

ORIGINAL ARTICLE

Biotransformation of water lettuce (*Pistia stratiotes*) to biohydrogen by *Rhodopseudomonas palustris*E. Corneli¹, A. Adessi² , E.J. Olguín³, G. Ragaglini^{1,4}, D.A. García-López³ and R. De Philippis²¹ Institute of Life Sciences, Scuola Superiore Sant'Anna, Pisa, Italy² Department of Agrifood Production and Environmental Sciences, University of Florence, Firenze, Italy³ Environmental Biotechnology Group, Institute of Ecology, CONACYT, Veracruz, México⁴ CRIBE – Centro Ricerche Interuniversitario Biomasse da Energia, Pisa, Italy**Keywords**

biohydrogen, lignocellulosic biomass, nitrogen stripping, photofermentation, water lettuce.

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Abstract**Aims:** Aim of the paper was to assess the feasibility of producing hydrogen as a biofuel by photofermentation of fermented water lettuce (*Pistia stratiotes* L.) waste biomass, after a nitrogen-stripping treatment.**Methods and Results:** A natural (42OL) and an engineered strain (CGA676, with low-ammonium sensitivity) of *Rhodopseudomonas palustris* were used for producing hydrogen. The stripping procedure was highly effective for ammonium removal, with an acceptable selectivity (91% of ammonium was removed; only 14% of total organic acids were lost). Both strains were able to produce hydrogen only in the nitrogen-stripped substrate. The natural strain *R. palustris* 42OL showed a higher Biochemical Hydrogen Potential (1224 ml l⁻¹ vs 720 ml l⁻¹; 50.0 mol m⁻³ vs 29.4 mol m⁻³), but at a lower rate (5.6 ml l⁻¹ h⁻¹ vs 7.3 ml l⁻¹ h⁻¹; 0.23 mol m⁻³ h⁻¹ vs 0.29 mol m⁻³ h⁻¹) than strain CGA676.**Conclusions:** Water lettuce waste biomass can be used for biofuel production, after hydrolyzation, fermentation and nitrogen stripping.**Significance and Impact of the Study:** The investigation on novel, low cost and sustainable biomasses as feedstocks for biofuel production is a priority. Aquatic plants do not compete for arable land. Moreover, water lettuce is a floating and invasive weed, thus its biomass must be harvested when detrimental, and can now be biotransformed in clean hydrogen.**Introduction**

Hydrogen gas is considered as one of the major energy carrier of the future, since it is a carbon, nitrogen and sulfur free fuel, and with the highest energy density per mass (142 kJ g⁻¹), which is 2.75 fold higher than that of hydrocarbon fuels (Holladay *et al.* 2009; Chandrasekhar *et al.* 2015). The conversion of raw biomasses into hydrogen through biological hydrogen production is a promising solution. Photofermentation is considered an efficient process, in particular when combined with dark fermentation in two-stage dark-photo fermentation processes (Keskin *et al.* 2011; Adessi and De Philippis 2012; Chandrasekhar *et al.* 2015; Corneli *et al.* 2016a). Photofermentation is carried out by purple non sulphur bacteria (PNSB), that

produce hydrogen through nitrogenase using light as energy source and simple organic acids as electron sources. Hydrogen biosynthesis occurs under nitrogen-limited conditions (Adessi and De Philippis 2014; Basak *et al.* 2014; Chandrasekhar *et al.* 2015). Commonly, biomass derived substrates are characterized by high nitrogen concentrations (Gómez *et al.* 2011; Keskin *et al.* 2011), hence some strategies for avoiding nitrogenase inhibition have been developed: engineered strains with ammonium-insensitive nitrogenase (Kars and Gunduz 2010; Adessi *et al.* 2012; Hallenbeck and Liu 2016), the use of zeolites for nitrogen removal (Wang and Peng 2010), nitrogen stripping (Bonmati and Flotats 2003; Aziz and Mojiri 2014), or medium dilution, which has as a major drawback the increase of the waste volume and handling costs (Keskin *et al.* 2011).

The investigation on renewable substrates for fermentation systems has been developed in recent years (Argun and Kargi 2011; Chandrasekhar *et al.* 2015). Currently, the use of conventional arable crops for bioenergy purposes need careful consideration about land availability and food demand. Hence, for promoting clean biofuel production and the subsequent decarbonization of energy sources the investigation of novel and low cost biomasses as feedstocks for the biological hydrogen production is required, and represents a key issue to promote the development of this technology (Keskin *et al.* 2011; Corneli *et al.* 2016a, 2016b). In this frame, an interesting opportunity for large scale sustainable application is represented by aquatic plants, which grow on water bodies, not competing with the use of arable land (Mishima *et al.* 2008). Furthermore, some species have been successfully used in large scale phytoremediation lagoons for treatment of water from polluted urban rivers and in floating wetlands for improving the quality of water and the provision of ecosystem services (Olguin *et al.* 2017a, 2017b). Thus, the harvested biomass of such aquatic plants used in these ecofriendly systems can be used for biohydrogen production.

Nevertheless, here are some considerations to take into account for successful use of aquatic plant for energy production. First, the low conversion of lignocellulosic material as feedstock for energy production is a limiting stage, therefore different authors suggest a previous stage aimed at degrading the lignocellulosic materials (Kumar *et al.* 2009). Some of the strategies described in the literature include the hydrolysis of cattail (*Typha latifolia*) by acid pretreatment (Zhang *et al.* 2011), the hydrolysis of water hyacinth (*Eichhornia crassipes*) by alkali pretreatment (Cheng *et al.* 2015), or by microwave heating (Cheng *et al.* 2013). Second, due to the high water content in the aquatic plant biomass (90–95%) and being the solid part lignocellulosic compounds, there is an imbalance in the C/N ratio during the fermentation of aquatic plant biomass as only substrate; thus co-fermentation or co-digestion are attractive alternatives to improve energy generation using aquatic plant as feedstock. In this context, Lay *et al.* (2013) explored the co-fermentation of water hyacinth and beverage wastewater for hydrogen production, reporting that the optimization of the C/N ratio is crucial to improve the hydrogen production during co-fermentation. Jacob and Banerjee (2016) tested the co-digestion of the aquatic plant *Pistia stratiotes* and potato waste for methane production and reported that the synergic effect of both substrates enhanced the methane yield by 76–45% compared to the condition of monodigestion. Third, another way to increase the energy conversion efficiency of lignocellulosic materials is through a two stage process; following this

strategy water hyacinth was converted into hydrogen and methane with high theoretical conversion efficiencies (Cheng *et al.* 2010; Su *et al.* 2010).

Water lettuce (*Pistia stratiotes* L.) is a typical macrophyte of tropical and sub-tropical environments, belonging to the *Araceae* family (Attionu 1976; Tripathi *et al.* 2010; Khan *et al.* 2014). It is a floating and invasive weed that usually covers the surface of water bodies, hindering the water flow and lowering the oxygen concentration and the light penetration in the underneath environment, which implies a serious damage for the aquatic flora and fauna (Attionu 1976; Khan *et al.* 2014). Thus, harvest and disposal of this biomass are necessary, when it becomes detrimental. Water lettuce can be used for several applications, like phytoremediation during its growth in water, otherwise the harvested biomass can be utilized for medicinal uses, biofuel production and as feed biomass for swine and buffalos (Tripathi *et al.* 2010; Khan *et al.* 2014). Concerning the use of water lettuce for biofuel production, only the production of biogas and bioethanol has been investigated (Nipanay and Panholzer 1987; Abbasi *et al.* 1991; Zennaki *et al.* 1997; Mishima *et al.* 2008; Pantawong *et al.* 2015), while hydrogen production processes have not been reported yet. The positive aspects concerning the use of this macrophyte in the bioenergy sector mainly rely on its high productivity (60–110 t ha⁻¹ year⁻¹) and on the fact that it is a no-food plant and that its production does not compete with the use of arable land, since it grows on water bodies (Mishima *et al.* 2008). To the best of the knowledge of the authors, no study yet assessed the biohydrogen potential of *P. stratiotes*.

The objective of this study was to investigate the production of biohydrogen via photofermentation using *P. stratiotes* hydrolyzed effluent, with and without ammonium removal (stripping), and using two PNSB strains of *Rhodospseudomonas palustris*, a natural strain (*R. palustris* 42OL) and a mutant strain with a low ammonium-sensitivity (*R. palustris* CGA676).

Materials and methods

Pistia stratiotes effluent

Pistia stratiotes effluent has been previously obtained by hydrolysis and acidogenesis using ruminal fluid as inoculum, as reported by Hernández-García *et al.* (2015): reactors of 2.6 l volume were operated in batch mode with an operational volume of 2.3 l. *Pistia stratiotes* biomass was added as substrate (plant leaves, roots, and stems cut to an average size of 5 mm using a conventional food processor and then homogenized), equivalent to a concentration of 30 gVS l⁻¹, finally 20% v/v of ruminal fluid as inoculum was added, tap water was used to reach

2.3 l. The reactors were kept in darkness inside an incubation chamber at $30 \pm 2^\circ\text{C}$ and were manually agitated once per day. pH was daily adjusted to pH found during rumen collection (pH 6.6), with 0.5 N NaOH. At the end of the experiment (9 days) COD was measured (APHA 1998), and resulted to be 23.78 g l^{-1} . The medium was dark-brown colored, and clear, without suspended particles.

A portion of the effluent (2 l) was then subject to ammonium stripping, according to the following procedure: the pH value was increased to 12 with the addition of NaOH (5N); the effluent was maintained at 25°C with an air flux of 3 l h^{-1} and the ammonium concentration was monitored until it reached a concentration lower than 50 mg l^{-1} (Aziz and Mojiri 2014). The effluents, stripped (ES) and not stripped (ENS), were centrifuged (10 min at 5 000 rpm, Eppendorf® centrifuge 5 810). Then, ferric citrate (0.005 g l^{-1}), sodium sulfate (0.23 g l^{-1}), phosphate buffer solution (K_2HPO_4 0.5 g l^{-1} and KH_2PO_4 0.3 g l^{-1}) were added to ES and to ENS and the effluents were autoclaved. The pH was adjusted to 6.8, before and after the autoclave.

Bacterial strains

The *R. palustris* 42OL strain (collection number CSMA73/42), which was originally isolated from a pond containing wastewaters of a sugar refinery (Adessi et al. 2016a), is deposited at the CSMA Culture Collection (WDCM number 147). The *R. palustris* CGA676, kindly provided by Prof. C.S. Harwood, has constitutive nitrogenase expression allowing it to produce H₂ in the presence of NH₄⁺ (McKinlay and Harwood 2010). The strain is available upon request to Prof. C.S. Harwood.

Inocula growth and acclimation

Rhodospseudomonas palustris strains were maintained in RPN medium in two 200 ml bottles for *R. palustris* 42OL and two 200 ml bottles for *R. palustris* CGA676 under the following growing conditions: temperature of 30°C and incandescent light of $150 \mu\text{mol (photons) m}^{-2} \text{ s}^{-1}$ (115 W m^{-2}) of intensity. Such quite high intensity was used due to the intense colour of the effluent. RPN medium (Adessi et al. 2016b) contained (g l^{-1}): lactic acid, 1.8; NH₄Cl, 0.5; K₂HPO₄, 0.5; KH₂PO₄, 0.3; MgSO₄·7H₂O, 0.4; NaCl, 0.4; CaCl₂·2H₂O, 0.075; Ferric citrate, 0.005; yeast extract, 0.4. Trace elements were provided by adding 10 ml per liter of a solution containing (mg l^{-1}): ZnSO₄·7H₂O, 10; MnCl₂·4H₂O, 3; H₃BO₃, 30; CoCl₂·6H₂O, 20; CuCl₂·2H₂O, 1; NiCl₂·6H₂O, 2; Na₂MoO₄·2H₂O, 30. The pH of the medium was adjusted at 6.8 with NaOH before autoclaving.

The phenotypic acclimation of strains was realized by centrifuging 3 ml of culture in two 1.5 eppendorf for 15 min at $1398 \times g$, the supernatant was discarded and the pellet was suspended into a tube with 20 ml of autoclaved *P. stratiotes* effluent (15% of inoculum). 12 tubes (20 ml) were set up with four different treatments as follows ($n = 3$): *R. palustris* 42OL in ES; *R. palustris* CGA676 in ES; *R. palustris* 42OL in ENS; *R. palustris* CGA676 in ENS. Tubes were put under the following growing conditions: temperature of 30°C and light of $150 \mu\text{mol (photons) m}^{-2} \text{ s}^{-1}$ (115 W m^{-2}).

Quantitative tests for hydrogen production

The photofermentation of *R. palustris* 42OL and *R. palustris* CGA676 were established with *P. stratiotes* effluent ES ($n = 3$) and ENS ($n = 3$). 100 ml rubber-stoppered glass bottles equipped with syringes for the detection of hydrogen were filled with 100 ml of autoclaved ES and ENS and an equal concentration of cells (measured as bacteriochlorophyll concentration) was suspended in each bottle. The initial bacteriochlorophyll *a* (BChl *a*) concentration in each replicate was equal to 0.3 mg l^{-1} for *R. palustris* 42OL and equal to 0.2 mg l^{-1} for *R. palustris* CGA676. Bottles were placed on the shaker (1 rpm) under anaerobic conditions, a controlled temperature (30°C), and light provided by an incandescent lamp ($200 \mu\text{mol (photons) m}^{-2} \text{ s}^{-1}$). Experiments lasted 14 days, gas production was collected continuously by water displacement method, and the amount of hydrogen in the gas phase was measured once when the gas production replaced all the air in the headspace of the bottle. Samples of the culture medium (3 ml per replicate) were taken 5 times from the beginning to the end of the experiment and stored at -20°C before chemical analysis: ammonium concentration and volatile fatty acids (VFAs) concentrations.

Analytical methods

BChl *a* concentration was determined as in Corneli et al. (2016a), measured with UV-Vis spectrophotometer model DR5000 (HACH Company, USA). The VFAs analysis was made following the method reported by Bianchi et al. (2010), using a High-Performance Liquid Chromatograph (HPLC) (1 200 series, Agilent Technologies, USA) equipped with Hi-Plex H for organic acids column ($300 \text{ mm} \times 7.7 \text{ mm}$) using a UV detector at 214 nm. Quantification of nine different organic acids (acetic, lactic, butyric, isobutyric, formic, propionic, valeric, isovaleric and isocaproic acids) in the *P. stratiotes*'s hydrolysate was carried out at an oven temperature of 60°C . As a mobile phase, 0.005 M sulphuric acid was

used at a flow rate of 0.8 ml min⁻¹. The concentration of hydrogen in the gas phase was determined with a gas chromatograph (7890A GC System, Agilent Technologies, USA) equipped with HayeSep Q Packed GC column (10 ft 1/8 2 mm 80/100 Ni, Agilent Technologies, USA) and a TCD filament detector, with N₂ as a carrier gas. The ammonium concentration in the effluent was determined both, during the stripping procedure and during the photofermentation experiments using Nessler method (APHA 1998).

Calculations and statistical analysis

The conversion yield of the carbon substrate to H₂ substrate conversion efficiency (SCE) was calculated according to Eq. 1 (Adessi and De Philippis 2014):

$$\text{SCE}(\%) = \frac{\text{molH}_2 \text{ obtained}}{\text{molH}_2 \text{ theoretical}} \times 100 \quad (1)$$

Substrate conversion efficiency is the ratio (%) between the amount of hydrogen really obtained and the amount of H₂ theoretically obtainable from the amount of substrate consumed. The moles of hydrogen produced were calculated from the volume of H₂ produced applying the ideal gas law at ambient conditions ($T = 25^\circ\text{C}$, $P = 1 \text{ atm}$).

Light conversion efficiency (LCE) for hydrogen production processes carried out by *R. palustris* was calculated as the free energy stored as hydrogen produced per unit of incident light energy.

Free energy was calculated as the amount of hydrogen produced by the standard enthalpy of combustion of H₂ (Eq. 2; Miyake and Kawamura 1987):

$$\text{LCE}(\%) = \frac{33.61 \times \rho_{\text{H}_2} V_{\text{H}_2}}{I \times A \times t} \times 100 \quad (2)$$

where 33.61 is the energy density of hydrogen (W h g⁻¹), ρ_{H_2} is hydrogen density (g l⁻¹), V_{H_2} is the volume of hydrogen produced (L), I is the intensity on the incident light (W m⁻²), A is the irradiated area (m²) and t is the duration of the process (h).

Biochemical hydrogen potential (BHP) was calculated as in Corneli et al. (2016a, 2016b) as the product of biochemical biogas potential (measured in ml of gas per l of effluent) and hydrogen concentration (measured by gaschromatography). BHP was expressed in ml l⁻¹, mol m⁻³ and NL kg VS⁻¹ in Table 2, to allow a wider comparison with literature data.

Independent *t*-test was used to analyze the differences in composition between ES and ENS for organic acids (lactic acid, acetic acid, propionic acid, butyric acid, and total) and ammonium. The results of photofermentation [organic acids consumption (Δ OA), ammonium

consumption (Δ NH₄⁺), final BChl *a* concentration, SCE, LCE, BHP, maximum and medium rates] were analyzed with two-way ANOVA, considering the strain and the substrate as factors. Bonferroni post test was applied to assess the significance of the differences between factors. All statistical analyses were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA.

Results

Chemical characteristics of *Pistia stratiotes* effluent and inocula acclimation

The ammonium stripping of *P. stratiotes* effluent (2 l) lasted 96 h. The starting ammonium concentration was equal to 282.3 mg l⁻¹, and after stripping it decreased to 24.3 mg l⁻¹. A significant, but moderate, decrease in organic acid concentration was also observed. The composition of ES and ENS, that were both subsequently used for culturing *R. palustris*, is reported in Table 1.

Rhodospseudomonas palustris 42OL acclimated faster than the strain CGA676. The BChl *a* concentration of strain 42OL after 9 days of acclimation was $6.2 \pm 3.7 \text{ mg l}^{-1}$ in ES and $5.6 \pm 3.9 \text{ mg l}^{-1}$ in ENS; the BChl *a* concentration of strain CGA676 after 27 days of acclimation was $4.1 \pm 3.3 \text{ mg l}^{-1}$ in ES and $4.8 \pm 1.8 \text{ mg l}^{-1}$ in ENS.

Photofermentation

During photofermentation, both strains grew in ENS but they did not produce hydrogen, while in ES they both grew and produced hydrogen. The growth of each strain,

Table 1 Concentrations of lactic acid, acetic acid, propionic acid, butyric acid, organic acids (as the sum of lactic acid, acetic acid, propionic acid and butyric acid) and ammonium of the effluent of fermented *Pistia stratiotes*, stripped (ES) and not stripped (ENS), at the beginning of the photofermentation experiments. Independent *t*-test results are indicated in the right-end column. Significance: ** ($P < 0.01$), **** ($P < 0.0001$), ns not significant

		ENS	ES	Independent <i>t</i> -test
Lactic acid	(g l ⁻¹)	0.29 ± 0.01	0.29 ± 0.01	ns
Acetic acid	(g l ⁻¹)	5.98 ± 0.09	5.64 ± 0.01	**
Propionic acid	(g l ⁻¹)	2.24 ± 0.02	1.71 ± 0.01	****
Butyric acid	(g l ⁻¹)	1.00 ± 0.01	0.55 ± 0.02	****
Tot organic acids	(g l ⁻¹)	9.51 ± 0.03	8.20 ± 0.01	****
NH ₄ ⁺	(mg l ⁻¹)	282.3 ± 2.5	24.3 ± 1.2	****

estimated as BChl *a* accumulation, was higher for *R. palustris* 42OL in ES and in ENS and for *R. palustris* CGA676 in ES (16.3, 15.2 and 12.3 mg l⁻¹, respectively), while it was significantly lower for *R. palustris* CGA676 in ENS (3.2 mg l⁻¹), as well as for ammonium consumption (Table 2). Two-way ANOVA indicated a very significant effect of the strain ($P = 0.0034$) and a significant effect of the medium ($P = 0.0307$) for what concerned growth, and a significant effect of the strain ($P = 0.0020$) and not significant effect of the medium for ammonium consumption. Bonferroni post-test indicated that the difference between the strains was significant in ENS but not significant in ES. Organic acids consumption followed a different pattern: in this case Bonferroni post-test indicated that the difference between the strains was significant only in ES, as happened for almost all the hydrogen-related parameters, since no hydrogen was produced in ENS. Both the effects of the medium and of the strain were very significant ($P < 0.0001$), both for organic acids consumption and for SCE (a derived parameter): *R. palustris* 42OL showed a higher organic acids consumption (37.5%) than *R. palustris* CGA676 (12.8%). SCE was higher in *R. palustris* 42OL (8.0%) than in *R. palustris* CGA676 (4.7%). The hydrogen production parameters (SCE, BHP, LCE, med and max rate) were dominated by the effect of the medium (always $P < 0.0001$, since hydrogen production was null in ENS) and alternatively by the effect of the strain. Indeed, only the maximum rate of hydrogen production was not significantly different between the two strains (on average 11.7 ± 2.9 ml l⁻¹ h⁻¹). BHP was higher for *R. palustris* 42OL than for *R. palustris* CGA676 (1224 ml l⁻¹ and

720 ml l⁻¹, respectively), while the medium rate was higher for *R. palustris* CGA676 (7.3 ml l⁻¹ h⁻¹) than for *R. palustris* 42OL (5.6 ml l⁻¹ h⁻¹), as well as for LCE (Table 2).

Hydrogen production, organic acids and ammonium concentration during the photofermentation in ES of *R. palustris* CGA676 and *R. palustris* 42OL are reported in Fig. 1a and b, respectively, and organic acids and ammonium concentration in ENS of *R. palustris* CGA676 and *R. palustris* 42OL are reported in Fig. 1c and d, respectively. Hydrogen production started after a lag phase of 118 h in *R. palustris* 42OL and of 238 h in *R. palustris* CGA676. After 14 days, the hydrogen production was in linear phase for both strains and the BHP was equal to 1224.0 ml l⁻¹ for *R. palustris* strain 42OL, which was significantly higher than the BHP of *R. palustris* strain CGA676, equal to 720.0 ml l⁻¹ (Table 2). The final organic acids and ammonium concentrations were 5.1 g l⁻¹ and 12.8 mg l⁻¹, respectively, for *R. palustris* 42OL and 7.2 g l⁻¹ and 10.7 mg l⁻¹, respectively, for *R. palustris* CGA676.

Discussion

It is well documented that an excess of ammonium-nitrogen inhibits hydrogen production on PNSB acting at several levels of nitrogenase regulation (Kim *et al.* 2008). This condition is commonly presented in waste effluents rich not only in carbon sources but also in nitrogen forms such as ammonia which is inhibitory for photofermentative hydrogen production (Keskin *et al.* 2011; Adessi *et al.* 2012). Thus, the ammonium stripping of

Table 2 Bacteriochlorophyll increment (Δ BChl) Organic acids consumption (Δ OA), ammonium consumption (Δ NH₄⁺), substrate to hydrogen conversion (SCE), light conversion efficiency (LCE), Biochemical Hydrogen Potential (BHP), maximum rate (Max rate) and medium rate (Med rate) at the end of the photofermentation of *Pistia stratiotes* in the effluent stripped using *Rhodospseudomonas palustris* strain CGA676 and *R. palustris* strain 42OL. nd (not detected). Two-way ANOVA results for each parameter are indicated. Significance: * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), **** ($P < 0.0001$), ns (not significant)

		CGA676 In ES	CGA676 In ENS	42OL In ES	42OL In ENS	Two-way ANOVA		
						Strain	Medium	Int
Δ BChl	mg l ⁻¹	12.3 \pm 1.2	3.2 \pm 3.0	16.3 \pm 4.0	15.2 \pm 3.5	**	*	ns
Δ NH ₄ ⁺	%	55.6 \pm 2.4	24.7 \pm 10.0	46.5 \pm 2.4	66.1 \pm 6.5	**	ns	***
Δ OA	%	12.8 \pm 2.7	8.0 \pm 5.0	37.5 \pm 2.3	12.6 \pm 2.2	****	****	***
SCE	%	4.7 \pm 0.0	0.0 \pm 0.0	8.0 \pm 0.1	0.0 \pm 0.0	****	****	****
LCE	%	0.7 \pm 0.1	0.0 \pm 0.0	0.5 \pm 0.0	0.0 \pm 0.0	**	****	**
BHP	ml l ⁻¹	720.0 \pm 71.1	0.0 \pm 0.0	1224.0 \pm 104.0	0.0 \pm 0.0	***	****	***
	mol m ⁻³	29.4 \pm 2.9	0.0 \pm 0.0	50.0 \pm 4.3	0.0 \pm 0.0			
	NL kgV S ⁻¹	22.0 \pm 2.2	0.0 \pm 0.0	37.4 \pm 3.2	0.0 \pm 0.0			
Max rate	ml l ⁻¹ h ⁻¹	9.6 \pm 0.6	0.0 \pm 0.0	13.7 \pm 3.4	0.0 \pm 0.0	ns	****	ns
	mol m ⁻³ h ⁻¹	0.39 \pm 0.02	0.0 \pm 0.0	0.56 \pm 0.14	0.0 \pm 0.0			
Med rate	ml l ⁻¹ h ⁻¹	7.3 \pm 0.7	0.0 \pm 0.0	5.6 \pm 0.5	0.0 \pm 0.0	**	****	**
	mol m ⁻³ h ⁻¹	0.29 \pm 0.03	0.0 \pm 0.0	0.23 \pm 0.02	0.0 \pm 0.0			

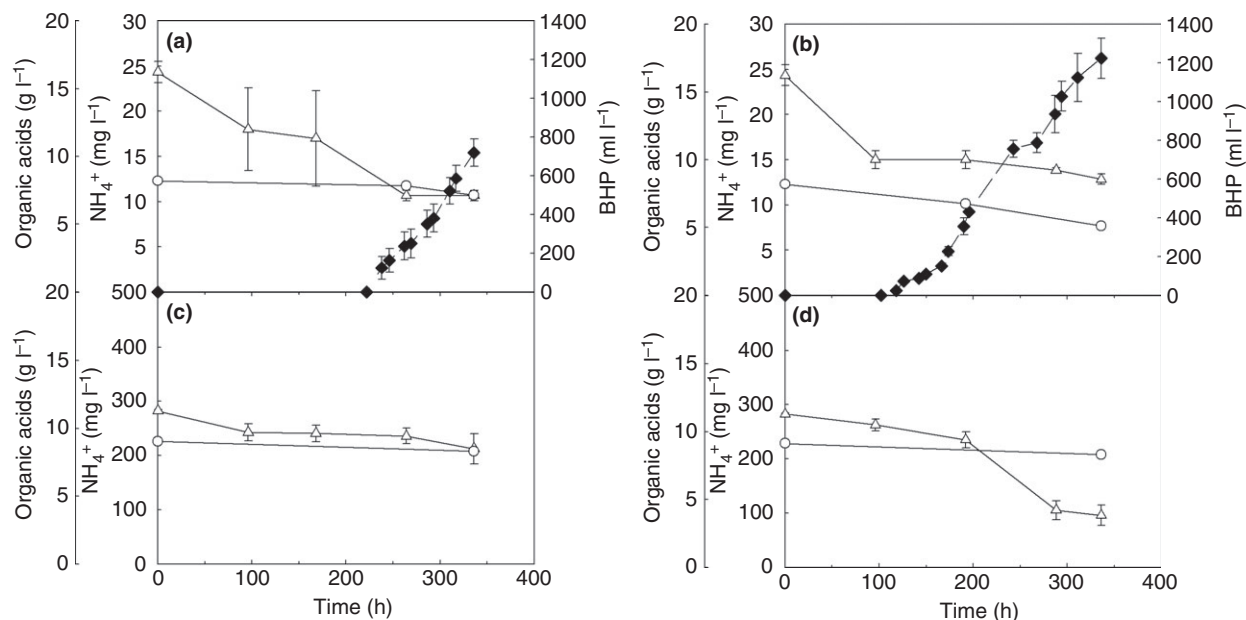


Figure 1 Biochemical hydrogen potential (BHP) (—◆—), ammonium concentration (—△—) and organic acids concentration (as the sum of lactic acid, acetic acid, propionic acid and butyric acid) (—○—) during photofermentation of *Pistia stratiotes* in the effluent stripped and not stripped using *Rhodospseudomonas palustris* strain CGA676 (a) and (c), respectively and *R. palustris* strain 42OL (b) and (d), respectively. Smaller scales were used for A and B than C and D in order to provide more details.

P. stratiotes effluent resulted to be an efficient pre-treatment that allowed hydrogen production with both strains tested, avoiding dilution of the effluent. Observing Table 1, the stripping procedure had a strong effect on the removal of ammonium (91% of the ammonium present was removed), but also had a minor effect on the volatilization of VFA (namely acetic, butyric and propionic acids). Indeed, it is in VFA nature to be volatile at pH above 3, thus organic acid total concentration decreased by about 14% during the stripping procedure. However, the amount of acids lost was less relevant than the benefit given by the substantial ammonium removal. Indeed, only the substrate subject to stripping (ES) was suitable for hydrogen production (Table 2).

The nitrogen stripping procedure avoided the need for dilution, thus the ES effluent kept its dark-brown color for the photofermentation tests. This arduous light penetration (though partially overcome with the relatively high light intensity used) could have been a reason for the long lag phase for hydrogen accumulation (Fig. 1). Indeed, initially cells couldn't get enough light to let accumulate a measurable amount of hydrogen; only after the number of cells increased the evolved gas could be measured.

Overall, *R. palustris* 42OL had a better performance in ES than *R. palustris* CGA676, probably due to its very high capability of acclimating to various environmental conditions giving high hydrogen rates, already shown in

previous studies (Adessi et al. 2016a). In ENS, *R. palustris* 42OL did not produce hydrogen, due to ammonium inhibition of nitrogenase, but it showed a growth similar to its growth in ES. *R. palustris* CGA676 grew scarcely in ENS, as shown by the low BChl *a* accumulation (Table 2), and confirmed by the low consumption of organic acids and of ammonium (Fig. 1c). Notwithstanding the low-sensitivity to ammonium, the strain did not produce hydrogen, most probably due to its clearly poor acclimation in such medium. Indeed, for both *R. palustris* 42OL and *R. palustris* CGA676 a longer acclimation on ENS may have been necessary in order to observe hydrogen production.

The results (Table 2) are comparable with other studies that used the same strains of *R. palustris* used in the present study, with similar substrates. Pintucci et al. (2013) used strain 42OL with diluted olive mill wastewater obtaining slightly lower results (1030 ml l⁻¹); the same strain on glucose-supplemented seawater (Adessi et al. 2016b) had better performances (14.7 ml l⁻¹ h⁻¹), but again in a diluted medium. Strain CGA676 was previously used in other processes using vegetable residues derived substrates, giving lower volumetric results: Adessi et al. (2012) reported a rate of 3.9 ml l⁻¹ h⁻¹ with an undiluted medium, while Corneli et al. (2016a) reported comparable or lower hydrogen gas accumulation (649 ml l⁻¹, 320 ml l⁻¹, 11 ml l⁻¹) using respectively wheat bran, ensiled maize, and ensiled giant reed.

However, in terms of initial biomass transformed, the results presented in this study (22.0–37.4 NL kgVS⁻¹) are closer to the results with giant reed biomass (7.5 NL kgVS⁻¹), than to wheat bran and maize (463.0 NL kgVS⁻¹ and 228.7 NL kgVS⁻¹, respectively). This is due to the nature of the substrates characterized by high fiber content and poor of easily fermentable carbohydrates (Corneli *et al.* 2016a). A study using water hyacinth, that can be considered a similar biomass to the one used in this study, reported higher biomass conversion results (522.6 NL kgVS⁻¹), using immobilized *R. palustris* (Su *et al.* 2010).

Up to now, few studies have been conducted on the use of water lettuce for energy production, and they all investigated anaerobic digestion for methane production (Nipanay and Panholzer 1987; Abbasi *et al.* 1991; Zenaki *et al.* 1997; Pantawong *et al.* 2015). Hydrogen, compared to methane, has a higher heat of combustion and is a carbon-free fuel. Here we reported for the first time the use of water lettuce biomass for hydrogen production, and the treatments needed to obtain a successful photofermentation. However, while anaerobic digestion is a mature technology, photofermentation is a process that needs to be improved and optimized (Keskin *et al.* 2011; Corneli *et al.* 2016a).

Different variables play a role in the bioprocess aimed to energy production, therefore to identify and optimize those variables is crucial to achieve high energy conversion from low cost materials. In particular, when using aquatic plants as the starting biomass, optimal substrate concentration or temperature may influence the yields of the process (Chuang *et al.* 2011). A possible solution for increasing the concentration of degradable compounds capable to be transformed into energy, can be provided by pretreatment of the lignocellulose material. Recently Chen *et al.* (2015) explored the alkali pretreatment of *P. stratiotes* before ensilage to reduce lignin content and they found that there was an increment in its biodegradability into organic compounds. This could be an attractive option to explore in the case of biohydrogen production from *P. stratiotes* biomass in order to increase the organic acid content in further experiments, since *R. palustris* has shown better hydrogen production profile in presence of some organic compounds such as malic, lactic or succinic acid (Bianchi *et al.* 2010; Adessi *et al.* 2012). Although no previous works have been reported using *P. stratiotes* for hydrogen production through photofermentation, there are several reports using water hyacinth for hydrogen production within a two stage process (Su *et al.* 2010; Cheng *et al.* 2013) or co-generation of methane and hydrogen (Chuang *et al.* 2011; Lin *et al.* 2015). Such increasing use of aquatic plants for energy generation confirms the importance of using low cost materials with

environmental benefits derived from the management of water weeds, mainly in developing regions where water weed species are commonly abundant in polluted water bodies.

Concluding, in order to increase the energy efficiency in future experiments using *P. stratiotes*, different strategies could be explored by separate or in combination, such as pretreatment for increasing hydrolysis-sacharification, co-fermentation or co-digestion, and two stage processes.

Developing an efficient hydrogen producing photofermentative process, based on *P. stratiotes* biomass, appears an interesting opportunity since the results obtained in the present study with *R. palustris* 42OL on nitrogen-stripped substrate are encouraging. Indeed, the substrate could be efficiently used with limited and inexpensive treatments.

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Conflict of Interest

No conflict of interest declared.

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