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Dark Fermentation of *Arundo donax:* Characterization of the Anaerobic Microbial Consortium

Giuseppe Toscano [1,*], Gaetano Zuccaro [2], Anna Corsini [3], Sarah Zecchin [3] and Lucia Cavalca [3]

[1] Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale (DICMaPI), Università degli Studi di Napoli Federico II, 80125 Napoli, Italy
[2] Nereus SAS, Le Pouget, 34230 Le Pouget, France
[3] Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente (DeFENS), Università degli Studi di Milano, 20133 Milano, Italy
* Correspondence: giuseppe.toscano@unina.it

Abstract: The dark fermentation of lignocellulose hydrolysates is a promising process for the production of hydrogen from renewable sources. Nevertheless, hydrogen yields are often lower than those obtained from other carbohydrate sources due to the presence of microbial growth inhibitors in lignocellulose hydrolysates. In this study, a microbial consortium for the production of hydrogen by dark fermentation has been obtained from a wild methanogenic sludge by means of thermal treatments. The consortium has been initially acclimated to a glucose-based medium and then used as inoculum for the fermentation of *Arundo donax* hydrolysates. Hydrogen yields obtained from fermentation of *A. donax* hydrolysates were lower than those obtained from glucose fermentation using the same inoculum (0.30 ± 0.05 versus 1.11 ± 0.06 mol of H_2 per mol of glucose equivalents). The hydrogen-producing bacteria belonged mainly to the *Enterobacteriaceae* family in cultures growing on glucose and to *Clostridium* in those growing on *A. donax* hydrolysate. In the latter cultures, *Lactobacillus* outcompeted *Enterobacteriaceae*, although *Clostridium* also increased. *Lactobacillus* outgrowth could account for the lower yields observed in cultures growing on *A. donax* hydrolysate.

Keywords: biohydrogen; dark fermentation; *Arundo donax*; lignocellulosic biomass; anaerobic consortia; *Clostridium*; *Klebsiella*; *Enterobacter*; lactic acid bacteria

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1. Introduction

Several technologies have been developed for the industrial production of hydrogen; nevertheless, most (>96%) hydrogen is still produced from fossil sources [1]. Steam reforming of natural gas associated with the water–gas shift reaction, coal gasification, and partial oxidation and cracking of hydrocarbons produce hydrogen that is used mainly in chemical synthesis processes. Since production processes based on fossil sources also give rise to a net release of carbon dioxide in the atmosphere, a wider adoption of hydrogen as the main energy carrier (the so-called "hydrogen economy") is bound to the development of carbon-neutral production technologies.

The most promising technologies based on renewable sources employ biomass feedstocks, such as non-food agricultural crops, agricultural and forestry wastes, livestock wastes, food industry wastes, and municipal wastewaters. Since plant biomass consumes atmospheric carbon dioxide during growth, it has a small net carbon footprint compared with fossil fuels. Considerable effort has been dedicated to the development of thermochemical processes based on gasification and pyrolysis of biomasses [2] as well as that of biological processes based on heterotrophic photo-fermentation and "dark fermentation" (anaerobic fermentation by hydrogen-producing microorganisms that do not require light for growth) [3].

Among the biological processes, dark fermentation seems to be the closest to practical application. It takes advantage of the simpler bioreactor configuration with respect to

photofermentation (it does not require lighting) and allows the use of mixed cultures for the degradation of complex substrates [4]. However, dark fermentation is still a very sensitive process that requires a careful control of several operating parameters (pH, retention time, organic load rate, etc.) for optimal hydrogen yield, generally limited to 1.2–2.0 mol of H_2 per mole of glucose [5]. Stoichiometrically, 12 moles of H_2 per mole of glucose could be produced. On the basis of thermodynamic considerations, it has generally been assumed that the physiological limit is four moles of H_2 per mole of glucose with the simultaneous production of two moles of acetate (also called the Thauer's limit, with reference to [6]). Recently, Thauer's limit has been exceeded with (1) a cell-free enzyme cascade [7]; (2) a genetically modified thermophile *Thermotoga maritima* [8]; and (3) artificial anaerobic consortia growing on optimized soils [9,10]. Nevertheless, in terms of practical application to continuous processes with non-sterile feedstock, the full exploitation of natural mixed consortia is still a challenge. Economic feasibility could still be obtained in a biorefinery framework, where hydrogen production is associated to the valorisation of other by-products (organic acids and solvents) or, alternatively, dark fermentation could be followed by the methanogenic fermentation of the exhaust stream [5].

Hydrogen-producing mixed cultures can be obtained from methanogenic consortia by various selection methods. In nature, macromolecules, such as proteins, nucleic acids, carbohydrates, and fats, are first hydrolysed to simple monomers and then catabolised into CO_2, H_2, NH_3, and volatile acids by fermentative bacteria. Proton-reducing acetogens catabolise volatile acids into CH_3COOH, H_2, and CO_2. Homoacetogens reconvert some H_2 and CO_2 into CH_3COOH. Finally, acetoclastic methanogens convert CH_3COOH into CH_4 and CO_2, while hydrogenotrophic methanogens convert H_2 and CO_2 into CH_4 and H_2O. The adaptation of a wild methanogenic consortia to the production of hydrogen involves the selection of hydrogen-producing bacteria (mostly from the families *Enterobacteriaceae* and *Clostridiaceae*) and the removal of hydrogen-consuming bacteria (homoacetogens and methanogens) and other fermentative bacteria, such as lactic acid bacteria, which are also detrimental because they compete for nutrients. Since methanogens are unable to form thermal resistance spores, a simple method of selection is a heat treatment, such as autoclaving the anaerobic consortium. Other bacterial selection methods are based on various stressors, such as freezing–thawing, UV-, IR-, or γ-irradiation, chemical agents, methanogenesis inhibitors, aeration, or electrical current [11].

Refined carbohydrates are good substrates for dark fermentation. However, sources of sugars and starch are also valuable food resources. Therefore, as happened for other fermentation processes for biofuel and biogas production, the exploitation of carbohydrates of lignocellulosic biomasses from energy crops (non-food woody or herbaceous plants, such as switchgrass and giant miscanthus, that can grow on marginal lands not used for food agriculture) or from agricultural wastes (straw, stover, wood chips) is currently under study for biohydrogen production [3,12,13].

The anaerobic digestion of lignocellulosic biomasses is usually preceded by a hydrolysis step to obtain readily fermentable carbohydrates [12]. After size reduction of biomass by cutting and milling, physicochemical and thermal pre-treatments are used to disrupt the lignocellulose structure and make it more accessible to hydrolysis. Hydrolysis is then catalysed by acid or base at high temperatures or by cellulolytic enzymes at milder conditions. Thermal pretreatments or harsh conditions of chemical hydrolysis can release degradation products (furans, phenols, acetic acid, and formic acid) that are known inhibitors of the microbial growth [14] and decrease hydrogen yields in dark fermentation [15,16]. The inhibitory compounds act more or less severely on the different microorganisms of anaerobic consortia, thus reducing the efficiency of the overall process. The effect is more pronounced on hydrogen-producing consortia, which have an artificially modified microbial composition compared with the wild methanogenic consortia [16].

In this study, *Arundo donax* (giant reed) was selected as a source of lignocellulosic biomass for the production of hydrogen by dark fermentation. *A. donax* is a non-food perennial crop that produces high amounts of biomass per hectare. Yields depend on

several factors, both climatic and agronomic. Annual yields of up to 30–40 tons of dry matter per hectare have been reported in Italy [17]. The plants do not have high soil quality requirements [18]. Resistance to heavy metal pollution makes it suitable for phytoremediation purposes [19] and compatible with its use as an energy crop. Bioenergy production from *A. donax* has been pursued not only through direct combustion of biomass but also through fermentative production of biofuels (biogas and bioethanol). Although biogas and bioethanol yields per unit dry matter of biomass are low compared with food crops, such as corn or sorghum, biofuel yields per hectare are higher due to the high productivity of *A. donax* crops [18,20]. Biofuel yields per hectare are also competitive with other energy crops, such as *Miscanthus* [17,21]. To date, however, very few results on biohydrogen production by dark fermentation from *A. donax* are reported in the literature [22–27].

In previous work on the dark fermentation of *A. donax* [22–25], biomass hydrolysis was carried out by steam explosion and subsequent enzymatic attack. The *A. donax* hydrolysate (ADH) was used as a carbon source in the culture medium, after the addition of nutrients (nitrogen and phosphorous) and trace elements. In cultures started with the same heat-treated inoculum, ADH fermentation showed a marked reduction of hydrogen yields (0.17–0.3 mol/mol) compared with glucose dark fermentation (1.0 mol/mol) [23]. By using a different inoculum selection technique (adaptation by culturing on glucose-based synthetic medium with short fermentation time), higher yields were obtained (2 mol/mol with glucose-based synthetic medium and 2.59 mol/mol with ADH) [25]. In the work of Vasmara et al. [26], yields as high as 2 mol/mol were achieved with an *A. donax* hydrolysate obtained by alkali treatment and enzymatic attack in cultures started with an inoculum without any previous adaptation, apparently only by optimizing the initial pH of the unbuffered culture.

In general, inoculum selection, biomass pretreatment, and control of operating conditions influence the hydrogen yields of mixed bacterial consortia. Generalization of the results is difficult given the interaction among different components of the process. In this work, we focused on how inhibitors generated by pretreatment can influence the process. Inhibitors can potentially act in two ways: (1) by changing the observed metabolism of individual species and (2) by changing the taxonomic composition of bacterial populations. To differentiate the two mechanisms, we aimed to highlight changes in the taxonomic composition of a hydrogen-producing anaerobic consortium in the transition from glucose to hydrolysed *A. donax* as the sole carbon source. The evolution of hydrogen and the production of volatile acids obtained with the two different fermentation media were matched with the taxonomic composition of the bacterial cultures assessed by molecular biology methods. The results could be of interest for the development of process optimisation criteria based on understanding the microbial ecology of the anaerobic hydrogen-producing consortium.

2. Materials and Methods

2.1. Anaerobic Consortium

The hydrogen-producing consortium was obtained by applying several selection techniques to a methanogenic consortium taken from a primary sludge digester at the municipal wastewater treatment plant in Nola (Italy). The sewage sludge was first autoclaved (121 °C, 15 min), then adapted to a glucose-based synthetic medium by performing several transplants. After the addition of 10% glycerol, the culture was frozen at −20 °C in a crimped vial under nitrogen atmosphere. After thawing, the culture was inoculated in a fresh medium to remove glycerol, which was used as a substrate. The obtained consortium [23] did not produce methane and was used as inoculum for the cultures in this work.

2.2. Arundo donax Hydrolysate

Giant reeds were gathered in a field in Torre Lama (Campania, Italy). The leaves were separated from the stems, washed, and dried overnight at 80 °C. The dried leaves were finely ground, and the powder was subjected to steam explosion (210 °C, 6 min) at the ENEA Research Center of Trisaia (Matera, Italy). The steam-exploded biomass was stored

at −20 °C. Before each fermentation batch, a fresh hydrolysate was prepared using cellulase from *Trichoderma reesei* ATCC 26921 (Celluclast® 1.5 L, from Novozymes, Denmark) and cellobiase from *Aspergillus niger* (Novozyme 188, from Novozymes) [11]. Cellulase activity was measured following the NREL filter paper assay [28,29] and reported in filter paper units (FPU). β-glucosidase (cellobiase) activity was measured using the method described by [30] and reported in cellobiase units (CBU). Hydrolysis was performed at 50 °C for 72 h on a basculating plate at 160 rpm (Minitron, INFORS HT, Switzerland). Dry steam-exploded biomass in water, 5% (*w/v*), was used, adding 750 FPU of Celluclast® 1.5L and 1500 CBU of Novozyme 188 per liter. The hydrolysate was vacuum-filtered, and the pH adjusted to 6.5 before use. Concentration of reducing sugars (mainly glucose) was approximately 22 g/L. The hydrolysate was added as sole carbon source in fermentation batches.

2.3. Fermentation Media

Fermentation media contained a carbon source (either glucose or *A. donax* hydrolysate), M9 minimal salts (Na_2HPO_4 7.0 g L^{-1}, KH_2PO_4 3.0 g L^{-1}, NaCl 0.5 g L^{-1}, and NH_4Cl 1.0 g L^{-1}), and trace elements. Resazurin (0.025%) was used as an anaerobiosis indicator. Glucose-based synthetic media contained 10 g L^{-1} of glucose as the sole carbon source. *A. donax* hydrolysate media contained approximately 10 g L^{-1} of total reducing sugars (mainly glucose).

2.4. Batch Cultures

Fermentations were conducted in 125 mL crimped Pyrex vials with perforable butyl rubber septa. Each vial was filled with 100 mL of culture obtained by mixing 80 mL of concentrated fermentation medium and 20 mL of the previously selected anaerobic consortium containing approximately 10 g TOC/L of organic suspended matter. Anaerobic conditions were ensured by sparging the medium with inert gases (nitrogen or helium). The vials were kept at 35 °C, with continuous stirring by a magnetic anchor. The initial pH of culture medium was 6.5, fixed by the buffering capacity of phosphate salts. Liquid samples were extracted from the crimped vials according to standard anaerobic techniques [31] for subsequent chemical analysis. Evolved biogas was collected by a water displacement method [23,32].

2.5. Chemical Analysis

Microbial growth was evaluated by the increase in culture turbidity at 600 nm. Reducing sugars and volatile acids in liquid samples were determined after centrifugation and filtration with 0.2 µm cut-off filters.

The concentration of reducing sugars was measured by Nelson–Somogyi method [33]. Concentration of organic acids (acetate, butyrate, and lactate) and ethanol was determined by gas chromatographic 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 Trajan, Australia).

In ADH fermentation media, total phenols were evaluated by the Folin-Ciocalteu reagent [34], furans by UV absorbance at 284 and 320 nm according to the method of [35], and formic acid by the colorimetric method of [36].

The collected biogas was analysed by gas chromatography using a Shimadzu GC-2014 equipped with a TCD detector and a molecular-sieve-packed column (Carboxen-1000, from Supelco, Bellefonte, PA, USA). The water displacement method underestimates the overall biogas volumes because of the high solubility of carbon dioxide in water [32]. Therefore, gas chromatographic analysis of the gas phase in the collection vial was used mainly to estimate hydrogen production.

2.6. Nucleic Acids Isolation

Total DNA was isolated using the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, San Diego, CA, USA), according to manufacturer's instructions.

2.7. Pyrosequencing of 16S rRNA

Pyrosequencing of 16S rRNA genes was performed from DNA isolated from the three consortia TS2, AD3, and AD4. Bacterial 16S rRNA was amplified with the universal bacterial primers 27F (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 1495R (5′-CTA CGG CTA CCT TGT TAC GA-3′) in triplicate in a 25 µL reaction volume containing 10 ng of DNA, 0.3 µM primers, and 1 × Taq PCR Master Mix kit (QIAGEN, MD, USA). The thermal incubation included a first denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 40 s, and elongation at 72 °C for 1 min and 40 s; the final elongation was performed at 72 °C for 10 min. Replicated amplicons were pooled and purified with MinElute PCR Purification kit (QIAGEN) to a final concentration of 20 ng μL^{-1}. Pyrosequencing was performed at Molecular Research LP (MRDNA, Shallowater, TX, USA) by bacterial Tag-Encoded FLX Amplicon Pyrosequencing (bTEFAP), using the primer 27F. The sequences were processed and analyzed with the QIIME tools [37]. Sequences with less than 200 bases of barcodes or primer biases, homopolymers, and chimeras were removed from the analysis. Sequences with a similarity higher than 97% were grouped in Operational Taxonomic Units (OTUs), and representative sequences for each OTU were aligned to the SILVA SSU Ref dataset [38] using the PyNAST method [39]. After taxonomic assignment OTU tables were generated for each sample. To measure the bacterial diversity within the samples, the OTU tables were rarefied, and different indices of alpha diversity were calculated (phylogenetic diversity, observed species, and Chao 1). To compare the bacterial diversity among the samples, weighted and unweighted Unifrac analysis of the rarefied OTU tables was performed. The OTU richness was estimated according to [40], assuming a sample size of 500.

2.8. Quantification of Hydrogen-Producing Species by Real-Time PCR

Along with 16S rRNA genes of total bacteria, other targets were quantified by Real-Time PCR and related to bacterial species relevant in hydrogen-producing consortia: 16S rRNA gene of *Klebsiella* sp. and the hydrogenase gene (*hydA*) of *Clostridium* spp. The primers were selected from the literature (see Table 1), and the thermal protocols were used accordingly.

Table 1. Primer set used in the present study to quantify target genes.

Gene Target	Reference Strain	Primer	Sequence (5′–3′)	Expected Amplicon (bp)	Reference
16S rRNA	Bacteria	EUB338F EUB518R	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	200	[21]
23S rRNA	*Klebsiella* sp.	1507F 1717R	AAG GCT GAG GTG TGA TGA CG CTA CAC ACC AGC GTG CCT TC	200	[22]
hydA	*Clostridium* spp.	L1F L1R	AAA TCA CCA CAA CAA ATA TTT GGT GC ACA TCC ACC AGG GCA AGC CAT TAC TTC	500	[23]

All reactions were set up in a 20 µL mixture volume containing 1× Titan HotTaq EvaGreen® qPCR Mix (Bioatlas, Tartu, Estonia), 200 nM of each forward and reverse primer, 20 ng of template DNA, and PCR-grade water. The qPCR was performed on a MJ Mini™ cycler equipped with a MiniOpticon™ system (Bio-Rad, CA, USA). The melting curves were calculated at the end of each run to determine the specificity of the amplification. No-template controls (NTC) were included in all runs. Each sample was run in triplicate. The PCR products were checked on 2% (*w*/*v*) agarose gel containing 0.01% (*v*/*v*) GelRed™ stain (Biotium, CA, USA) and visualized using the GelDoc image analyzer system (Bio-Rad).

To determine the limits of detection, the reaction efficiencies (90% < E < 105%) and linear ranges of amplification (R^2 > 0.98), standard curves were generated using serial dilutions of total DNA of clones and reference strains (Table 2). Each dilution was run in triplicate. For each standard, the logarithm of concentration of the diluted DNA was plotted against the threshold cycle value (C_T). For evaluation of PCR amplification efficiencies,

the slope of the standard curves was calculated by performing a linear regression analysis with CFX Manager Software (version 1.5, Bio-Rad). The amplification efficiency E was calculated from the slope s of the standard curve using the equation $E = (10^{-1/s} - 1) \times 100$, where a reaction with 100% efficiency would generate a slope s of −3.32.

Table 2. Range of concentrations for reference DNAs used to generate standard curves.

Target	Reference DNA	Range of Standard Curve	
		ng DNA μL^{-1}	Gene Copies μL^{-1}
16S rRNA Bacteria	*Aliihoeflea* sp. Strain 2WW (Acc. Num. AYOD00000000)	18.5×10^{-6}–18.5×10^{-2}	8.44×10^{4}–8.44×10^{8}
23S rRNA *Klebsiella pneumoniae*	clone K7 (Acc. Num. KU985052)	20.4×10^{-6}–20.4×10^{-2}	1.55×10^{4}–1.55×10^{8}
hydA of *Clostridium* spp.	clone AD3–1	5.4×10^{-7}–5.4	1.64×10^{2}–1.64×10^{9}

The gene copy number was calculated as follows:

$$\text{Copy number (molecules } \mu L^{-1}) = \text{DNA concentration (g } \mu L^{-1})/[\text{average genome size (bp)} \times 660] \times A$$

where 660 is the average molecular weight of one base pair, and $A = 6.022 \times 10^{23}$ molecules $(\text{mole})^{-1}$ is the Avogadro's number.

Obtained data were subjected to one-way analysis of variance (ANOVA) with Bonferroni tests using SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA). All analyses were performed at the $p < 0.05$ level.

2.9. Clone Library Preparation

To create the clone libraries, standard PCR was performed with the same thermal protocol described for qPCR, mixing 1 × Taq PCR Master Mix (QIAGEN) with the primers, 20 ng of template DNA, and PCR-grade water to a final volume of 25 µL. The PCR products were cloned using the TOPO® TA Cloning® Kit (Invitrogen, MA, USA), with TOP10 chemically competent *E. coli* cells and pCRTM2.1-TOPO® vector, according to manufacturer's instructions. The positive clones were sequenced, and the plasmid was extracted with UltraClean™ 6 Minute Mini Plasmid Prep Kit™ (MO BIO Laboratories).

2.10. Comparative Sequence Analysis

The 16S rRNA gene sequences were first compared with sequences available in GenBank using Nucleotide BLAST [41]. Subsequently, the sequence and those with high similarities were aligned using the online aligner SINA in SILVA [42] and imported into the software program ARB [43]. Subsequently, a phylogenetic tree was calculated using the SILVA database version 10.8 within ARB. Hydrogenase gene sequences *hydA* were compared with sequences available in GenBank using BlastX. Phylogenetic analysis of the deduced amino acid sequences was performed using MEGA software [44] version 4. Phylogenetic trees were constructed using the neighbour-joining distance method based on p-distance. A total of 1000 bootstrap replications were calculated.

3. Results

3.1. Batch Cultures

The hydrogen-producing consortium was selected starting with a methanogenic consortium sampled from a primary sludge digester. The hydrogen-producing microorganisms were acclimated to a synthetic medium containing 10 g/L glucose as the sole carbon source, as described in Materials and Methods (Section 2.3). The same culture was used as inoculum for the dark fermentation of *A. donax* hydrolysates. A suitable volume of ADH was added to the fermentation medium to obtain a final concentration of reducing sugars of approximately 10 g of glucose equivalents per L. In addition to reducing sugars (mainly

glucose and lesser amounts of xylose), the ADH contained phenols, formic acid, and furans (furfural and hydroxymethylfurfural) originated from thermal degradation of lignin and carbohydrates during the steam explosion pre-treatment of lignocellulosic biomass (see Section 2.2). Concentrations of lignocellulose degradation compounds in the fermentation medium were: phenols 0.62 ± 0.11 g L^{-1}, formic acid 4.97 ± 0.30 g L^{-1}, and furans 0.14 ± 0.02 g L^{-1}.

Two glucose-based cultures (labelled TS2 and TS3) and three ADH-based cultures (labelled AD2, AD3, and AD4) were carried out according to the sequence of Figure 1, by using each culture as an inoculum for the following one: the TS2 culture on glucose was used to inoculate both the AD2 culture on ADH and the TS3 culture on glucose; the TS3 culture was then used as inoculum for an additional culture on ADH, named AD3; and the AD2 served as inoculum for a third culture on ADH, named AD4. Reducing sugars conversion, soluble products, and hydrogen production were evaluated for all the batches. Community analysis of the initial glucose-based culture TS2 and ADH-based terminal cultures AD3 (inoculated from a previous glucose-based culture) and AD4 (inoculated from a previous ADH-based culture) was conducted to detect a change in the taxonomic composition of the mixed microbial population.

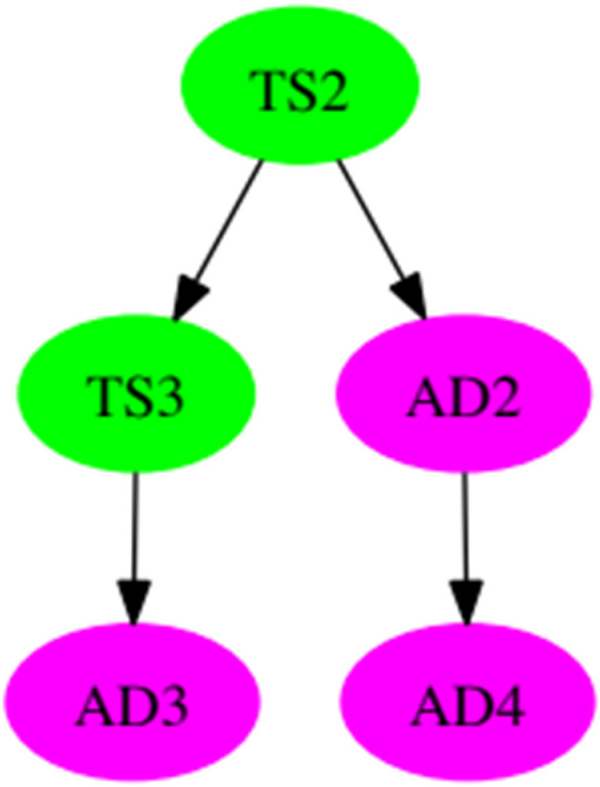

Figure 1. Sequence of batch cultures: TS2 and TS3 cultures on glucose-based medium (green); AD2, AD3, and AD4 cultures on ADH-based medium (purple).

In batch cultures with a 10 g/L glucose-based medium, hydrogen production and microbial growth occurred during the first 48 h. CH_4 was not detectable in the collected biogas, which consisted entirely of H_2 and CO_2. The main soluble product was butyric acid. Overall glucose removal (mmol), hydrogen production (mmol), microbial turbidity (AU at 600 nm), and overall increase in soluble end products (mmol) at the end of biogas production for the batch cultures with glucose-based medium (TS2 and TS3) are shown in Figure 2.

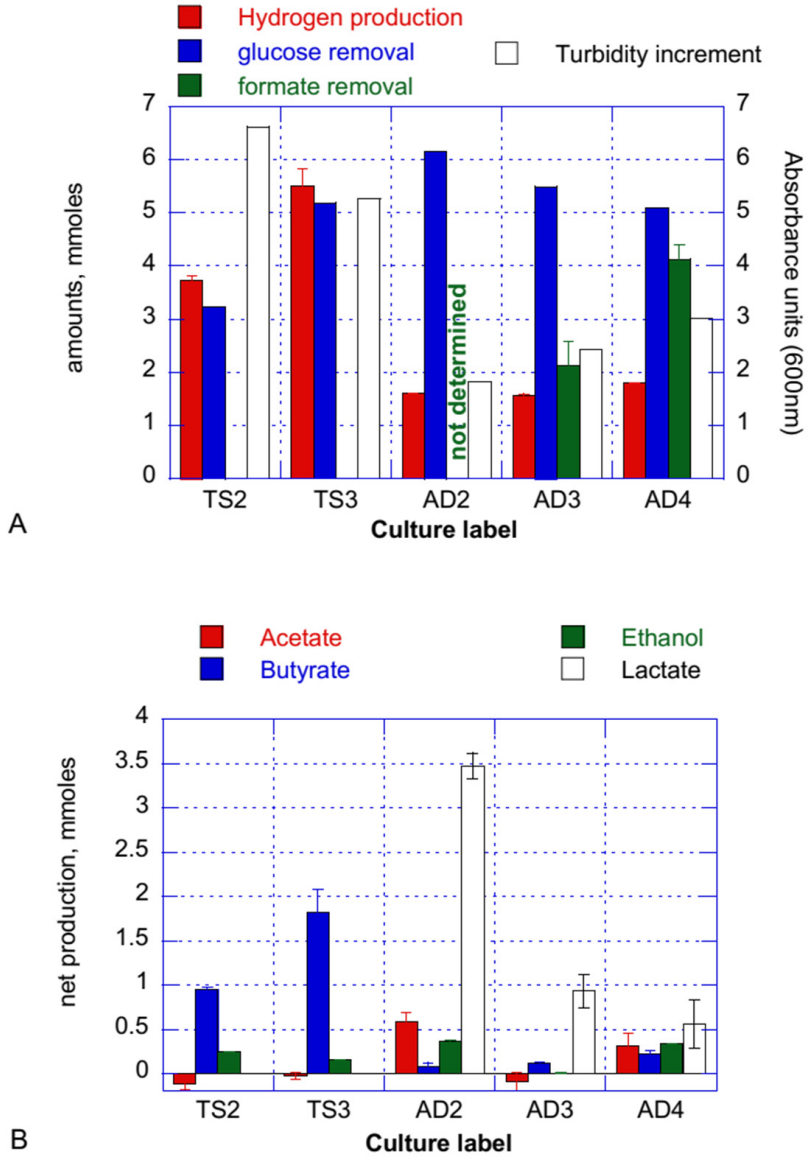

Figure 2. (**A**) Overall substrate removal (mmol), hydrogen production (mmol), and turbidity (AU at 600 nm) increment; (**B**) net increase in soluble end products (mmol) in the five cultures at the end of biogas production (error bars represent standard deviations; negative values in (**B**) are due to initial presence of soluble end products carried by the inoculum from the preceding culture).

In the batch cultures with ADH medium, hydrogen production and microbial growth occurred during the first 24 h. Collected biogas consisted entirely of H_2 and CO_2. While phenols and furans were not degraded throughout the culture formic acid was consumed but apparently not used (or used only in part) for hydrogen production (see discussion below). In contrast to glucose-based batch cultures, the main soluble product was lactic acid. Overall reducing sugars removal (glucose eq mmol), hydrogen production (mmol), microbial turbidity (AU at 600 nm), and overall increase in soluble end products (mmol) for the batch cultures with ADH-based medium (AD2, AD3, and AD4) are shown in Figure 2.

The overall volume of collected biogas from ADH-based cultures was much lower than that from the glucose-based medium, and a marked reduction of microbial growth can be estimated by comparison of the turbidity increment at the stationary phase. From the data of Figure 2A, H_2 yields (H_2 mmol per glucose mmol) and microbial yields (AU per glucose mmol) can be evaluated (Table 3). The H_2 yields for TS cultures are significantly higher than those for AD cultures (two-group *t*-test, *p*-value = 0.0002). The hydrogen production

of cultures on glucose-based medium corresponds to an average yield of approximately 1.11 ± 0.06 mol of H_2 per mol of converted glucose, whereas for cultures on ADH-based medium, the average yield is 0.30 ± 0.05 mol of H_2 per mol of converted glucose equivalents, both in the low range of values reported in the literature (see Introduction). Analogously, microbial yields for TS cultures are significantly higher than those for the AD cultures (p-value = 0.0369). The average microbial yield for the cultures on glucose-based medium is 1.53 ± 0.73 (AU per glucose mmol), whereas it is 0.44 ± 0.14 (AU per glucose mmol) for the cultures on the ADH-based medium.

Table 3. Hydrogen and microbial yields.

Culture Label	H_2 Yields (H_2 mmol per glucose eq mmol)	Microbial Yields (AU per Glucose eq mmol)
TS2	1.15	2.05
TS3	1.07	1.01
AD2	0.26	0.30
AD3	0.28	0.44
AD4	0.35	0.59

Variation in the type and amount of end products can be explained by a change of relevance of different catabolic routes. The release of butyric acid and acetic acid is associated to known routes of biological hydrogen production [45], characteristic of strict anaerobes, such as *Clostridiaceae*:

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2\,CO_2 + 2H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow 2\,CH_3COOH + 2\,CO_2 + 4H_2 \quad (2)$$

Facultative anaerobes, such as *Enterobacteriacae*, produce hydrogen by the pyruvate-formate hydrogen-lyase pathway [45]:

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3 \quad (3)$$

$$C_3H_6O_3 + HSCoA \rightarrow CH_3CO\text{-}SCoA + HCOOH + H_2O \quad (4)$$

$$HCOOH \rightarrow CO_2 + H_2 \quad (5)$$

(HSCoA: coenzyme A)

Incidentally, the formic acid in the ADH medium is consumed but apparently not used (or only partially used) for hydrogen production through the formate hydrogen-lyase reaction (5), otherwise hydrogen production would have exceeded that observed with the glucose-based medium.

Ethanol and lactic acid are produced in fermentative routes not involved in hydrogen production or consumption:

$$C_6H_{12}O_6 \rightarrow 2\,CH_3CH_2OH + 2\,CO_2 \quad (6)$$

$$C_6H_{12}O_6 \rightarrow 2\,CH_3CH(OH)COOH \quad (7)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH(OH)COOH + CH_3CH_2OH + CO_2 \quad (8)$$

The last two reactions, (7) and (8), are typical of *Lactobacillales* catabolism [46].

The marked change in soluble products associated with the reduction in hydrogen yield and microbial growth could be attributed to the selective inhibition of hydrogen-producing bacteria and consequent outgrowth of resistant lactic acid bacteria. Indeed, inhibition of bacterial growth by phenols and furans produced during pre-treatment and hydrolysis of lignocellulose is a known problem in the exploitation of woody biomasses for hydrogen production [15]. Inhibitors affect also *Lactobacilli* [47], but several resistant strains have been isolated [48,49]. Lactic acid bacteria, such as *Bifidobacteria*, *Lactobacilli*

and *Lactococci*, have been frequently detected in hydrogen-producing cultures [49]. Their negative role in H_2 production, due to substrate competition or inhibitor production, has been demonstrated by several authors in similar studies [50,51]. In order to validate the hypothesis of a taxonomical composition change of bacterial population, samples from the stationary phase of the initial (TS2) and final cultures (AD3 and AD4) of the batch sequence have been checked by molecular methods (pyrosequencing of 16S rRNA genes and qPCR of target genes).

3.2. Taxonomic Composition of the Bacterial Consortia

3.2.1. Pyrosequencing of 16S rRNA Genes

The composition of the microbial communities of TS2, AD3, and AD4 consortia were characterized by pyrosequencing of 16S rRNA genes (Figure 3). On average, 12,000 reads for each sample were produced, of which 10,000 passed the quality check and were used for the analysis. Taxonomic assignments were obtained by comparison with the database of 16S rRNA sequences deposited in SILVA [38] (Table 4).

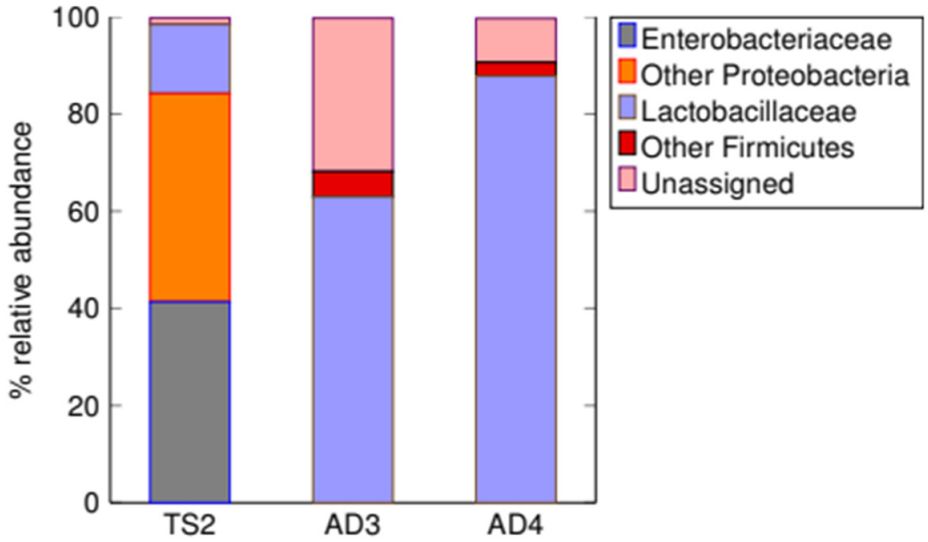

Figure 3. Relative abundance of main bacterial groups as obtained by barcoded sequencing of 16S rRNA amplified from hydrogen-producing consortia TS2, AD3, and AD4.

Table 4. Detailed analysis of the bacterial community by pyrosequencing. Taxonomic assignments obtained by comparison with the database of 16S rRNA sequences deposited in SILVA [38]. Values are indicated as percentage.

Phylum	Class	Order	Family	Genus	TS2	AD3	AD4
Unassigned	Other	Other	Other	Other	1.30	31.56	9.04
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	0.00	0.00	0.01
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Enterobacter*	41.35	0.00	0.00
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	Other	0.20	0.00	0.00
Proteobacteria	Other	Other	Other	Other	42.87	0.00	0.01
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	0.01	0.21	0.32
Firmicutes	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Lactococcus*	0.00	0.04	0.00
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	14.23	62.96	87.81
Firmicutes	*Bacilli*	*Bacillales*	*Sporolactobacillaceae*	*Sporolactobacillus*	0.03	0.00	0.00
Firmicutes	*Bacilli*	Other	Other	Other	0.00	5.21	2.82
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	0.00	0.01	0.00
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	Other	Other	0.00	0.01	0.00

The TS2 bacterial community selected in the presence of glucose is characterized by the co-dominance of *Enterobacteriaceae* (41.5%) and *Lactobacillus* (14.23%). The AD3 and AD4 bacterial communities selected in the presence of ADH are characterised by the domi-

nance of *Lactobacillus* (63% and 87.81% in AD3 and AD4, respectively), with *Enterobacter* being completely outcompeted. In these communities, *Firmicutes* increased as well as *Clostridium* (<0.5% of the total).

The presence of easily fermentable glucose likely promoted the increase of *Enterobacteriaceae* family of the *Proteobacteria* in the TS2 culture. On the contrary, *Lactobacillus* and *Clostridium* populations were likely more adaptable to the presence of ADH inhibitory compounds, thus developing in such culture conditions. Adaptation is also confirmed by the observation that, upon recultivation on ADH-based medium (AD2 → AD4), no further population changes were evidenced.

3.2.2. Quantification of Hydrogen-Producing Species by qPCR

The standard curves of 16S rRNA of Bacteria, of 23S rRNA of *Klebsiella* sp., and hydA of *Clostridium* spp. were linear, more than seven order of magnitude (R^2 = 0.990, 0.991 and 0.988 respectively), with amplification efficiencies of 100, 100.6, and 102.9%, respectively.

Gene quantification of 16S rRNA of Bacteria, 23S rRNA of *Klebsiella* sp., and hydrogenase *hydA* of *Clostridium* spp. was performed in TS2, AD3, and AD4 consortia (Figure 4). Nucleotide sequence analysis confirmed that the amplified fragments corresponded to the searched targets.

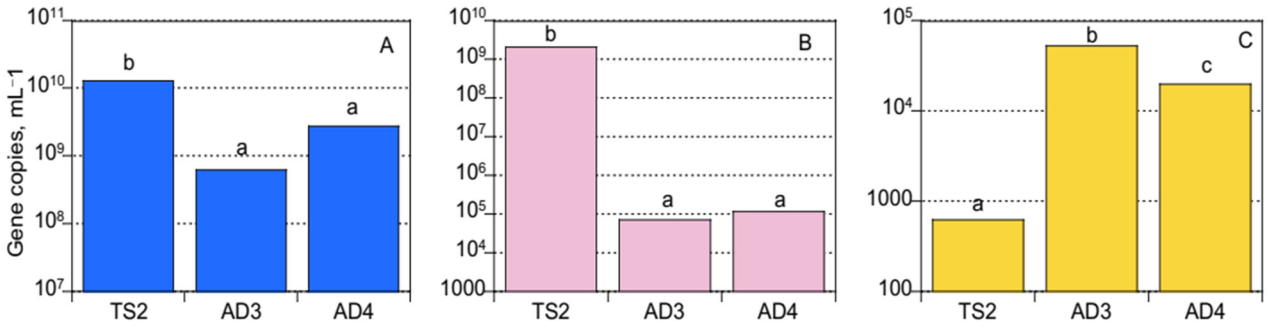

Figure 4. Quantification of target genes in TS2, AD3, and AD4 consortia: (**A**) 16S rRNA of Bacteria, (**B**) 23S rRNA of *Klebsiella* sp., and (**C**) hydrogenase *hydA* of *Clostridium* spp. Different lowercase letters indicate significantly different results (ANOVA, $p < 0.05$).

The use of ADH medium causes a 10-fold reduction in copy number of Bacteria, 10,000-fold reduction in copy number of *Klebsiella* sp. 16S rRNA, and a 100-fold increase in copy number of *Clostridium* sp. *hydA*. The data confirm a severe inhibition of H_2-producing *Enterobacteriaceae*.

The *hydA* copy number mirrors the relative increase of *Clostridium* spp. detected in barcoded 16S rRNA gene libraries: 0.01% in TS2, 0.21% in AD3, and 0.31% in AD4 (Table 4).

3.2.3. Phylogenetic Analysis of hydA Genes

The *hydA* sequences present in the consortia cluster with three different hydrogenases belonging to the *Clostridium* genus (Figure 5). The first type has 99% homology to the hydrogenase gene of *Clostridium* sp. strain CCH-2004.2 (Acc. Num. AY652730), and it was retrieved in TS2, AD3, and AD4; the second type has 99% homology to that of *Clostridium butyricum* strain CGS5 (Acc. Num. EF450251) retrieved in AD3; and the third type has 99% homology to FeFe-hydrogenase of *Clostridium tyrobutyricum* strain JM1 (Acc. Num. FJ226584) present in AD4.

It is possible to evidence that the presence of complex substrates, such as ADH, has exerted a positive effect on the enrichment of different *Clostridium* strains not detected in the initial consortium grown on glucose.

Figure 5. Phylogenetic relationships of deduced amino acid sequence of hydrogenase *hydA* of clones from TS2, AD3, and AD4 consortia. The evolutionary history was inferred using the neighbour-joining method. Solid circles are bootstrap values between 75 and 100. The bar indicates 1% sequence difference. The sequence of *Clostridium pasteurianum* NRRLB-598 (ETD68259) was used as an outgroup.

4. Discussion

Hydrogen production is consistently reduced immediately after the transition from glucose to ADH medium. At the same time, the composition of volatile acid products is also affected. The changes suggest a shift from catabolic pathways that lead to H_2 production along with acetic and butyric acids to pathways that do not produce hydrogen with lactic acid and ethanol as end products.

Medium composition may influence the distribution of end products by activating different metabolic pathways in a stable mixed population or by changing the taxonomic distribution resulting in the proliferation of species with different metabolism. In fact, the low hydrogen yields and the simultaneous production of lactic acid observed during the dark fermentation of *A. donax* hydrolysates point out a shift in the taxonomic composition of the original microbial consortium. The inhibition of hydrogen-producing bacteria, resulting in the unwanted prevalence of lactic acid bacteria, is confirmed by the analysis of the bacterial community by molecular biology methods.

Indeed, from the analysis of the bacterial community by 16S rRNA gene pyrosequencing and qPCR quantification, the prevalence of *Enterobacteriaceae* and of *Clostridium* sp. in the presence of either glucose or *A. donax* hydrolysates, respectively, was highlighted. This is in accordance with [49], who reported H_2 production by glucose or cellulose degradation by these bacterial taxa. *Lactobacillaceae* show greater adaptability to the ADH medium and constitute the majority at the end of carbohydrate fermentation. Lactic acid bacteria have often been detected in H_2-producing consortia as well [49]. qPCR quantification of target genes also confirms the shift in the composition of microbial consortium with the ADH medium. The inhibition is severe for *Enterobacter* and *Klebsiella* spp. On the other side, the ADH substrates seem to have a beneficial effect on number and diversity of *Clostridium* spp.

Preliminary tests on the effects of adding known inhibitors present in lignocellulosic hydrolysates (p-coumaric acid and furfural) on the dark fermentation of a glucose medium with the same anaerobic consortium used in this study showed a strong decrease in overall H_2 production and an increase in lactic acid production [35]. Although a change in taxonomic composition was not checked by molecular methods, the results support the view that the low yields of hydrogen generally observed with anaerobic consortia growing on lignocellulose hydrolysates may be due to the direct action of inhibitors on the growth of hydrogen-producing bacteria and to the competing outgrowth of more resistant lactic bacteria.

Improvements in hydrogen yields could be obtained by the optimization of inoculum selection techniques [25]. Alternatively, since each species is differently affected by changes in process parameters, such as retention time, pH, and partial pressure of H_2 and CO_2, careful choice of operating conditions may be used to control the distribution of end products. In particular, pH seems to be the most important parameter in dark fermentation [52]. Preventing the acidification of medium indeed had a beneficial effect in preliminary tests [24]. This could be explained by the fact that the acid resistance of lactic acid bacteria [46] confers an advantage at acidic pHs over other competing species. In the work of Vasmara et al. [26], the use of an initial alkaline pH (8.7) improved the hydrogen yield, likely by avoiding premature acidification during fermentation.

Future work will deepen the impact of process conditions on the hydrogen yields and their correlation with the taxonomic composition of anaerobic consortium.

5. Conclusions

Community analysis highlighted the dramatic effect of the lignocellulosic hydrolysate components on the taxonomic composition of hydrogen-producing mixed cultures. The development of rational strategies for the optimization of dark fermentation of lignocellulosic hydrolysates should take into account the susceptibility to the inhibitors of the different bacterial groups of the anaerobic consortium. As an alternative to prior detoxification of hydrolysates, inoculum selection and process control techniques designed to favour more resistant H_2-producing bacterial groups could be envisaged.

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