CHEMICAL ENGINEERING TRANSACTIONS
VOL. 43, 2015

A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet

Chief Editors: Sauro Pierucci, Jiří J. Klemeš
Copyright © 2015, AIDIC Servizi S.r.l.,
ISBN 978-88-95608-34-1; ISSN 2283-9216

DOI: 10.3303/CET1543065

Biohydrogen Production by Dark Fermentation of *Arundo donax* for Feeding Fuel Cells

Angelo Ausiello*, Luca Micoli, Domenico Pirozzi, Giuseppe Toscano, Maria Turco

Department of Chemical, Materials and Production Engineering, University of Naples Federico II, PleTecchio 80, 80125, Naples, Italy.
angelo.ausiello@unina.it

Hydrogen may be considered as an alternative source of energy since it can be produced from several thermochemical and biological processes. The hydrogen obtained can be feed to fuel cells, which are the most promising devices to produce electric energy with high efficiency and very low environmental impact.
The present work studies the biological H_2 production by dark fermentation of Arundo donax (AD) inoculated with sewage sludge in mesophilic condition (38°C). The substrates used are AD without pretreatment (AD),and AD treated from steam explosion (AD exp) both supplemented with a nutrient medium. A synthetic medium was used as reference model. Fermentation process produce products. The liquid phase contains mainly acetic acid, butyric acid, propionic acid and ethanol that. The biogas phase contains mainly of hydrogen and carbon dioxide. Liquid and gaseous phases are analyzed by GC technique. The fermentation process is monitored reducing sugars and microbial biomass by spectophotometric analysis.
Best results are obtained with AD sample as substrate that gives yield to hydrogen up to 1.34 mol H_2/mol glucose fed. Biogas produced has H_2 concentration up to 74% by volume being CH_4 present in traces amount. It can be potentially feed directly to a PEMFC (Proton Exchange Membrane Fuel Cell), since it does not contain CO.

1. Introduction

The constant increase in energy demand associated with the decrease in the availability of fossil fuels, as well as the universally recognized need to reduce greenhouse gas emissions and the environmental pollution, are directing energy production processes towards the use of renewable fuels.
Fuel Cells (FCs) are considered the solution of the future for the problem of the production of electricity and for automotive transportation. The FCs are electrochemical devices that convert the chemical energy of a fuel, mainly H_2, directly into electricity, without an intermediate heat cycle, thus obtaining efficiencies higher than conventional thermal systems. Further advantages are related to the extremely reduced polluting emissions, the high power density and a modular construction that allows a power ranges from a few kW to some MW. Hydrogen produced can be used as an energy carrier and feed a FCs to produce energy with low environmental impact (Cigolotti et al., 2009).
Hydrogen can be produced from several kinds of biomasses by thermochemical or biological processes (Hallenbeck et alt., 2012).The biological production of hydrogen (biohydrogen) is the microbiological conversion of water and organic substrates into hydrogen, through the action of the enzyme hydrogenase (Hasi) or nitrogenase (Nasi)(Chong et al., 2009). The biological production of H_2 can be carried out in presence of light using green algae, photosynthetic bacteria and cyanobacteria, or in the absence of light through the process named Dark Fermentation (DF), using heterotrophic bacteria (like as Clostridium)(Saratale et al., 2008). The DF process transforms sugars, starches and other carbohydrates or fermentable organic substrates into biogas containing mainly H_2 and CO_2 together with organic acids, alcohols and other by-products according to the scheme shown in Figure 1 (Liu and Whitman, 2008).
Some limits exist at present in the use of biohydrogen as fuel for FCs, due to the low H_2 yields obtained in the DF process using different substrate, like as glucose (Hussy et al., 2005) or sucrose (Lin et al., 2011).

Purpose of the present work is to improve the H_2 yield of DF process by optimizing the biomass pretreatment and fermentation procedure with the aim of enhancing hydrogenophilic bacteria to the detriment of methanogenic ones.
In this study Arundo donax, a non-food and giant reed plant, has been selected as a source of lignocellulosic biomass for the production of biohydrogen by the DF process (Toscano et al., 2013). Optimization of pre-treatment of the raw lignocellulosic material is essential to obtain better yields to H_2 in the fermentation process. Harsh conditions of hydrolysis can release inhibitors of the cellular growth (such as furfural, acetic acid, etc.) in the fermentation medium (Taherzadeh and Karimi, 2007). Therefore, a pre-treatment of AD has been carried out by steam explosion and subsequent enzymatic attack by cellulase and cellobiase, in order to avoid the production of inhibitory compounds typical of acid hydrolysis. The use of an anaerobic mixed culture has been preferred to the use of selected strains in view of the application to non-sterile reactor operation.

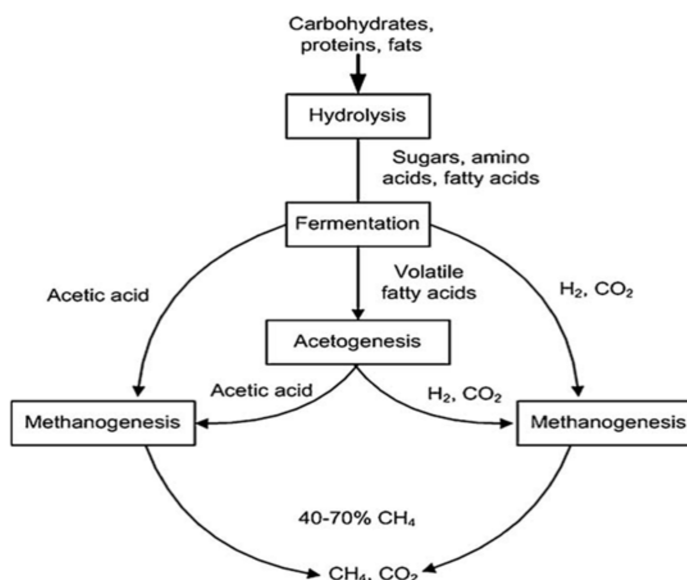

Figure 1: Anaerobic digestion scheme

2. Materials and Methods

2.1 Anaerobic mixed culture

Sewage sludge used as inoculum was obtained from a primary wastewater digester of Nola (Naples). In the first fermentation the sludges were not subjected to treatments and used directly as inoculum with a filtration to remove coarse particles. Starting from the second inoculation, the sludge was treated with a nutrient medium to support the growth of bacteria hydrogen producer (such as Clostridium) and to eliminate the methanogenic bacteria.
Then 20 mL of the first inoculum has been treated under following condition: heating at 100 °C for 5 min, centrifugation for 20 min at 4000 rpm, separation of the bottom product (biomass) from the supernatant, resuspension of the biomass in a medium of nutrients to obtain a volume of 20 mL. After that the inoculum was treated at 100 °C for 5 min, and then has been a new fermentation was carried out.

2.2 Preparation of nutrient medium

Nutrient medium was prepared dissolving in 500 mL of H_2O NH_4Cl (0.5 g), K_2HPO_4 (0.15 g), KH_2PO_4 (0.15 g), $MgCl_2$*6 H_2O (0.1 g), $CaCl_2$*2 H_2O (0.05 g), NaCl (5 g), KCl (0.05 g), cysteine (0.25 g), CH_3COONa (0.25 g), yeast extract (1 g), tripeptone (1 g), NaOH solution at pH 11 (5 mL), resazurin (0.05 g).
The pH of the solution was increases up to 8.0 adding drop by drop a solution 10M of KOH and kept in N_2 atmosphere for 45 minutes and then heated at 100°C for 45 min. After leaving the whole under stream of nitrogen, after that was heating at 100 °C for the same time. The solution was then autoclaved at 121 °C before to inoculation.

2.3 Synthetic medium

Synthetic medium, employed for the first strand of the tests performed, was prepared in a Pyrex glass vial (125 mL). The volume of liquid phase in the vial is 100mL. Synthetic medium was prepared with 19.6 mL of distilled water, 0.4 mL of resazurin solution 0.025% w/v; 10 mL of mineral solution (Na_2HPO_4 7 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L, NH_4Cl 1 g/L and trace elements). Resazurin is used as an indicator of anaerobiosis. After sterilization in autoclave (SMEG HV-85L) at 121°C for 20 minutes, 50 mL of solution 20 g/L of glucose (autoclaved separately); 250 μL of saline solution 400x; 20 mL of inoculum were added to the medium. Then the vial is clamped with butyl rubber stopper pierced equipped with an aperture ring. Finally, the anaerobic conditions were obtained keeping the vial under stream of nitrogen for 30 min.

2.4 *Arundo donax* hydrolysis

Arundo donax was collected from Torre Lama (Campania, Italy) agro-land. Leaves were separated from stems, washed, dried overnight at 80°C and minced with a chopper.
The tests were carried out with Arundo donax not treated (AD), and with Arundo donax treated by steam explosion at 210 °C for 6 min (ADexp) (supplied by ENEA in Trisaia (Matera, Italy)). AD and ADexp were subjected to enzymatic hydrolysis by the action of cellulase (Celluclast 1.5L, from Novozymes) and cellobiase (Novozyme 188, from Novozymes). Ratios of 15 filter paper units of Celluclast and 30 cellobiose units of Novozyme 188 per gram of lignocellulosic biomass have been used according to(Gong et al., 2013). Hydrolysis has been performed at 50°C for 72 h with 5% (w/v) of biomass in water and samples of hydrolyzed AD (ADH) and ADexp (ADHexp) have been obtained. The hydrolyzate has been filtered (with filter paper) and the pH adjusted to 6.5 before the use.

2.5 *Arundo donax* fermentations

The tests performed with ADH and ADHexp were performed with initial reducing sugars concentration of 20 g/L. The media were supplemented with 0.4 mL of 0.025% w/v resazurin solution, 10 mL of mineral solution (Na_2HPO_4 7 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L, NH_4Cl 1 g/L and trace elements). The procedure adopted is similar that used for the synthetic medium (see paragraph 2.3).

2.6 Analytical tecniques

Sampling of liquid and gaseous phases from crimped vials was performed according to standard anaerobic procedures (Strobel, 2009).
The biomass concentration was monitored by measuring the optical absorbance of liquid samples at 600 nm. After centrifugation at 3000 rpm for 5 minutes and filtration with 0.2 μm cut-off filters, the liquid sample was analyzed for residual substrate content (glucose or total reducing sugars) and soluble fermentation products (organic acids, alcohols).
The concentration of glucose was measured following a modified Nelson-Somogyi method for reducing sugars (Nelson, 1944). Concentration of acetic, butyric, propionic acids and ethanol was measured by GC technique, using a Shimadzu instrument GC-17A equipped with FID detector and a capillary column containing a PEG stationary phase (BP20, 30 m by 0.32 mm i.d., 0.25 μm film thickness, from SGE).
Biogas (H_2 and CO_2) composition was determined by GC analysis, using a HP 5890 series II equipped with a TCD detector and a double packed molecular sieves-porapack column.

2.7 Biogas production

The crimped vial is a standard technique in anaerobic studies, even though it does not allow an easy evaluation of biogas volumes. The culture medium contained in the vial is placed in continuously stirring (800 rpm), inside an electrical oven at a constant temperature of 38 °C. A capillary tube connects the vial to an overturned vial which contains 100 mL of distilled water (Figure 2). The tubular connection allows the flow of the biogas produced from the first to the second vial where it accumulates in the top of the vial, thus allowing the dripping of the liquid phase through a bullet hole. The volume of liquid that is collected corresponds to the volume of biogas produced. The system then responds to the Mariotte’s law (volume of liquid dripped =volume of gas produced) (Toscano et al., 2014).

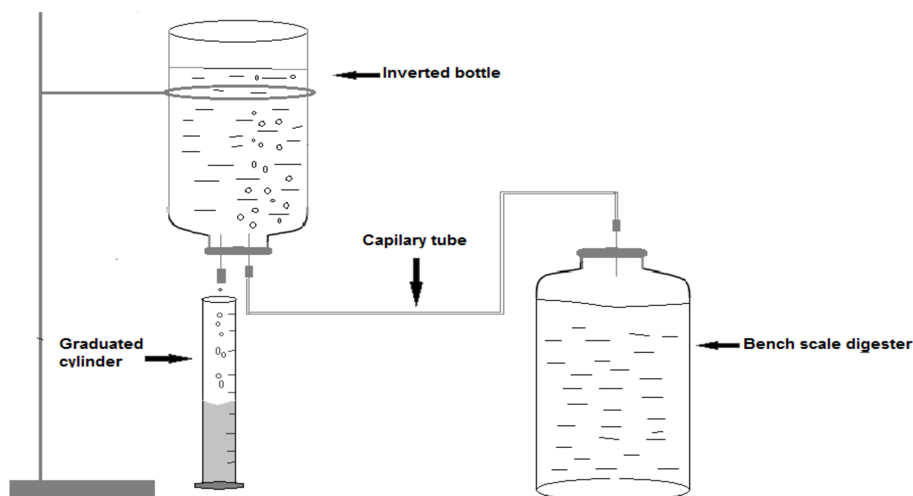

Figure 2: The experimental apparatus for the anaerobic fermentation process using Mariotte's law and crimped vials.

3. Results and Discussion

The biohydrogen production using *Arundo donax* as substrate has been investigated and compared to a synthetic medium as reference. In the first adaptation the microbial consortium contains both methanogenic and hydrogen-forming bacteria. The focus of this work is to stimulate the growth of the latter bacteria to the detriment of the methanogenic ones. To this purpose successive adjustments of the substrates were required. In particular, every fresh substrate (see paragraph 2.3 and 2.5) was inoculated with a microbial consortium deriving from the previous adaptation. Each new inoculum from a previous adaptation has been subjected to treatment with a nutrient medium (see paragraph 2.2) to promote the formation of Clostridium bacteria, that are responsible of the formation of biohydrogen. Fermentation tests have shown that this procedure gives an improvement and stabilization of *dark fermentation* after nine adaptations.

Figure 3 (a) and (b) show the results obtained with the synthetic medium. The glucose concentration (Figure 3 (a)) decreases to zero reaching negligible contents after 48 h. As a consequence the biogas production is limited to the first two days of fermentation. This can be due to the depletion of glucose and to the acidification of the solution. It is known that dark fermentation takes place optimally with a pH of about 6.5-7. In fact, as shown in the Figure 3 (b), the presence of acetic acid and propionic acid in the culture medium increases leading to the acidification of the solution, with consequent reduction of the efficiency of the process. This is confirmed by the microbial biomass trend reported in Figure 3 (a), showing that the microorganisms growth increases in the first 24 h reaching a maximum, after which slowly decrease.

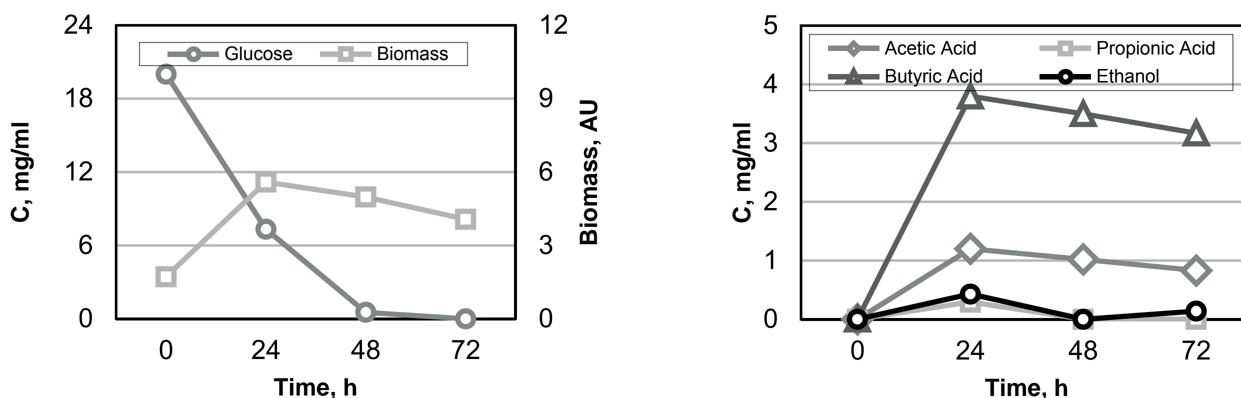

Figure 3 (a) and (b): Concentration of glucose, biomass and soluble fermentation products for synthetic medium as function of time

The results for the ADH and ADHexp are shown in Figures 4 and 5. The trends are similar to those obtained for the synthetic medium. In this case the concentration of reducing sugars is negligible in the first 24 h, while the development of the biomass is completely similar to that of the synthetic medium. In the liquid phase, besides acetic acid and butyric acid, also ethanol is present due to the alcoholic fermentation.

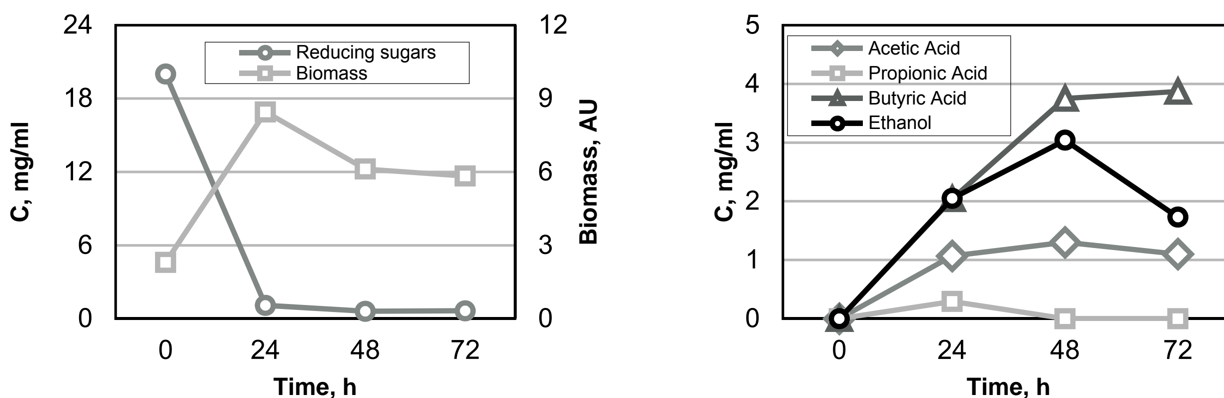

Figure 4 (a) and (b): Concentration of reducing sugars, biomass and soluble fermentation products for ADH as function of time

Biogas composition and H_2 yield obtained after 72 h of fermentation are reported in Table 1 for synthetic medium, ADH and ADHexp. It can be observed that ADH and ADHexp samples give a higher production of biohydrogen. In fact H_2 yields have been obtained between 1.14 (ADHexp) and 1.34 (ADH). These are acceptable results considering that the maximum yields are between 2 and 3 (Thauer et al., 1977). This is due to the composition of hydrolyzed *Arundo donax* that contains monomers and reducing sugars that can be separated more easily than the glucose present in the synthetic medium leading to an enhanced fermentation. ADH gives H_2 yield higher than ADHexp probably because of during the process of steam explosion (ADHexp) there is an accumulation of inhibitory substances (i.e. phenols) which hinder the dark fermentation process.

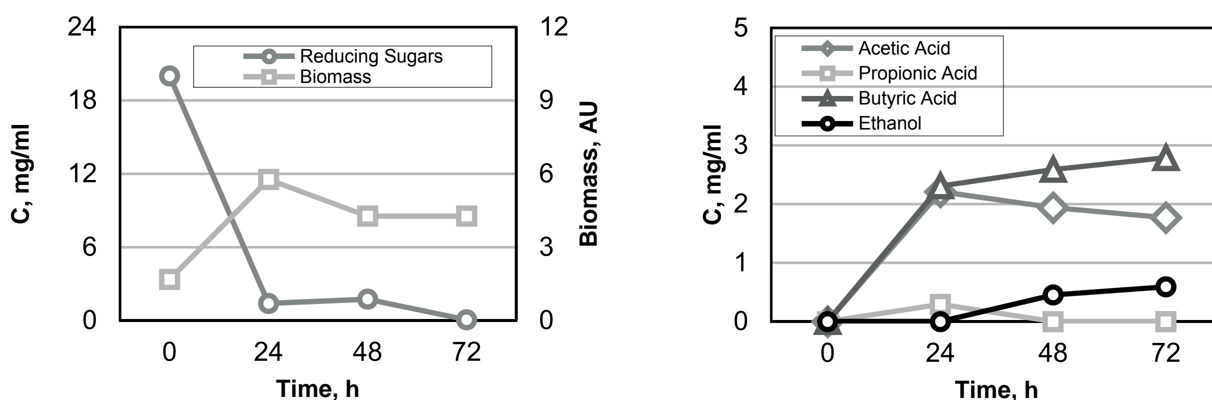

Figure 5 (a) and (b): Concentration of reducing sugars, biomass and soluble fermentation products for ADHexp as function of time

Table 1: Biogas composition and biohydrogen yield after 72 h of fermentation.

Sample	$V_{biogas}/V_{culture}$ (L/L)	H_2 (vol %)	CO_2 (vol %)	H_2 yield ($mol_{H_2}/mol_{glucose}$)
Synthetic M.	2.50	36	64	0.89
ADH	2.85	74	26	1.34
ADHexp	2.30	69	31	1.14

4. Conclusions

Results obtained in this work have shown that the dark fermentation of *Arundo donax* is a promising process to obtain biogas with high H_2 content. Treatment of AD by steam explosion have been investigated, however this treatment does not improve the H_2 yield due to the production of inhibiting substances (i.e. phenols). H_2 yields obtained with ADH and ADHexp are higher than Synthetic Medium and comparable to the theoretical values. These results represent a very promising starting point. In fact, the quality of the biogas obtained is very satisfactory in view of the application of dark fermentation in the FCs technology. Nevertheless the process of *dark fermentation* could be improved by controlling the production of volatile acids, especially butyric acid, that causes the acidification of the liquid phase. Additionally further studies are needed to optimize the hydrolysis process avoiding the formation of inhibiting compounds for bacteria hydrogen producers.

References

Chong M.L., Sabaratnamb V., Shiraic Y., Hassana M.A., 2009, Biohydrogen production from biomass and industrial wastes by dark fermentation. Int. J. Hydr. Energy, 34, 3277–3287.

Cigolotti V., McPhail S., Moreno A., Nonconventional fuels for high-temperature fuel cells: status and issues. J Fuel Cell SciTechnol. 2009;6:0213111e8.

Gong Z., Shen H., Wang Q., Yang X., Xie H., K Zhao Z., 2013, Efficient conversion of biomass into lipids using the simultaneous saccharification and enhanced lipid production process. Biotechnol. Biofuels, 6:36, 1-12.

Hallenbeck P.C., Abo-Hashesh M., Ghosh D., 2012, Strategies for improving biological hydrogen production. Bioresource Technology, 110, 1–9.

Hussy I., Hawekes F.R., Dinsdale R., Hawekes D.L., 2005, Continuous fermentative hydrogen production from sucrose and sugarbeet, Int. J. Hydr. Energy, 30, 471-483.

Lin PJ., Chang JS., Yang LH., Lin CY., Wu SY., Lee KS., 2011, Enhancing the performance of pilot-scale fermentative hydrogen production by proper combinations of HRT and substrate concentration, Int. J. Hydr. Energy, 36, 14289-14294.

Liu Y., Whitman W. B., 2008, Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea, Ann. NY Acad. Sci., 1125(1), 171–189.

Nelson N., 1944, A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 153(2), 375–380.

Saratale G.D., Chen S.D., Lo Y.C., Saratale R.G., Chang J.S., 2008, Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation – a review. J. Sci. Ind. Res., 67, 962–979.

Strobel H., 2009, Basic laboratory culture methods for anaerobic bacteria. In Mielenz, J. R., editor, Biofuels, Vol. 581 of Methods in Molecular Biology, 247–261. Humana Press c/o Springer Science+Business Media, LLC, New York, USA. ISBN 978-1-60761-213-1 DOI 10.1007/978-1-60761- 214-8_16.

Taherzadeh M.J., Karimi K., 2007, Acid-based hydrolysis processes for ethanol from lignocellulosic materials: A review. BioResources, 2, 472–499.

Thauer R.K.., Jungermann K., Decker K.,1977, Energy conservation in chemotrophic anaerobic bacteria, Bacteriological Reviews, 41, 100-180.

Toscano G., Ausiello A., Micoli L., Zuccaro G., Pirozzi D., 2013, Anaerobic digestion of residual lignocellulosic materials to biogas and biohydrogen, Chemical Engineering Transactions, 32, 487–492, DOI:10.3303/CET1332082.

Toscano G., Zuccaro G., Ausiello A., Micoli L., Turco M., Pirozzi D., 2014, Production of hydrogen from giant reed by dark fermentation, Chemical Engineering Transactions, 37, 331-336, DOI: 10.3303/CET1437056.