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Argentine Navy Icebreaker Ship "Almirante Irizar" Sludge Microbial Composition Analysis for Biohydrogen Production

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Abstract

Sludge from the wastewater treatment plant of the Argentinean Navy icebreaker ship "Almirante Irizar" was used as inoculum for biohydrogen production. The bacterial community was monitored throughout the fermentation, by sequencing 16S rRNA amplicons, to establish the microbial dynamics of the bioreactor over time. The established operating procedure assured a hydrogen content, along the process, in the range of 59.2–70.0%. The predominant species found were *Clostridium* sensu stricto and *Sporolactobacillus* sp. *Clostridium* showed higher values in the beginning of the fermentation with more than 90% of relative abundance. Conversely, *Sporolactobacillus* reached values close to 20% at its end. Additional topics discussed are the role of lactic acid bacteria in fermentative biohydrogen production systems and a series of in-process parameters that would allow control of this population. The results obtained allow supporting the use of this type of sludge as a source of hydrogen-producing bacteria.

Keywords Anaerobic · Metabarcoding · Fermentative · Biogas · Renewable · Energy

Introduction

A great fraction of the global energy is produced from fossil fuels, resulting in CO_2 emissions associated with climate change. The increasing demand for energy, due to population growth and rapid industrialization [1], triggered the emergence of gas-based fuels as an alternative to fossil fuels, becoming a natural, easy, and largely available clean form of energy [2].

The profound environmental damage generated by traditional fuels, creates an urgent need to develop sustainable energy systems that do not depend on their consumption for energy generation. In this sense, it is important to highlight the relevance of hydrogen (H_2) gas as an energy carrier [3]. In order to point out the prominent role that hydrogen will have in the future, it can be mentioned that the USA, the European Union, and the People's Republic of China have shown interest in the research and development of fuel cell and hydrogen technologies. In the case of the United States Department of Energy (DOE), through the "Hydrogen and Fuel Cells program" with a budget of US\$ 3.7×10^6 for the period 2004–2020 [4]. The People's Republic of China, since 2001 has invested US\$ 105.6×10^6 in the research and development of such technologies within the framework of the programs "973—National Basic Research Program of China" and "863—National High-tech R&D" [5].

The European Union has implemented the Fuel Cells and Hydrogen Joint Undertaking (FCH JU). The program intends to accelerate the market introduction of fuel cell technologies and the generation, distribution, and use of hydrogen gas, taking advantage of their potential as an instrument to

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achieve a low-carbon energy system. In order to meet these goals, the European Union will invest around € 80×10^9 within the framework of the Horizon 2020 call [6].

Hydrogen gas is known as an eco-friendly, efficient energy carrier [7], and it is considered by many as the fuel of the future. One of hydrogen advantages over other fuels is that it can be produced through a number of renewable methods such as water electrolysis and biomass biological conversion [8]. Also, when used in a proton exchange membrane (PEM) fuel cell to generate electricity [9], only water and heat are produced as waste, instead of greenhouse gases [10]. Biological production of hydrogen can be accomplished through several methods such as direct and indirect biophotolysis, photofermentation, microbial electrolysis, and dark fermentation. There are several advantages that support the election of dark fermentation over other biological hydrogen production methods. Dark fermentation does not depend on light conditions, has a high rate of hydrogen production [11], and the bioreactor design is simple and relatively easy to control. In addition, a broad variety of carbonic rich waste can be used as substrates [12].

Nowadays, researchers are focusing on circular bio-economy, using sludge waste, animal manure, wastewater, among others as substrates, allowing simultaneous management of these residues for the production of hydrogen [13]. The utilization of waste as a carbon source for bio-hydrogen production makes the process more economical and sustainable [14].

Sewage sludge in particular presents great potential as substrate due to its low cost and vast worldwide availability [15]. When used in this manner, it must be considered however that activated sludge is composed mainly of proteins. Hydrogen yields from this source are inferior to the observed yields for the organic fraction of municipal solid waste (OFMSW) rich in carbohydrates and only slightly superior to those achieved when fermenting protein rich OFMSW [16]. Sludge can, as it is in this study, be used as a source of bacteria for biohydrogen production. It contains numerous microorganisms capable of producing hydrogen gas. Moreover, mixed cultures show better adaptive capacity to environmental changes than pure cultures and they can establish synergistic interactions, which might result in improved substrate degradation and enhanced hydrogen production [17].

Research and development of biological hydrogen production technologies take on special relevance if one considers that it is estimated that by the year 2040 the production value of the biological hydrogen industry will reach US$ 8.97×10^9 in the USA, while for the People's Republic of China, that value is estimated to be US$ 7.5×10^9 [18].

Optimization of the biological hydrogen production process is of crucial importance to the economic and scale-up feasibility of this technology [19]. Knowing which microorganisms are present in the bioreactor and how their population changes during the fermentative process contributes in this sense. It paves the way for better understanding the variables that shape bacterial community interactions and help determine the conditions that should be established for microorganisms recognized as good hydrogen producers to become dominant in the culture. Therefore, in this research, the changes of the bacterial community along time in four hydrogen production assays were studied through 16S rRNA amplicon sequencing to depict the bioreactor microbial dynamics. As well, the hydrogen percentage in gas samples, generated throughout the fermentative process, was determined. The impact of the heat pre-treatment on the microbial population was analyzed and the function of the observed dominant microorganisms was discussed.

Through this research, the Argentine Navy intends to acquire the know-how that would eventually allow the production of renewable fuel on the high sea or in isolated guard posts, in a manner compatible with environmental care and in compliance with international treaty protocols such as the Antarctic one [20]. This aims to reduce Navy's dependency on traditional fuels and the harmful effects related to their use.

Materials and Methods

Bacterial Inoculum

Sludge from the anaerobic decantation chamber of the Deltabio PRB-2940 wastewater treatment plant (Detegasa, Galicia, Spain) installed on board of the Argentine Navy ship Icebreaker "Almirante Irizar" was used as the source of inoculum for all batch experiments. This type of inoculum was selected, as adequate hydrogen production was observed in a prior study in which sludge from a wastewater plant treating black and gray water from human origin was used [21]. Furthermore, in eighteen batch assays (not published) performed previously in the Biostat A-plus bioreactor with inoculums from similar sources, average hydrogen percentage in the gas mixture was determined to be superior to sixty percent, an acceptable result for this production process. A diagram of the three-stage treatment plant along with a description of its main features can be found at Detegasa [22]. A heat pre-treatment was applied to all 5×10^{-1} L sludge aliquots prior to inoculation. Aliquot samples were placed in Erlenmeyer flasks and exposed to 75 °C on a BS-655H water-bath (Faeta S.A., Buenos Aires, Argentina) for 50 min.

Batch Fermentation Conditions

All fermentations were performed in a 5 L (final working volume) Biostat A-plus bioreactor (Sartorius, Stedim Biotech, Germany). Temperature was set at 37 °C since

adequate hydrogen production was observed in the mesophilic range, particularly at 35 °C and 38 °C versus 45 °C, at which temperature, no production of hydrogen was detected [21]. Other studies also support the selection of this temperature when considering optimal growth [23] and hydrogen production conditions [24]. Agitation to 50 rpm and an oxygen free atmosphere was established inside the bioreactor by nitrogen gas sparging. pH sensor was calibrated using pH 4 and 7 reagents from Cicarelli Laboratories. Culture medium was prepared as previously described by García et al. [21] (Table 1). Sucrose was used as substrate given that the objective of the assay was to identify the microorganisms present in the bioreactor under optimal conditions, not to evaluate the hydrogen production potential of other substrates.

Biogas Volume and Hydrogen Content Determination

Two biogas samples were taken from the bioreactor headspace for each time point (Table 2), and the hydrogen percentage was determined using a PEM fuel cell as described in Martinez et al. [25]. Biogas generated in the bioreactor was stored in three acrylic cylinders (Fig. 1), and the total amount produced in each batch was determined on the basis of the volume occupied in them.

Sample Collection for 16S rRNA Amplicon Sequencing

1×10^{-2} L sample was collected through the harvest pipe embedded in the bioreactor at each time studied (Table 2). Additionally, the 0-h (T0) samples taken from the sludge stock were included prior to the heat treatment. This schedule was designed so that microbial population through the entire batch process could be studied, before, during, and after hydrogen production peaks. Samples were stored at – 20 °C until DNA extraction.

Table 1 Culture medium reagent concentration

Reagent	Concentration (g/L)
$C_{12}H_{22}O_{11}$	25
NH_4HCO_3	2
KH_2PO_4	1.2
$MgSO_4·7H_2O$	0.2
$Na_2MoO_4·2H_2O$	0.2
$CaCl_2·2H_2O$	0.2
$MnSO_4·H_2O$	0.2
$FeSO_4$	0.2
$C_2H_3O_2Na$	6.8

Table 2 Sampling schedule for Biogas volume and Hydrogen content determination

Batch	Time (hours)				
	T1	T2	T3	T4	T5
1	19	24	43	51	116
2	19	24	43	50	138
3	7	25	95	-	-
4	18	23	42	-	-

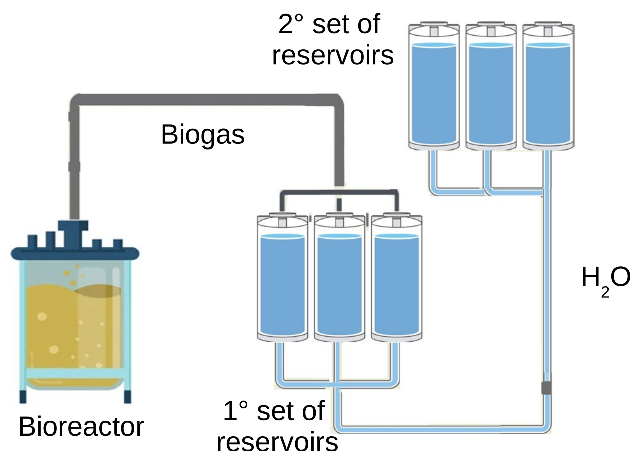

Fig. 1 Biogas storage system. Biogas produced in the bioreactor is stored in the first set of acrylic reservoirs, which are initially filled with water that gets displaced to the second set when biogas flows in

DNA Extraction

1×10^{-2} L of sample were concentrated on a SpeedVac (Savant, USA) overnight at room temperature up to 1×10^{-3} L. Total genomic DNA was obtained using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA concentration and purity were assessed in NanoDrop ND – 1000 spectrophotometer (NanoDrop Technologies, DE, USA), and DNA was stored at – 20 °C until further analysis. A total of 20 DNA samples were used for 16S rRNA gene-based microbial community analysis.

Illumina 16S rRNA Gene Region Amplification and Sequencing

PCR amplification was performed using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, USA) in combination with the Roche High Fidelity Fast Start Kit (Roche, Basel, Switzerland) following Lange et al. [26]. Briefly, a 3×10^{-6} L PCR master mix was prepared for each sample containing 5×10^{-7} L 10× buffer mix without magnesium chloride ($MgCl_2$) (Roche High Fidelity Fast Start Kit), 9×10^{-7} L 25×10^{-3} M $MgCl_2$, 25×10^{-8} L DMSO, 25×10^{-8}

L 20× Access Array Loading Reagent (Fluidigm Corporation, South San Francisco, USA), 1×10^{-7} L 10×10^{-3} M dNTPs each (Roche High Fidelity Fast Start Kit), 5×10^{-8} L Fast Start High Fidelity Enzyme Blend (5 U/ µL) (Roche High Fidelity Fast Start Kit) and 1×10^{-6} L target specific primers mix for V3-V4 region of the 16S rRNA gene (250×10^{-9} M each, 341F: 5′-CCT ACGGGNGGCWGCAG-3′ and 805R: 5′-GACTACHVGGG TATCTAATCC-3'). 3×10^{-6} L PCR master mix, 1×10^{-6} L DNA and 1×10^{-6} L dual-index barcodes (2×10^{-6} M equimolar mix of index 1 (i7) and index 2 (i5)) were mixed. Index primers were obtained from Lange et al. [26].

The PCR was performed on a Veriti thermal cycler (Applied Biosystems) using the protocol described in Table A. 1 (see Supplementary information). Equal amounts of each PCR amplicon were pooled and cleaned using Agencourt AMPure XP beads (Beckman Coulter) following the manufacturer's instructions. The concentration of the pooled library was quantified using Qubit and analyzed on a Fragment Analyzer (Agilent, Santa Clara, CA, USA). The final concentration of the library was diluted to 6×10^{-12} M with ~ 20% PhiX spiked in to account for the low base-diversity library. The final pooled library was sequenced on Illumina MiSeq with a MiSeq Sequencing Reagent Kit v2 to obtain 250-bp paired-end reads at the Unidad de Genómica (UGB) of the Instituto Nacional de Tecnología Agropecuaria (INTA, Hurlingham, Buenos Aires, Argentina).

Data Analysis of 16S rRNA Gene Amplicons and Statistical Analysis

Raw reads were processed by QIIME2 software package (version 2018.2). Quality control was performed using Divisive Amplicon Denoising Algorithm 2 (DADA2) [27] by removing chimeras and residual PhiX reads, and low-quality regions of sequences. Forward and reverse reads were truncated to 240 bp for DADA2 analysis based on the average quality scores determined. After quality filtering, dereplication was performed by DADA2, which combines identical reads into amplicon sequence variants (ASVs).

Representative sequences were classified using a pre-trained naive Bayes classifier for the V3–V4 region of the 99% SILVA v.128 database.

The resulting ASVs were aligned with MAFFT [28] and placed into a phylogenetic tree with FastTree [29]. Metrics of alpha diversity (observed ASVs, Faith PD, Shannon diversity index and Pielou's evenness), beta diversity (weighted and unweighted UniFrac [30]), and a principal coordinate analysis (PCoA) were estimated after samples were rarefied to the minimum sequence depth observed in any given sample.

Statistical analyses for alpha- (Kruskal–Wallis) and beta-diversity (PERMANOVA) as inputs for assessing group significance, and plotting principal coordinate analysis (PCoA), charts were completed using QIIME2.

Results and Discussion

Hydrogen Production

The hydrogen content obtained in this study showed an average H_2 percentage in the gas samples in the 59.2–70% range (Table 3). This result was comparable to other studies that also used sucrose as an energy source. For example, Zhang et al. [31] reported a gas-phase H_2 concentration averaging 74 ± 3% for all conditions tested, using glucose as the carbon source, a pure culture of *Clostridium acetobutylicum* and working under a continuous regime. Cripa et al. [32] used samples from anaerobic ponds of a poultry slaughterhouse as inoculum and sucrose (13.56 COD g O_2/L) as substrate, obtaining methane free biogas which contains 50–60% H_2. Rodríguez-Valderrama et al. [33] informed an average hydrogen content of 69.04% in their study, using methanogenic granules obtained from brewery industry anaerobic sludges as inoculum. Lutpi et al. [34] investigated biohydrogen production enhancement under thermophilic conditions using anaerobic sludge from a palm oil mill effluent treatment plant. In their study, the H_2 content ranged from 48 to 50% of the total biogas.

Table 3 Hydrogen percentage average in the gas mixture for each time-point (T1–T5). Mean value of hydrogen percentage, cumulative production of biogas and hydrogen, during batches 1–4

Batch	H_2[a] (% v/v)					Mean H_2 (% v/v)	Biogas volume (L)	Hydrogen volume (L)
	Time							
	T1	T2	T3	T4	T5			
1	N/A	65.0 ± 7.1	65.0 ± 0.0	70.0 ± 0.0	70.0 ± 0.0	67.5 ± 2.9	25.8	17.4 ± 0.7
2	68.8 ± 1.8	67.5 ± 3.5	70.0 ± 0.0	65.0 ± 0.0	70.0 ± 0.0	68.3 ± 2.1	23.8	16.3 ± 0.5
3	45.0 ± 0.0	70.0 ± 0.0	62.5 ± 3.5	-	-	59.2 ± 12.8	14.1	8.3 ± 1.8
4	65.0 ± 0.0	75.0 ± 0.0	70.0 ± 0.0	-	-	70.0 ± 5.0	21.3	14.9 ± 1.1

H_2[a] (% v/v) is expressed as mean (number of gas samples tested for each time point = 2) ± SD

Interestingly, hydrogen percentage in the gas mixture in studies in which no sucrose but a variety of waste have been used as substrate is still high. Xing et al. [35] reported a maximal hydrogen content of 38.6% using dairy manures with acidification. Tang et al. [36] achieved 32% of H_2 content in the biogas produced from cattle wastewater. Di Cristofaro et al. [37] used different mixtures of digested and fresh buffalo manure and determined that the potential hydrogen concentration was 20%. Other sources from food waste were described in literature, showing values between 52 and 66% H_2 (52% H_2 from Palm oil mill effluent [38], 38–44% H_2 from sonicated food waste [39], 53.35% H_2 from chicken manure [40]). Taking into consideration the mentioned range of H_2 production, the performance of this system is an indication that the sludge from the wastewater treatment plant of the Argentine Navy Icebreaker ship "Almirante Irizar" gives rise to an efficient inoculum. Further studies will be necessary to assess the requirements for scaling-up.

Medium pH

Medium pH evolution was monitored online throughout the fermentation process. Results for all four batches are shown in Fig. 2. As expected, pH decreased in all batches since hydrogen production is concomitant with proton release. In this study, two phases of production are observed. At the beginning, after the fermentation process has started, pH decreases abruptly during the first 20 h reaching nearly a plateau subsequently. The second phase occurs between the 42 and 138 h along with a smooth pH decrease. The decay range was from 7.3 to 3.8 (batches N° 1 and 3) and 7.2 to 4 (batches N° 2 and 4).

Microbial Community Analysis

The composition of the bacterial communities present at different times in each bioreactor was assessed and compared. The criterion for sampling time-point selection was to be able to follow population changes along the fermentation process. Therefore, T0 samples allowed knowing which microorganisms were present in the inocula before the heat treatment and samples taken on time-points one through five (T1–T5) allowed understanding how microbial population evolved as hydrogen production and culture media acidification occurred.

After sequencing and sample filtering, 1,487,751 quality reads (mean 74,387.55 ± 43,402.19) were obtained. The community structures at phylum and family level could be observed in Fig. 3.

Relative abundance of Firmicutes phylum dominated the whole process from T1, particularly Clostridiaceae and Sporolactobacillaceae families (Fig. 3b). Indeed, *Clostridium* sensu stricto and *Sporolactobacillus* sp. were the

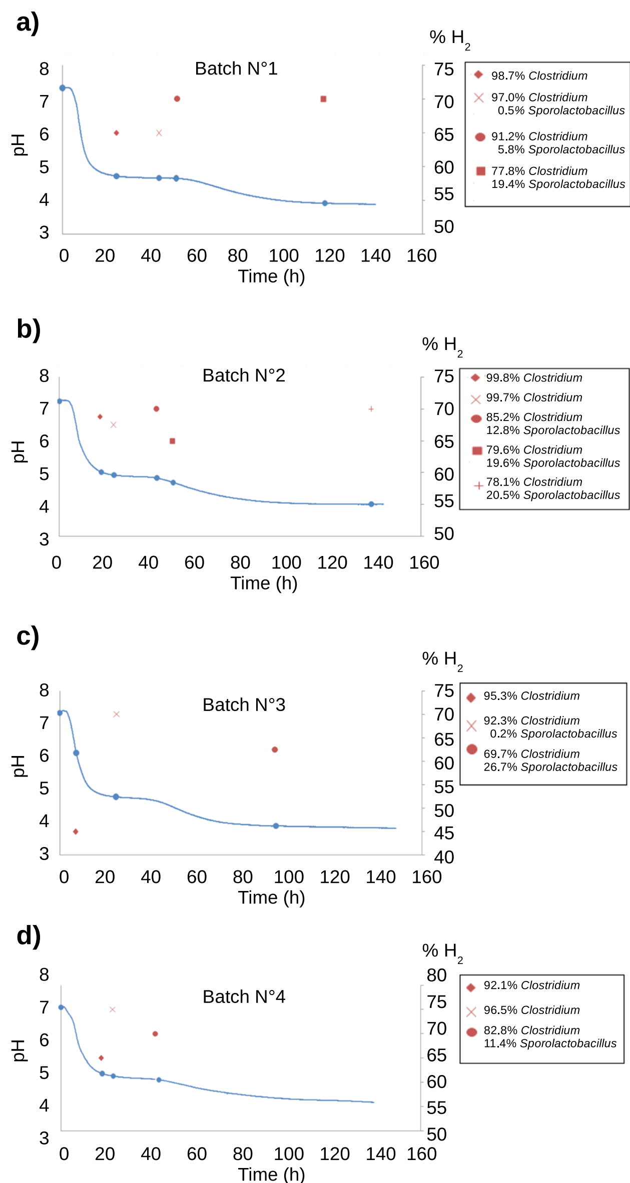

Fig. 2 pH evolution through time (hours) for all batches. Blue dots indicate sampling time for 16S rRNA sequencing in each batch. Blue line indicates pH value (left axis). Red data points indicate hydrogen percentage (right axis) and dominant microorganism relative abundance (attached black square)

predominant genera along the biogas production process (Fig. 4). *Clostridium* showed higher values in the beginning of the fermentation process with more than 90% of the total bacterial present at T1–T2 and a slight decrease over time, reaching values among 70–80%. Conversely, *Sporolactobacillus* newly appeared at T3 achieving values near to 20% of relative abundance at the end of the process.

Clostridia are well known and extensively studied for their capability to produce hydrogen from various carbohydrates [41–43]. They are strict anaerobes, extremely sensitive to oxygen and are commonly considered as the most

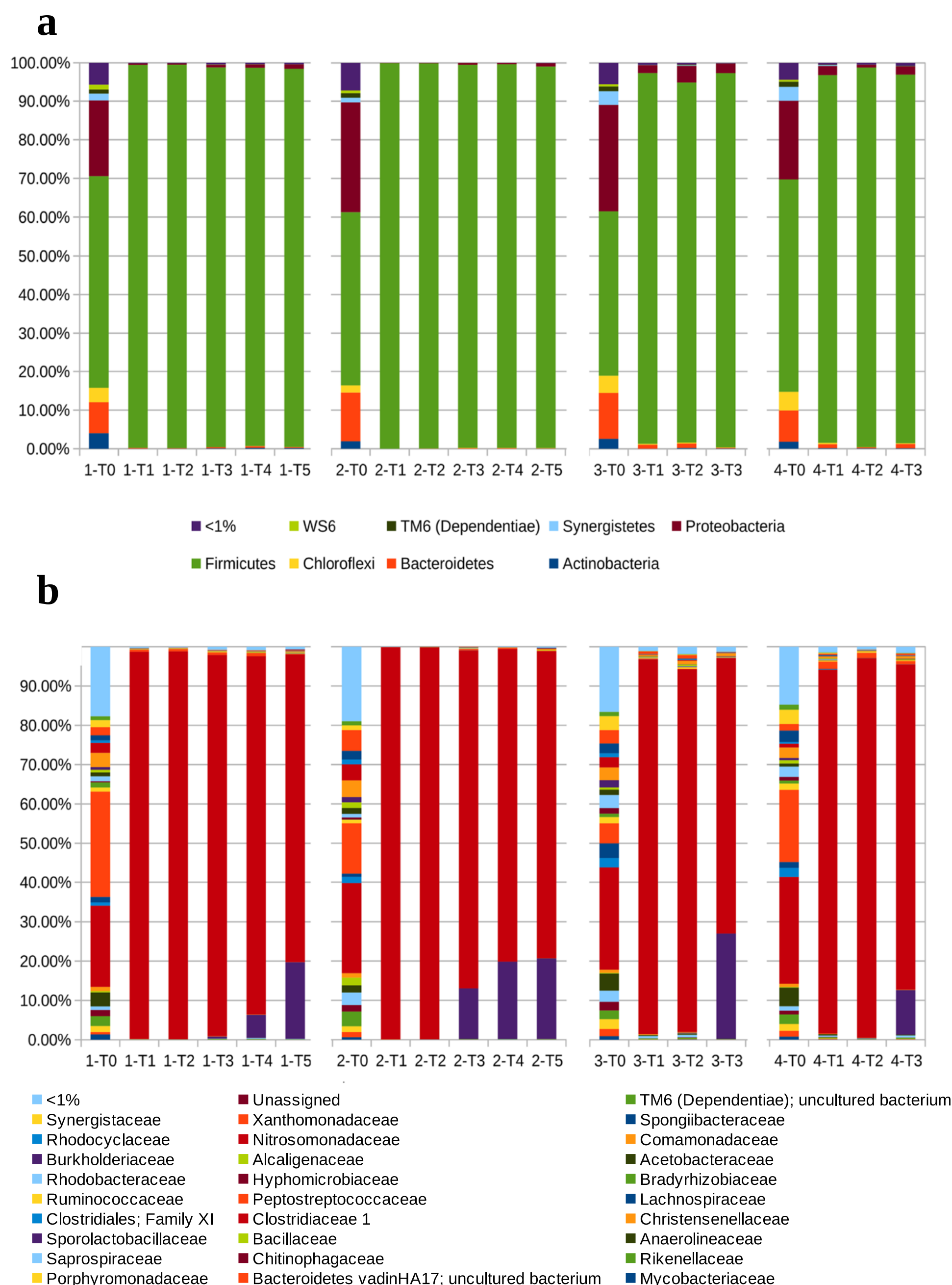

Fig. 3 Taxonomic analysis of microbial community at phylum (a) and family (b) level. Different colors are used to indicate each individual taxon along the time-point curve from T0 up to T5, for each batch (1–4)

abundant and efficient H_2 producers in bioreactors, usually found to be predominant during periods of high hydrogen production efficiency [44–47]. In this study, the presence of *Clostridium* sp. population is simultaneous with production of H_2 and a remarkable decrease in pH (Fig. 2). This result is supported by previous reports showing *Clostridium* sensu stricto as the major H_2 producer [10] or its presence had a significant positive correlation with hydrogen yield. In that study, enhancement of biohydrogen production from macroalgae through iron supplementation was analyzed [48]. Yang and Wang [49] investigated the changes in microbial community structure dark fermentative hydrogen production and determined through the Spearman correlation that *Clostridium* sensu stricto contributed the most to hydrogen production performances. Kumar et al. [50] evaluated optimal hydrogen production conditions through an experimental design approach and found that *Clostridium* sp. was dominant when the most favorable conditions were established.

A truly interesting feature of this group of bacteria, especially if on-site generation of energy is planned, is that they are ubiquitous in nature [51], being found and isolated from multiple sources including melted snow or ice from Antarctica, dry desert sand, alkaline and acidic

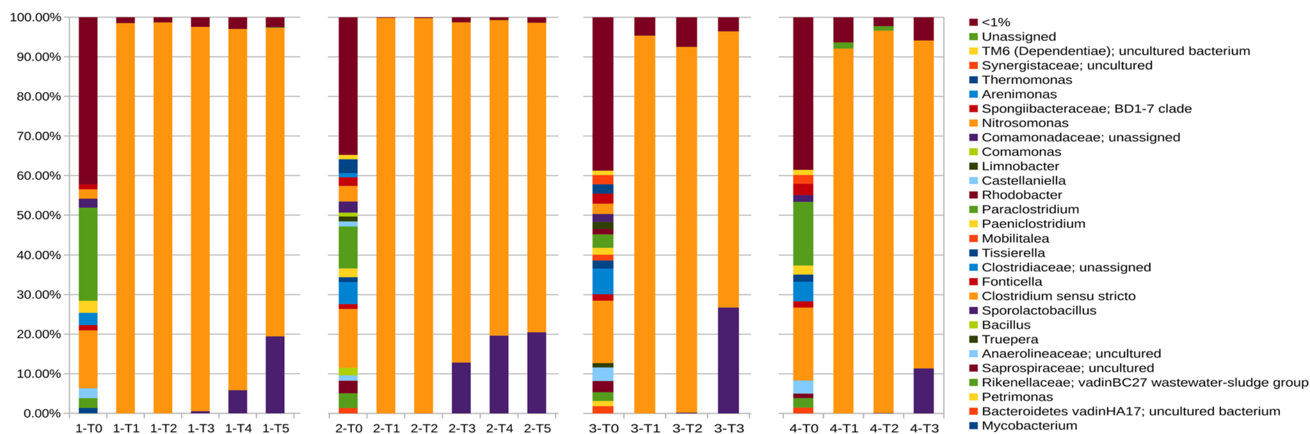

Fig. 4 Taxonomic analysis of microbial community at genus level. Different colors are used to indicate each individual taxon along the time-point curve from T0 up to T5, for each batch (1–4)

hot spring water and mud, mesobiotic and psychrobiotic river and lake water or sediments, and intestinal tracts of animals and insects [52]. Biohydrogen could be generated on-site in a bioreactor and then compressed and stored for further use. Regarding biohydrogen and purification for use on PEM fuel cell systems, if a carbohydrate such as pure sucrose is used exclusively as substrate, no contaminant gases for the PEM fuel cell catalysis seem to be produced (data not shown). Therefore, it might be possible to use hydrogen produced on-site through fermentation directly on a PEM fuel cell system for electric energy generation without polluting the cell. Considering the cost of hydrogen purification and the high purity requirements of PEM fuel cell systems, this approach might be worthwhile researching and exploring.

The differences observed in the composition of the community structure between more diversity sludge samples and post heat treatment *Clostridium*-predominant samples were confirmed using alpha and beta-diversity analysis, after rarefied samples to 13,500 sequences depth. The bacterial richness and diversity along time are shown in Fig. 5.

Sludge samples showed the greatest richness, giving rise to a rather homogenous community structure after heat treatment and during the fermentation process. Shannon and Pielou's indexes for species diversity and evenness from sludge or treated samples were also significantly different. This indicates that species diversity between both types of samples decreases, showing lower values for treated samples with losses of taxa after heat treatment.

Multidimensional scaling analysis PCoA plots for all metrics used in beta-diversity analysis, showed two distinct groups for treated or sludge samples. The arrangement that arose the PCoA based on weighted and unweighted UniFrac distances of the 16S rRNA gene clearly showed (Fig. 6) the

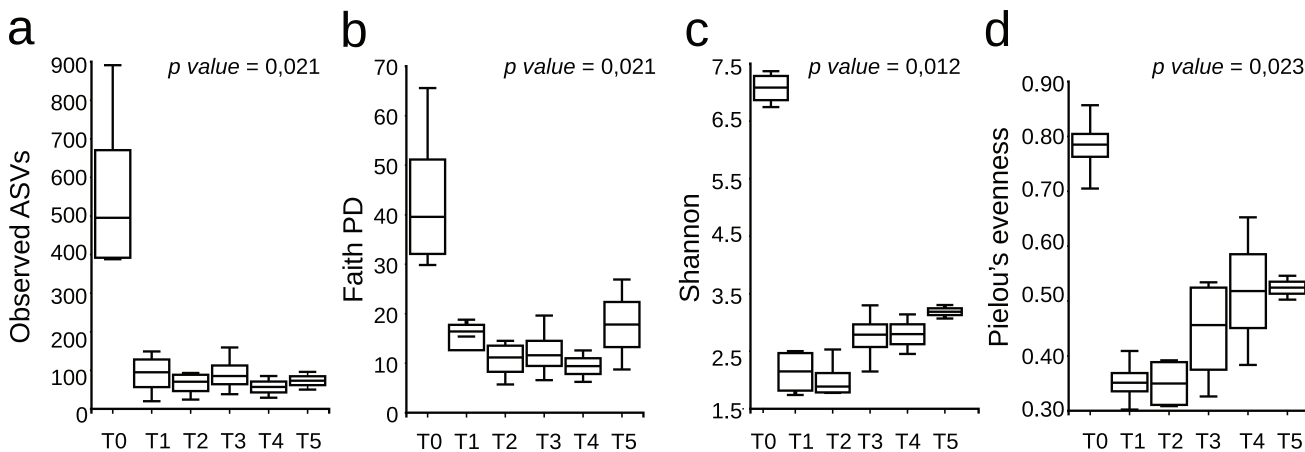

Fig. 5 Boxplots of alpha diversity metrics along the experiment for the four batches. They include the interquartile range, the median value of the data set, and *p* values for the Kruskal–Wallis test among the time-point groups. Statistical significance ($p < 0.05$) was detected among sludge (T0) and treated samples (T1–T5)

distinction between sludge samples or H_2-producing ones, with PC1 of 81.18% or 35.03% and PC2 of 11.89% and 14.57%, respectively, of explained variability.

UniFrac is a metric of the phylogenetic distance between sets of taxa in a phylogenetic tree. This measure captures the total amount of evolution that is unique to each state, presumably reflecting adaptation to one environment that would be deleterious in the other [30]. Weighted Unifrac gives more importance to the most abundant bacteria as it takes into account sequence abundance per ASVs, while unweighted Unifrac gives similar weight to all bacterial ASVs present in the samples. In both PCoA plots, the effect of the heat pre-treatment against sludge and bioreactor samples as a selection factor was observed.

Heat treatment has been demonstrated to be successful in eliminating non-spore forming H_2-consuming bacteria (e.g. methanogenic bacteria) [53, 54] while selecting for H_2-producing bacteria, like Clostridia, that can form protective spores under extreme conditions. After heat treatment, the community structure evaluated in this study showed a decrease in diversity with the predominance of *Clostridium* and to a lesser extent of *Sporolactobacillus*, obtaining a stabilized H_2-producing consortium. This indicates that heat treatment is an adequate strategy to select for H_2-producing bacteria when using the inocula evaluated in this study, especially, if considering that heat treatment is a simple, low cost, effective method that can limit methanogenic activity [55]. Examples of different heat pre-treatment protocols and their effectiveness can be found at Bundhoo et al. [56].

The role of lactic acid bacteria (LAB) and in particular *Sporolactobacillus* in relation to biohydrogen production is not yet well defined. Many authors have found LAB to be detrimental to biohydrogen production owing to substrate competition or secretion of bacteriocins [43]. Cieciura-Włoch et al. [57] attributed the observed instability in their hydrogen production system to the dominance of the *Lactobacillaceae* family, which accounted for 59% of all present microorganisms in one of their experimental set-ups. They also correlated this to high concentration of lactic acid. In accordance, Park et al. [58] reported abrupt failure of hydrogen production due to inhibitory concentration of lactic acid (920×10^{-3} g COD/L). Conversely, there is evidence that the presence of LAB correlated with higher hydrogen production [59, 60]. It was proposed that lactic acid bacteria might be involved in hydrogen production, by providing extra substrate to H_2-producing bacteria in the form of lactate, or by directly converting lactate to hydrogen. Lactate can promote hydrogen production through a beneficial synergic interaction between lactic acid and H_2-producing bacteria [61]. Furthermore, lactate conversion to butyrate with H_2 production has been observed in mixed culture systems [62–64]. Another way in which lactate might favor hydrogen production is by unbalancing the conversion of pyruvate to lactate, therefore generating an accumulation of the reduced form of nicotinamide adenine dinucleotide (NADH). These molecules will, in turn, be oxidized and hydrogen generated [65].

Lactic acid bacteria are common contaminants of biohydrogen production systems and are frequently found in bioreactors even after treatment of the inocula. Despite this, the first strategy usually employed to control the population of bacteria detrimental to hydrogen production is pre-treatment of the inocula. Several authors have reported LAB inhibition when heat was applied. However, this is not the case for other studies in which methods such as freezing–thawing or exposure to acid were evaluated and survival of bacteria belonging to *Lactobacillus* sp. was observed [66]. Since there are benefits related to the presence of LAB in the mixed culture, a good

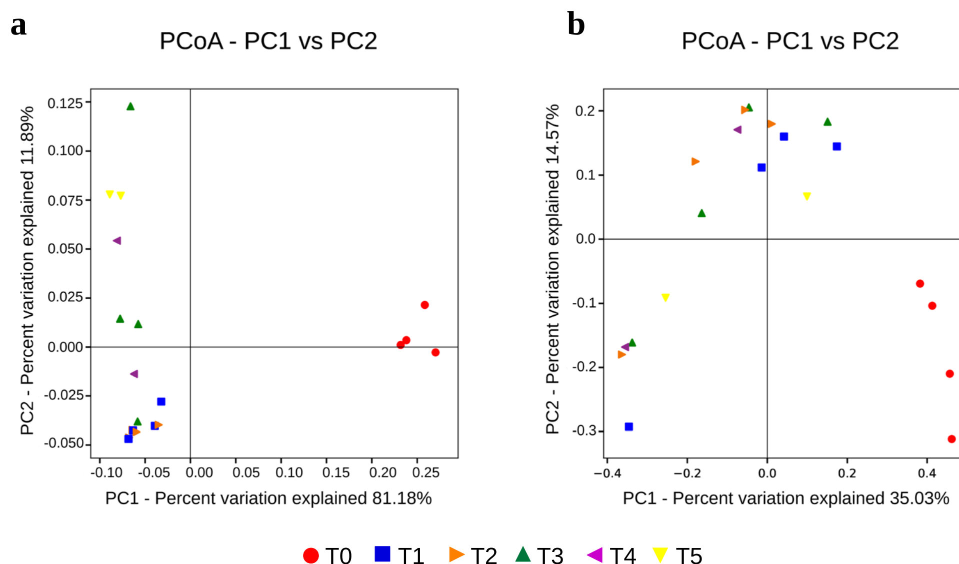

Fig. 6 Principal Coordinate Analysis of weighted (a) and unweighted (b) UniFrac

strategy might be to perform a pre-treatment of the inocula in order to get rid of most injurious microorganisms and then, aim to limit persistent detrimental populations by controlling fermentation parameters and conditions.

For example, Park et al. [67] evaluated the effect of substrate concentration on the competition between LAB and *Clostridium* during hydrogen production. They observed that *Lactobacillus casei* outcompeted *Clostridium butyricum*, when initial glucose concentration was low (< 1 g/L) and concluded that the competition between these bacteria was dependent on substrate concentration. Kim et al. [68] studied the effect on hydrogen production of various initial concentrations of lactic acid on a system dominated by *Clostridium* sp. and found that hydrogen yield increased when the initial concentration of lactic acid was increased from 0 to 8 g/L in batch tests. However, when concentration was increased to 16 g/L, glucose consumption efficiency and hydrogen yield decreased. Gomes et al. [69] analyzed the role of homo and heterofermentative LAB on hydrogen producing reactors operated with cheese whey wastewater. They observed the prevalence of the homofermentative *Lactococcus* spp. when the organic load rate (OLR) was low (8.5 and 12 kg/m d) and this was associated with higher hydrogen production. Conversely, prevalence of potentially heterofermentative LAB from *Lactobacillus* spp. was observed when OLR was high (15 and 40 kg/m d) and associated with lower hydrogen yields. Romão et al. [70] performed semi-continuous assays using lactose from cheese way permeate as carbon source and found that short medium removal times (24 and 12 h) resulted in less lactic acid being detected in the media, they suggested that this indicates that short removal times might inhibit LAB limiting their capacity to release bacteriocins, which would be detrimental to hydrogen production.

García-Depraect and León-Becerril [71] investigated the effects of solids content and substrate concentration on hydrogen production performance from tequila vinasse via the lactate-acetate pathway. They reported that the highest hydrogen production rate to occur in their experimental setup when total solids content was low (34.1 ± 0.2 g/L). In addition, they observed a direct correlation between higher hydrogen production and substrate concentration (from 7.9 to 57.7 g COD/L). The authors argue that the optimal conditions found in their study exploit and harness the beneficial effects related to the presence of LAB, favoring stable hydrogen production. Niño-Navarro et al. [72] observed that different carbon/nitrogen (C/N) ratios influenced not only the amount of hydrogen, but also the acids and microorganism profile. In their study, lower C/N ratios result in higher cumulative hydrogen production. When C/N = 39, the culture was comprised of 56% hydrogen producing bacteria and 13% LAB, this bacterial composition showed a far superior performance when compared to the one resulting from establishing a C/N = 82 ratio. In this case, LAB accounted for 71% of the microbial population whereas hydrogen producing bacteria represented only 26%. These results indicate that an adequate mixed culture composition can be set up by controlling fermentation conditions. In this situation, LAB might play an important role in maximizing hydrogen production when their presence does not compete but rather stimulates it.

To conclude, the previous examples suggested that the presence of LAB contributes to an efficient production of hydrogen along the whole fermentation process even when their role is not fully understood.

Conclusions

The hydrogen content obtained under laboratory conditions was comparable to other studies, leading to supporting the use of sludge from the “Almirante Irizar” Icebreaker ship wastewater treatment plant as a source of H_2-producing bacteria. This is of particular interest to the Argentinean Navy given that being able to produce fuel, or even better, renewable fuel on-site could represent a viable solution when resources are otherwise depleted. In order to achieve this milestone, scaling up procedures and protocols is still required. Also, heat treatment proved to be an adequate strategy to select for H_2-producing bacteria when using this inocula, since it was possible to establish a consortium dominated by *Clostridium*, a species considered a good hydrogen producer. Regarding the presence of lactic acid bacteria in the culture, the extent of the beneficial or detrimental effect of their presence was not assessed in this study. However, the review of relevant scientific literature here detailed, reveals that the beneficial effects of these microorganisms relative to hydrogen production might be harnessed if proper culture conditions are established.

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Author Contribution RG and NPV performed the analysis and interpretation of data, drafted the manuscript, and substantively revised it. RG, FAG and VN carried out the experiments. AP advised on methodology. MJL and MF conceptualized the project, advised on methodology and data interpretation, supervised the project, and were involved in the drafting and editing of the manuscript. All authors have read and approved the final draft of the manuscript prior to submission.

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Availability of Data and Materials The dataset supporting the conclusions of this article are available in the NCBI Sequence Read Archive database under the BioProject accession number PRJNA633652.

Declarations

Ethics Approval and Consent to Participate Not applicable for this study.

Consent for Publication Not applicable for this study.

Competing Iinterests The authors declare no competing interests.

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