have been reported when using *S. obliquus* as substrate [3].

4. Conclusions

This study has enabled to establish the most appropriate

operational conditions for the biological production of hydrogen from a strain of *E. aerogenes*, using *Scenedesmus obliquus* microalga as substrate. The effect of the initial gas-liquid volume ratio, as well as the bacterial growth and inoculation conditions, have proved to be determining factor on the biohydrogen production yield. Sterilization by autoclave can be effective for extracting the microalgae sugars, especially for alga concentrations below 10 g/L, avoiding additional pre-treatment steps which are time consuming and energy demanding. Regarding the fermentation kinetics, for 2.5 g/L alga substrate concentration, short lag phases (1 h) and early stationary phases (4.5 h) were identified. Moreover, the maximum hydrogen production potential was higher when using wet biomass, which is an advantage due to the high energy demanding algal biomass drying step being skipped/avoided. Overall, these results can be useful for providing insight and extrapolation to dark fermentation studies with other microalgal species.

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