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Production of Hydrogen from Giant Reed by Dark Fermentation

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Arundo donax hydrolysate (ADH), obtained by steam explosion and enzymatic treatment, was exploited as a substrate for anaerobic digestion aimed to the production of hydrogen. Mixed cultures of hydrogen-forming anaerobic bacteria selected from a primary sludge digester were used as inocula. Methanogens were removed from the wild consortium by the use of thermal treatments (autoclaving, freezing and thawing). Either with glucose or ADH as carbon sources, the soluble anaerobic fermentation products were butyric acid, lactic acid, acetic acid, formic acid and ethanol. The collected biogas consisted mainly of hydrogen (86-97%) and carbon dioxide. Hydrogen yield with ADH was lower than that found with glucose fermentation with the same inoculum. Outgrowth of lactic acid bacteria during ADH fermentation was presumably the cause of the low observed yield. Further optimization of ADH pre-treatment and stabilisation of microbial consortium are necessary in order to improve hydrogen yield on ADH.

1. Introduction

The diffusion of the hydrogen as a fuel is still limited by the high costs of production and storage (Pant and Gupta, 2009). The biological processes offer a possible approach to obtain a renewable supply of hydrogen (Hallenbeck et al., 2012). The biological production of hydrogen could in principle be based on the direct exploitation of the photosynthetic activity of algae (biophotolysis) or bacteria (photofermentation) (Kapdan and Kargi, 2006). However, the development of these processes toward a practical application will likely require a long term (Hallenbeck et al., 2012). On the other side, dark fermentation (anaerobic digestion without photosynthesis) can be carried out using a technology similar to that of anaerobic digestion with methanogenesis, widely employed at industrial scale and consequently easier to be developed in the short term at industrial level (Chong et al., 2009). Dark fermentation appears to be suitable for the exploitation of agricultural wastes or non-food crops consisting of complex substrates that can be degraded biologically only by microbial consortia (Guo et al., 2010).
Many efforts have been directed to the development of efficient technologies to obtain renewable energy from lignocellulosic biomasses, by recycling a large range of agricultural wastes (non-food parts of crops: stems, leaves, husks, etc.) and industry wastes (woodchips, skin and pulp from fruit pressing, etc.). Anaerobic digestion with methanogenesis is a microbial process that has already found wide application for the reduction and stabilization of agricultural wastes (Ward et al., 2008). In the last years, different papers have been concerned at the dark fermentation of lignocellulosic feedstocks (Saratale et al., 2008).
The *anaerobic food chain* begins with the bacterial hydrolysis of complex organic compounds (Figure 1). Insoluble organic polymers, such as carbohydrates and proteins, are broken down to soluble derivatives that become available for other bacteria. Acidogenic bacteria then convert sugars and amino acids into carbon dioxide, hydrogen, ammonia, and organic acids. During acetogenesis, other bacteria convert these resulting organic acids into acetic acid, along with additional ammonia, hydrogen, and carbon dioxide. Finally, methanogens convert these products to methane and carbon dioxide. The adaptation of the

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methanogenic microbial consortia to the production of hydrogen requires the imposition of a selective pressure in favour of hydrogen-producing bacteria (mainly belonging to the genera *Enterobacter* and *Clostridia*) to the detriment of hydrogen-consuming bacteria (acetogenic and methanogenic) and of bacteria competing for nutrients (such as lactic acid bacteria).

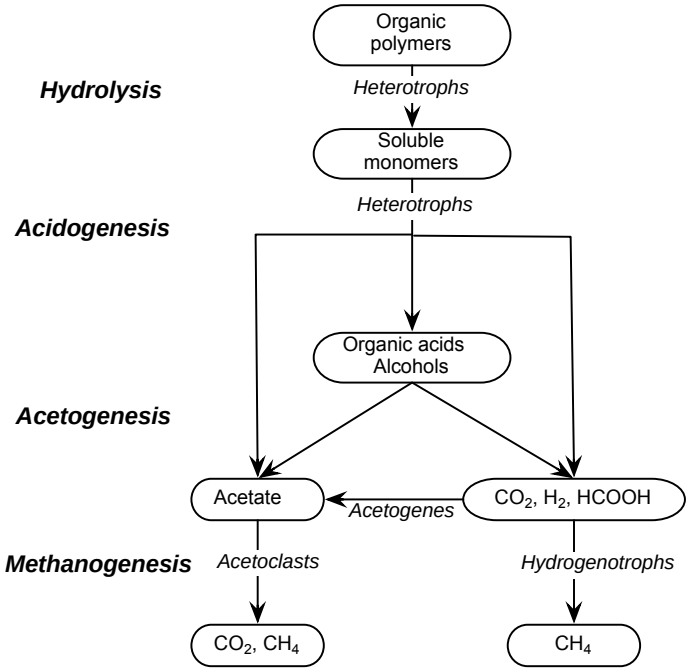

Figure 1: The anaerobic food chain (redrawn with modifications from Liu et al 2008).

In this study, a non-food plant, giant reed (*Arundo donax*), has been selected as a source of lignocellulosic biomass for the production of biohydrogen by dark fermentation. Optimisation of pre-treatment of the raw lignocellulosic material is essential to obtain better yields in fermentations. 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 *A. donax* 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.

2. Materials and Methods

2.1 Anaerobic mixed culture

Sewage sludge was obtained from a primary sludge digester of a municipal wastewater treatment plant (Nola, Italy). As a first attempt to increase the hydrogen yield, the inoculum was autoclaved (121 °C, 15 min) to selectively eliminate microorganisms unable to form heat-resistant spores (as most of methanogens) in favour of spore-forming hydrogen-producing genera like *Clostridia*. The anaerobic consortium was adapted to a synthetic medium (see §2.2) by performing several transplants. Since the methanogens survived autoclaving (Toscano et al. 2013), the mixed culture was submitted to a further selective stress by freezing and thawing. After the addition of 10% glycerol, the mixed culture was frozen at –20°C in a crimped vial under nitrogen atmosphere. After thawing, the culture was inoculated anaerobically in a fresh medium.

2.2 Synthetic medium fermentations

Synthetic medium contained glucose 10 g/L as sole carbon source, supplemented with Na_2HPO_4 7.0 g/L, KH_2PO_4 3.0 g/L, NaCl 0.5 g/L, NH_4Cl 1.0 g/L, and trace elements. Resazurin (0.025%) was also added as anaerobiosis indicator.

Fermentation was carried out in 125 mL crimped Pyrex vials with perforable butyl rubber septa. Each vial was filled with 80 mL of concentrated culture medium, and inoculated with 20 mL of the anaerobic

consortium (see §2.1). Anaerobic conditions were ensured by sparging the medium with helium gas. The vials were kept at 35°C with continuous stirring by a magnetic anchor.

2.3 *Arundo donax* hydrolysis

Giant Reeds (*Arundo donax*) were collected from Torre Lama (Campania, Italy) agro-land. Leaves were separated from stems, washed, dried overnight at 80^0C and minced with a chopper. The powder was pre-treated in the steam explosion plant of ENEA Reserch Center of Trisaia (Matera, Italy). The steam-exploded lignocellulosic biomass (treated at 210 °C, 6 min) was then subjected to enzymatic hydrolysis by the action of cellulase (Celluclast 1.5L, from Novozymes) and cellobiase (Novozyme 188, from Novozymes). Following Gong et al. (2013), the optimal ratios of 15 filter paper units of Celluclast and 30 cellobiose units of Novozyme 188 per gram of lignocellulosic biomass have been used. Hydrolysis has been performed at 50°C for 72 h with 5% (w/v) of dry steam-exploded biomass in water. The hydrolysate has been filtered (with filter paper) and the pH adjusted to 6 before use.

2.4 *Arundo donax* fermentations

Fermentation medium contained *A. donax* hydrolysate as sole carbon source (amount equivalent to 10 g/L of reducing sugars), supplemented with Na_2HPO_4 7.0 g/L, KH_2PO_4 3.0 g/L, NaCl 0.5 g/L, NH_4Cl 1.0 g/L, and trace elements. Resazurin (0.025%) was also added as anaerobiosis indicator.

Fermentation tests were carried out following the same procedure adopted with synthetic medium.

2.5 Analytical techniques

Sampling of liquid and gaseous phases from crimped vials was performed according to standard anaerobic techniques (Strobel, 2009).

The biomass concentration was monitored by measuring optical absorbance of liquid samples at 600 nm. After centrifugation and filtration with 0.2 µm cut-off filters, the liquid sample was analysed 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 organic acids (acetic acid, butyric acid, acetic acid, formic acid, lactic acid) was determined by HPLC analysis, using an Agilent HP1100 equipped with DAD detector and a SUPELCOGEL H column (25 cm x 4.6 mm, from Supelco).

Ethanol was determined by GC analysis, using a Shimadzu GC-17A equipped with a FID detector and a capillary column with a PEG stationary phase (BP20, 30 m by 0.32 mm i.d., 0.25 µm film thickness, from SGE).

Biogas (H_2, CO_2, and CH_4) composition was determined by GC analysis, using a HP 5890 equipped with a TCD detector and a molecular sieve wide bore capillary column.

2.6 Biogas production

The crimped vial is a standard technique in anaerobic studies, even though it does not allow an easy evaluation of biogas volumes. In this study, a volumetric method was attempted by connecting with a capillary tube the headspace of the fermentation vial to an inverted vial filled with water (Figure 2).

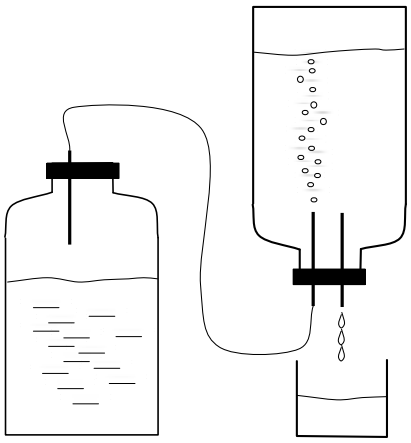

Figure 2: A simple apparatus for the measurement of biogas production by anaerobic fermentation using crimped vials. Left vial: fermentation vessel; right vial: gas collection vial

The biogas volume was measured by weighing the water displaced through a second needle in the septum of collection vial. The collection vial was periodically replaced and collected biogas analysed by GC. Biogas production was somewhat underestimated by the method, because of the high solubility of carbon dioxide in water (Walker et al., 2009). An approximate estimate of the amount of dissolved carbon dioxide was attempted by the use of known thermodynamic equilibrium data for the system CO_2-H_2O (Rittman and McCarty 2001).

3. Results and Discussion

The anaerobic consortium used in this study was sampled from a primary sludge digester. The sludge was first autoclaved, in order to reduce the content of methanogens unable to form thermal resistance spores. The surviving microorganisms were then acclimated to a synthetic medium containing glucose as a sole carbon and energy source. Since the treatment did not remove fully methanogens (Toscano et al 2013), the acclimated microorganisms were subjected to a further stress selection by freezing and thawing. The thawed microbial biomass was used as inoculum for the anaerobic fermentation of *A. donax* hydrolysates supplemented with nitrogen and phosphorous sources plus trace elements to balance the element requirements. For comparison, the same inoculum has been cultured with the glucose synthetic medium, in order to evaluate the effect of inhibitors in the lignocellulosic hydrolysate.

Glucose and soluble products trends in the batch fermentation with synthetic medium are shown in Figure 3. Near complete conversion of 10 g/L glucose is attained in less than 2 days. Prevailing soluble products are butyric acid and acetic acid. Collected biogas was entirely constituted by hydrogen and carbon dioxide whereas methane was not detectable. About 86% of collected biogas was hydrogen, but carbon dioxide content was underestimated due to the high solubility in water. By taking into account dissolved carbon dioxide and bicarbonate in the medium of fermentation vial and in the water of gas collection vial, the evolved biogas composition should have been about 57% in hydrogen. 2-2.5 L of biogas (at 35 °C) were collected per L of culture medium, corresponding to an overall yield of about 0.9-1.1 mol H_2 / mol glucose.

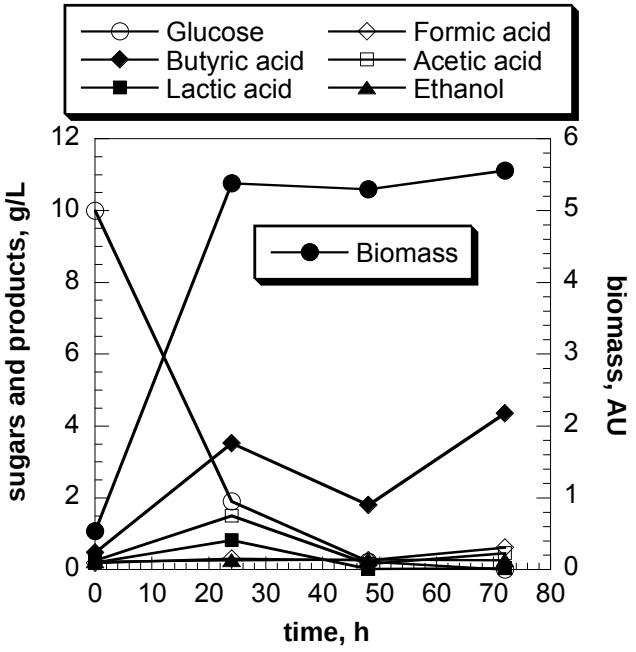

Figure 3: Time courses of glucose, soluble fermentation products, and biomass concentrations during glucose fermentation

Fermentable sugars from *A. donax* were obtained by a steam explosion pre-treatment followed by enzymatic hydrolysis with cellulase and cellobiase. Steam-explosion is a thermomechanical treatment widely used to destructure lignocellulosic biomasses by solubilizing most of hemicellulose fraction and leaving residual lignocellulose more accessible to enzyme attack. Final concentration of reducing sugars (mainly glucose, xylose, arabinose, mannose, galactose) in the fermentation medium was about 10 g/L. In the batch fermentation with ADH medium, conversion of reducing sugars was only slightly slower than in

the case of synthetic medium (Figure 4). In contrast to the former batch, the main soluble product were lactic acid and acetic acid. About 97% of collected biogas was hydrogen (75% by taking into account dissolved carbon dioxide), but the overall volume was much lower, 0.25-0.5 L of biogas per L of culture medium, corresponding to an approximate yield of 0.17-0.3 mol H_2 / mol glucose equivalents. Apparently, the low hydrogen yield with *A. donax* hydrolysate is due to the outgrowth of lactic acid bacteria in the microbial consortium. The overall biomass yield is nearly halved (as can be estimated by the comparison of final turbidity values in the two batches): growth inhibition of hydrogen-forming bacteria by some hydrolysis products (furfural, phenols) of lignocellulose is likely to be the cause.

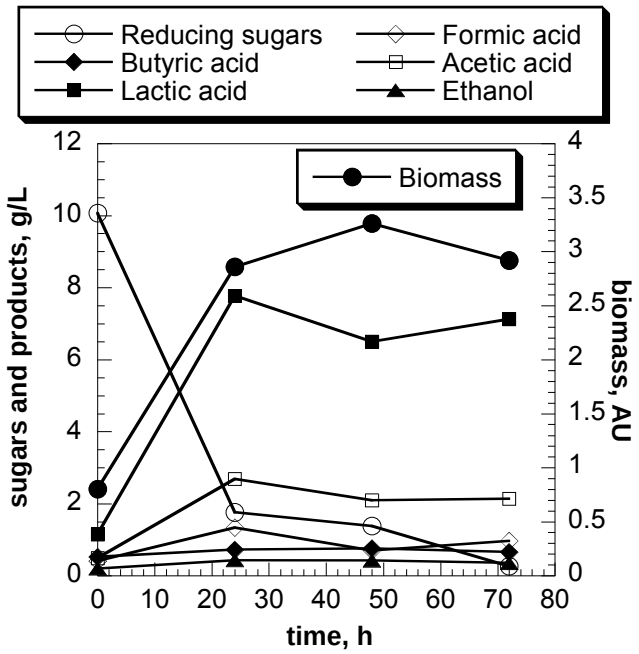

Figure 4: Time courses of reducing sugars, soluble fermentation products, and biomass concentrations during Arundo donax hydrolysate fermentation

4. Conclusions

The addition of a further stress selection treatment by freezing was successful in removing most of methanogens from the wild anaerobic sludge consortium, as shown by the analyses of biogas evolved from both glucose and ADH fermentation batches. However, the low hydrogen yield and the outgrowth lactic acid bacteria observed with ADH hydrolysate point out the negative effects of unrecognised hydrolysis products.

Apparently, the substitution of steam explosion and enzymatic hydrolysis to acid hydrolysis is not enough to fully prevent the production inhibitory compounds for microbial growth. Hydrogen-forming bacteria seem particularly sensitive and the presence of inhibitors shifts the composition of microbial consortium, leading to the undesired prevalence of lactic acid bacteria.

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