Sigma Journal of Engineering and Natural Sciences
Web page info: https://sigma.yildiz.edu.tr
DOI: 10.14744/sigma.2026.2073

Research Article

Investigation of microbial communities and [FeFe]-hydrogenase gene by different dry-dark fermentation processes on hydrogen production

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ARTICLE INFO

Article history
Received: 08 October 2024
Revised: 05 February 2025
Accepted: 06 April 2025

Keywords:
Biohydrogen; *Clostridium butyricum*; Dark Fermentation; *hydA* Gene; Next Generation Sequencing; *Lactobacillus* spp.

ABSTRACT

Hydrogen production by dark fermentation is a promising approach for sustainable energy production. However, the effect of substrate pretreatment and microbial community dynamics on hydrogen yield remains an area requiring further research. This study aimed to investigate the changes in microbial profiles in a hydrogen-producing bioreactor with three different operating parameters. Furthermore, [FeFe]-hydrogenase, an enzyme notoriously difficult to detect in mixed cultures, was characterized according to the dominant species in the reactors. In the bioreactors, differences in the hydrogen-producing microbial population were monitored, focusing on the effects of particle size and heat pretreatment of fruit and vegetable waste. These microbial differences were analyzed using next-generation sequencing. Species-specific primers were designed to target the *hydA* gene of [FeFe]-hydrogenase in *Clostridium butyricum* in mixed cultures. Phylogenetic relationships of [FeFe]-hydrogenase partial amino acid sequences were evaluated. The results showed that members of the genera *Clostridium* and *Lactobacillus* predominated in thermally pretreated waste reactors with higher hydrogen yields. *C. butyricum* (46.93%) was predominant in reactors containing waste particles larger than 5 cm, while *Lactobacillus fermentum* (28.28%) was the most abundant species in reactors with waste particles smaller than 5 cm. Phylogenetic analysis revealed that the [FeFe]-hydrogenase of *C. butyricum* showed close similarity to those of *Clostridium saccharobutyricum*, *Clostridium* sp., and *Clostridium chromiireducens*. These findings provide new insights into hydrogen-producing microbial dynamics under different operating conditions and support the use of species-specific *hydA* gene characterization in mixed cultures.

Cite this article as: Vural C, Vural C, Azbar N, Ozdemir G. Investigation of microbial communities and [FeFe]-hydrogenase gene by different dry-dark fermentation processes on hydrogen production. Sigma J Eng Nat Sci 2026;44(3):1925–1939.

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This paper was recommended for publication in revised form by Editor-in-Chief Ahmet Selim Dalkilic

INTRODUCTION

Environmental pollution and diminishing energy resources are among the most important issues. Global warming and climate change have led to increased research into renewable energy technologies [1]. The development of these technologies is particularly important for the production of hydrogen with zero emissions and high energy efficiency [2,3]. Efficient biohydrogen production requires a better understanding of microbial processes. Advances in microbial ecology can contribute to the development of biotechnological approaches to clean and sustainable energy solutions in this field [4,5].

Biohydrogen is produced as a metabolic by-product by microorganisms under both phototrophic and dark fermentation conditions [6]. Biological methods such as direct and indirect biophotolysis, photofermentation, dark fermentation, hybrid systems and microbial fuel cells are used for its production [5]. Studies in the literature focus mainly on dark fermentation processes, in which microorganisms ferment organic matter to produce hydrogen gas [4]. This process is usually carried out using facultative (*Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii,* etc.) and obligate anaerobic bacteria (*C. butyricum*, *C. tyrobutyricum*, *C. pasteurianum*, *Ethanoligenens harbinense,* etc.) [7]. Specifically, *Clostridium* species are widely preferred in hydrogen production studies due to their high production yields and environmental stability[8].

One of the major challenges in H_2 production by dark fermentation is the presence of hydrogen-consuming microorganisms, such as methanogens, in the medium. To overcome this problem, most studies have applied heat pretreatment to inhibit non-spore-forming and hydrogen-consuming microorganisms. In the literature, heat pretreatment is usually performed by applying temperatures between 90°C and 100°C for periods ranging from 15 to 60 minutes. As a result of heat pretreatment, hydrogen-producing spore-forming species such as *Clostridium* are selected. However, it can be difficult to maintain the stability of microbial communities under operating conditions due to variables such as pH, substrate structure and temperature. For all these reasons, continuous monitoring of microbial changes is recommended to ensure sustainable hydrogen production and yield [9].

Both culture-dependent and culture-independent methods are used to characterize microbial communities. Culture-independent methods target the DNA and RNA of microorganisms, enabling the detection of a wide microbial spectrum and non-culturable species [10]. Recent studies have shown that Next Generation Sequencing (NGS) techniques enable the rapid and cost-effective identification of microbial communities in environmental samples [11]. These methods target the 16S rRNA gene region, which is found in most bacteria, allowing for the phylogenetic classification of bacterial species [12,13]. Arisht et al. [14] identified *Thermoanaerobacterium thermosaccharolyticum* as the dominant species producing biohydrogen in bacterial community study by NGS analysis and also identified bacteria belonging to the classes Clostridia, Bacilli, Bacteroidia, Thermoanaerobacteria, and Gammaproteobacteria.

In hydrogen production through dark fermentation, hydrogenase enzymes play a crucial metabolic role. [FeFe]-hydrogenases are found in anaerobic bacteria (e.g. *Clostridium*, *Thermotoga*) and some green algae and show 10 to 100 times higher activity compared to [NiFe]-hydrogenases [15–19]. The *hydA* gene encodes the catalytic site of the [FeFe]-hydrogenase enzyme and therefore the presence and distribution of the *hydA* gene can be used as a functional biomarker within microbial communities. However, detection of the *hydA* gene in mixed culture is often difficult and false results can be obtained due to alternative binding [16,20]. This calls for more extensive characterization of [FeFe]-hydrogenase in mixed culture.

In this study, the similarities and differences between microbial populations under different operating parameters in reactors producing hydrogen by dark-dry fermentation were investigated. The hydrogen-producing dark-dry fermentation reactor system, in which microbial changes were evaluated, stands out as an alternative dark fermentation method that has high solids loading capacity, can operate in smaller reactor volumes, and operates with minimal water requirements. This study has two main objectives. First, the effect of the particle size of the fermented substrate on the hydrogen yield was investigated. For this purpose, fermenters with substrates of different particle sizes were operated. Second, the effect of the removal of hydrogen-consuming microorganisms on hydrogen production was investigated [21]. Accordingly, the effect of heat pretreatment of fruit and vegetable wastes (FVWs) and particle size on microbial dynamics in three bioreactors operating under mesophilic conditions was evaluated. In addition, the presence and characterization of the *hydA* gene was analyzed in detail. The identification of the [FeFe]-hydrogenase enzyme with species-specific primers could provide a new contribution to the literature to understand its distribution in mixed cultures.

MATERIALS AND METHODS

Supply of Biomass Responsible for Hydrogen Production

Biomasses were obtained from a variety of H_2-producing reactors that were utilized in a previous study [21]. The reactor system and experimental setups employed in the previous study are outlined below. The reactor system comprised a 15 L percolation tank (PT) and a 55 L dry fermenter (DF). Experiments were conducted under mesophilic conditions at 37±2°C, with an inoculum/substrate ratio maintained at 1:1. A mini pump system was employed to regulate the pH, which was kept at approximately 8.45. FVWs were collected from the main hall of Izmir Municipality (Turkey) and utilized as the primary substrate for hydrogen production.

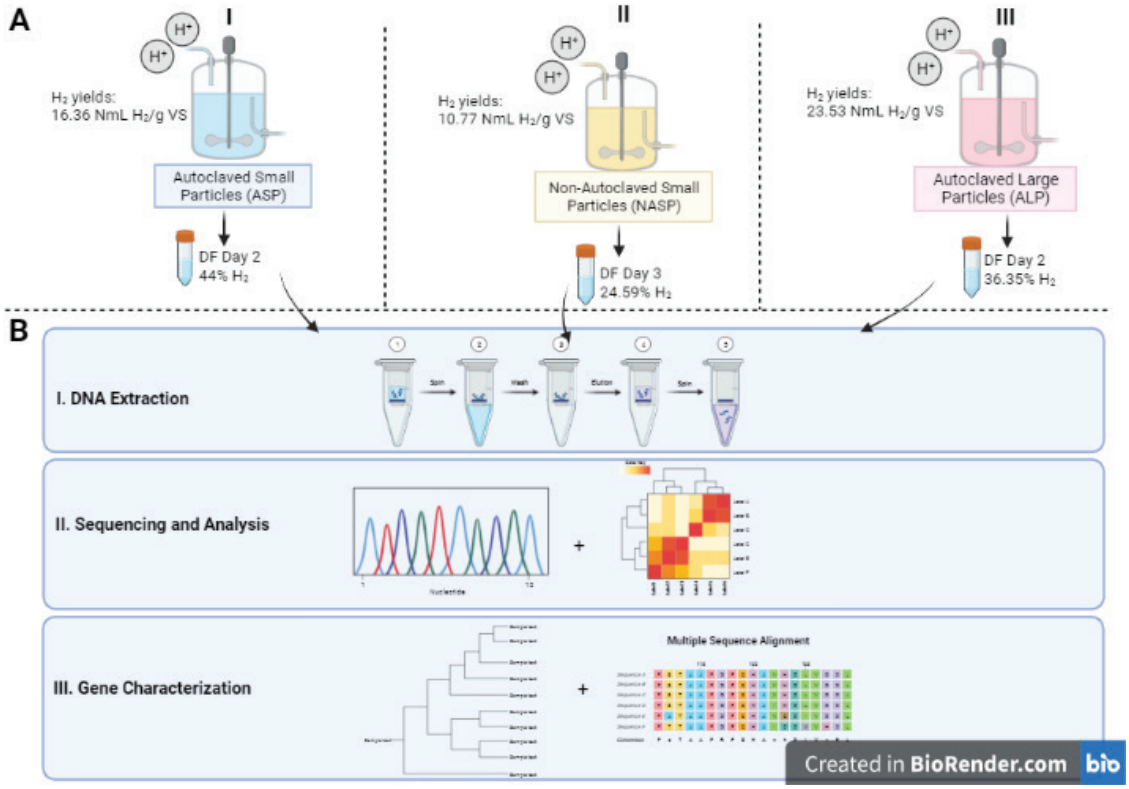

Figure 1. Sources of biomass and a brief summary of the study. A) Our previous work [21] in shows reactors, hydrogen yields, and days when the headspace gas percentage is maximum. B) Summary of the analyses performed in this study.

The inoculum was obtained from a biogas digester at a dairy plant and thermally pretreated at 105˚C for 10 minutes prior to use [21].

Three distinct dry fermentation experiments were conducted to investigate the effects of particle size, autoclaving, and substrate conditions on hydrogen production:

1. *Autoclaved Small Particles (ASP):* The FVWs were chopped into particles smaller than 5 cm and then autoclaved at 121 °C and 15 psi (100 kPa) for 20 minutes. H_2 yield: 16.36 NmL H_2/g VS, the maximum percentage of hydrogen in the headspace was 44% in the DF.
2. *Non-Autoclaved Small Particles (NASP):* The FVWs were chopped into particles smaller than 5 cm but not autoclaved. H_2 yield: 10.77 NmL H_2/g VS, the maximum percentage of hydrogen in the headspace was 24.59% in the DF.
3. *Autoclaved-Large Particles (ALP):* The FVWs were chopped into particles measuring more than 5 cm and autoclaved under the same conditions as ASP (121˚C, 15 psi, 20 min). H_2 yield: 23.53 NmL H_2/g VS, the maximum percentage of hydrogen in the headspace was 36.35% in the DF.

All the FVWs were pre-chopped and ground to achieve target particle sizes before autoclaving. During fermentation, temperature, pH, and biogas production were monitored regularly to assess the effect of substrate conditions on hydrogen yield. A schematic representation of the experimental setup is depicted in Figure 1. Further details concerning the configuration of the reactor and the operational conditions can be found in [21].

During the operation of the dry fermentation system, 50 mL samples were collected in sterile falcon tubes from the reactors on the first day, the day of maximum H_2 production, and the day of the end of H_2 production for each new parameter experiment. They were stored at -20˚C for molecular analysis.

Total Genomic DNA Extraction

Total genomic DNA extraction samples were taken from the DF when the maximum percentage of H_2 was in the headspace (ASP – Day 2, NASP – Day 3, and ALP – Day 2). Prior to genomic DNA extraction, samples were allowed to lyse in a sterile environment at room temperature. They were then sonicated to ensure a homogeneous mixture. Homogenization was performed using an ultrasonic homogenizer (Bandelin SONOPULS™ GM 2070, Germany). The process was applied to each sample at 90% power for 10 seconds with 3 cycles in cooling ice. Homogenized samples were then subjected to DNA extraction using the ZR Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's instructions. Extracted DNA samples were then stored at -20˚C until use.

Library Preparation

For 16S rRNA gene sequencing, the V3-V4 regions were determined using universal primer sets (Table 1). Two-step PCR was performed using KAPA HiFi HotStart ReadyMix from Roche GmBH. The first PCR step consisted of 25 cycles with initial denaturation at 95°C for 3 min, amplification step at 95˚C for 30 s, 55˚C for 30 s, 72˚C for 30 s, and cooling step at 72˚C for 5 min for one cycle. In the second PCR step, 8 cycles were performed using the Illumina Nextera XT Index Primer 1 and Nextera XT Index Primer 2 sets; initial denaturation was 3 minutes at 95˚C, amplification step was 30 seconds at 95˚C, 30 seconds at 55˚C, 30 seconds at 72˚C, 30 seconds at 72˚C, and cooling step for one cycle was 5 minutes at 72˚C. Purification was performed using the Beckman Coulter™ Agencourt AMPure XP kit. The Illumina 16S Metagenomic Sequencing Library was then used for sequencing library preparation.

16S rRNA Gene Sequencing

Sequencing analysis was performed on Illumina's iSeq 100 next-generation sequencing system. This system includes the iSeq 100 i1 reagent kit and 2x150 base pair paired-end reads and was processed according to the manufacturer's protocols. After sequencing, bioinformatic analysis and quality control steps were performed.

As part of the sequencing protocol, FastQC version 0.11.9 software was used to ensure data quality. In the quality assessment process, low-quality reads in the data set (a Phred score below Q20 within 30 base pairs) were eliminated. In addition, low quality base calls, potential adapter contamination and chimeric sequences at the ends of reads were cleaned using Trimmomatic according to the Genomes OnLine Database (GOLD) guidelines.

For taxonomic profiling, reads were aligned to target organisms using the Ribosomal Database Project (RDP) Classifier based on data in the Greengenes database [23]. Operational Taxonomic Unit (OTU) groups were then defined for each sample after alignment.

Characterization of the *hydA*

The *hydA* gene ($hydA_{cbutyricum}$) was characterized due to the dominance of *C. butyricum* species in the ASP – Day 2, NASP – Day 3, and ALP – Day 2 samples. The full genome of the $hydA_{cbutyricum}$ was searched in the National Center for Biotechnology Information (NCBI) and the Universal Protein Resource (UniProt) databases and the nucleotide and amino acid sequences were obtained. As a result of multiple alignment of nucleic acid sequences using the MEGA-X database, species-specific primers were designed using the Primer3 database (Table 2). These designed primers are 1648 bp lenght. The *hydA* primers verified using DNA from pure *C. butyricum* culture. For this purpose, the *C. butyricum* strain (DSM-10702) was purchased from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH (Germany). It was incubated in Differential Reinforced Clostridial Medium (DRCM) at 37˚C.

The reaction mixture was prepared using Xpert Fast Hotstart Mastermix (2x) with Dye (Grisp, Portugal) according to the manufacturer's instructions. PCR conditions were set up for 40 cycles; the initial denaturation at 95˚C for 3 min, the amplification stage was at 95˚C for 30 sec, at 54˚C for 15 sec, and at 72˚C for 25 sec, cooling stage for one cycle at 72˚C for 3 min, respectively.

Bioinformatic Analysis of the *hydA*

After amplification, the nucleic acids were sequenced in both directions. Sequences were cleaned using DNA Dragon and BioEdit. The NCBI Blastx database was then searched to transform amino acid sequences and determine similarities. Sequences between 70% and 100% similarity were selected and obtained in FASTA formats and multiple alignments were performed using the MEGA-X program. Phylogenetic trees were then constructed using the maximum likelihood method with the DNAMAN 6.0 program. The maximum likelihood statistical method and the bootstrap phylogeny test method with 1000 replicates were used to construct the phylogenetic tree.

Table 1. The universal primer set

Primers	Sequence (5' – 3')	Reference
16S-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	[22]
16S-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC	

Table 2. Primer set used for characterization of the $hydA_{cbutyricum}$

Primers	Sequence
B-HYDAF	5' – GGTACATCTATCATTCTAGCTGCA– 3'
B-HYDAR	5' – GGGCTTTATGTTGTCCTGGCAT– 3'

Multiple alignments of the amino acid sequences of *hydA* with the phylogenetically most closely related *Clostridium* species for $hydA_{cbutyricum}$ and the *Clostridium* species found in the reactors according to the NGS analysis results were performed using the BioEdit database. In addition, peptide similarities were searched using the UniProt database to identify conserved regions in amino acid sequences and closely related species. This search using the UniProt database (https://www.uniprot.org/peptidesearch/) provided detailed information on peptide similarities and conserved regions.

RESULTS AND DISCUSSION

Changes in Microbial Structure in Bioreactors

NGS analyses were performed to determine changes in microbial profiles in reactor samples operating at different operational parameters (Fig. 2). As a result, a total of 50 phyla and 1350 genera were identified in all samples. The graph in Figure 2 shows the phylum distribution according to their relative densities, with the top 30 phyla having the highest proportion. The phylum Firmicutes was found to be dominant in all samples. While the proportion of Firmicutes phylum was 54.15% in the inoculum, it increased to 81.44% in the NASP - Day 3 sample, 90.91% in the ASP - Day 2 sample, and 97.44% in the ALP - Day 2 sample. Firmicutes phylum was dominant in the inoculum followed by Bacteroidetes (15.41%) and Proteobacteria (11.14%). However, these phyla were detected at low abundance in H_2-producing reactors.

Figure 3 shows the heat map showing the distribution of the 30 genera with the highest density. *Hydrogenispora* (14.17%) was the most abundant genus in the inoculum. In the ASP - Day 2 sample, the dominant genus was *Lactobacillus*, in the NASP - Day 3 sample *Anaerostipes* was the dominant genus, and in the ALP - Day 2 sample, the dominant genus was *Clostridium* sensu stricto (Fig. 3).

The differences and similarities in the microbial profiles of the three DFs in which the effect of particle size and heat pre-treatment under mesophilic conditions were investigated are discussed in the graph in Figure 4 together with the H_2 production amounts. *Hydrogenispora* was the most abundant genus with 14.17% in the inoculum, but decreased below detection limits with increasing H_2 production in the reactors. In the reactor samples with high H_2 production (ASP - Day 2 and ALP - Day 2), the predominant genera are *Clostridium* and *Lactobacillus*. In the ASP - Day 2 sample, the dominant genera were *Lactobacillus* (29.75%), *Clostridium* IV (18.2%), and *Clostridium* sensu stricto (17.59%). In the NASP - Day 3 sample, *Anaerostipes* (11.97%) was the dominant genus. Finally, in the ALP - Day 2 sample, the dominant genera were *Clostridium* sensu stricto (30.2%), *Lactobacillus* (23.2%), *Lysinibacillus* (16.8%), and *Solibacillus* (15.11%) (Fig. 4). In contrast to the ASP - Day 2 reactor sample, *Bacillus*-like genera are more prevalent in the microbial profile of the ALP - Day 2 sample. The only difference between these two reactors is the size of the substrates. FVWs sizes were obtained by passing through a shredder to be <5 cm in ASP, and it was cut with a knife to be >5 cm in ALP and loaded into a DFs. Regarding the effect of particle size, it is seen that higher H_2 yields are obtained with particles larger than 5 cm. A yield of 16.36 NmL H_2/g VS was obtained from the ASP with particles smaller than 5 cm, while a yield of 23.53 NmL H_2/g VS was observed in the ALP with larger particles. This

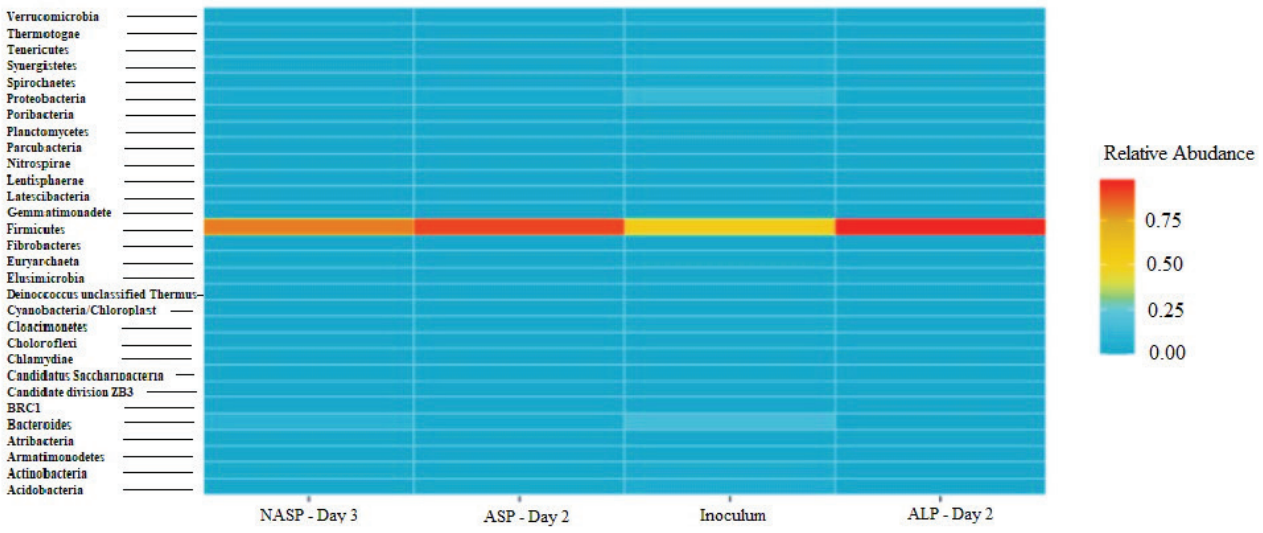

Figure 2. Relative abundance of OTUs at the phylum level in the microbiotas from inoculum and reactors operated under different conditions.

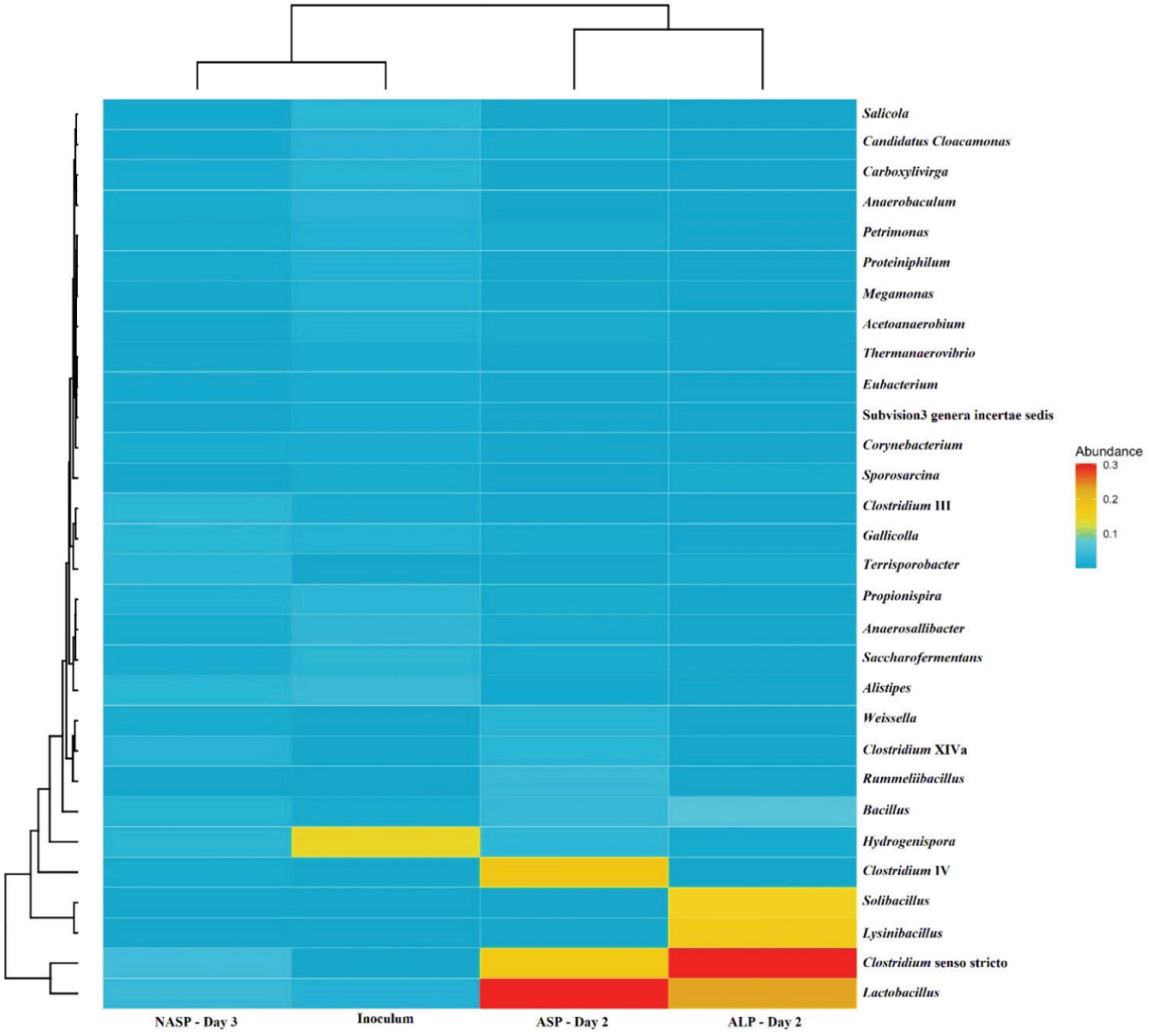

Figure 3. Relative abundance of OTUs at the genus level in the microbiotas from inoculum and reactors operated under different conditions.

difference is believed to be due to the excessive amount of water released from FVWs during their disintegration and thermal pre-treatment. The FVWs juice released during the thermal pre-treatment of large particulate waste contains a high proportion of simple sugars, making it easily degradable and therefore a source of high H_2 production efficiency. Considering the maximum percentages of H_2 in the headspace of the DF, 44% of H_2 was observed as produced gas in the ASP - Day 2 while 36.35% of H_2 was detected in the ALP - Day 2. Overall, it was observed that the higher H_2 yield in the ALP reactor and the use of particles larger than 5 cm in terms of energy consumed during fractionation resulted in higher production in the dry fermentation system [21].

The results showed that the inoculum used in the DF reactors exhibited significant microbial diversity. The predominant genus *Hydrogenispora* in the inoculum belongs to the phylum Firmicutes and includes obligate anaerobic, non-motile, spore-forming bacilli. They can ferment various sugar molecules but are unable to ferment amino acids or aromatic compounds. Genera such as *Clostridium* senso stricto (1.09%), *Bacillus* (1%), and *Lactobacillus* (1.91%), which were found in relatively low abundance in the inoculum, were detected with high intensity in the reactor samples by NGS analysis (Fig. 5). It was observed that the microbial diversity of the inoculum and the NASP Day 3 sample could be relatively similar. These two samples were not heat pretreated. In addition, lower H_2 production efficiency was observed in the NASP reactor compared to the other reactors. It can be assumed that H_2 consuming

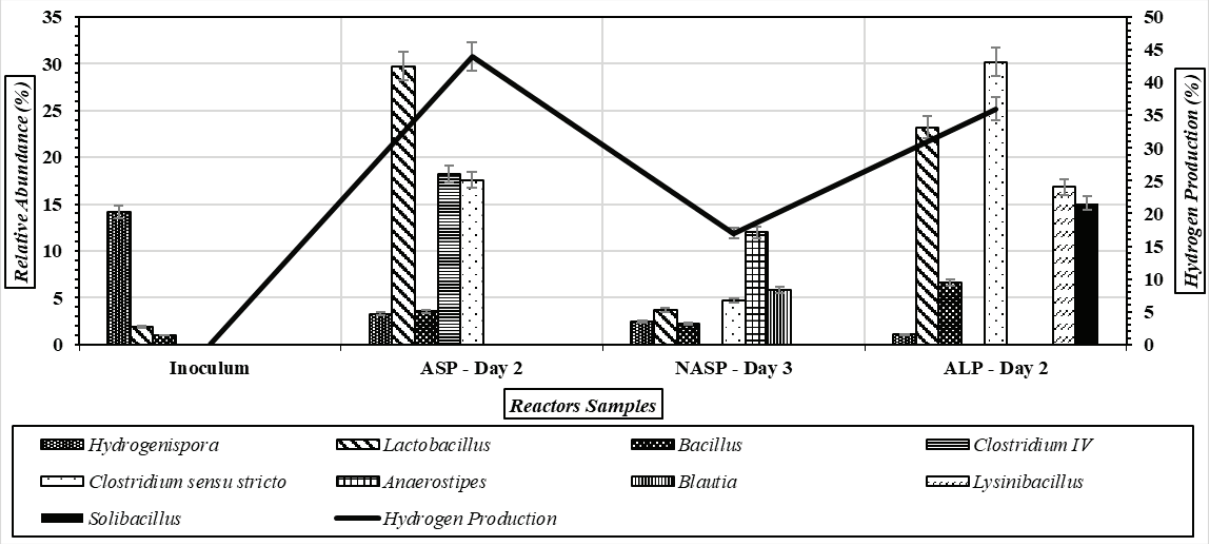

Figure 4. Comparison of Dominant Genera and H_2 Production.

microorganisms are the biggest problem during H_2 production. Therefore, the removal of these groups of microorganisms is crucial for a high production yield. Successful removal of these microorganisms has been achieved by heat pre-treatment in H_2 production by mixed culture [24]. Some studies have used the boiling method for this purpose while others have eliminated potential H_2 consuming microorganisms using the drying technique [25–28]. Within this concept, our heat pre-treatment applications show similar results to the literature. In this study, inoculum pre-treated at 105°C for 10 min. and FVWs were autoclaved at 120°C and 15 psi (100 kPa) for 20 min. in ASP and ALP reactors, where H_2 yields were high.

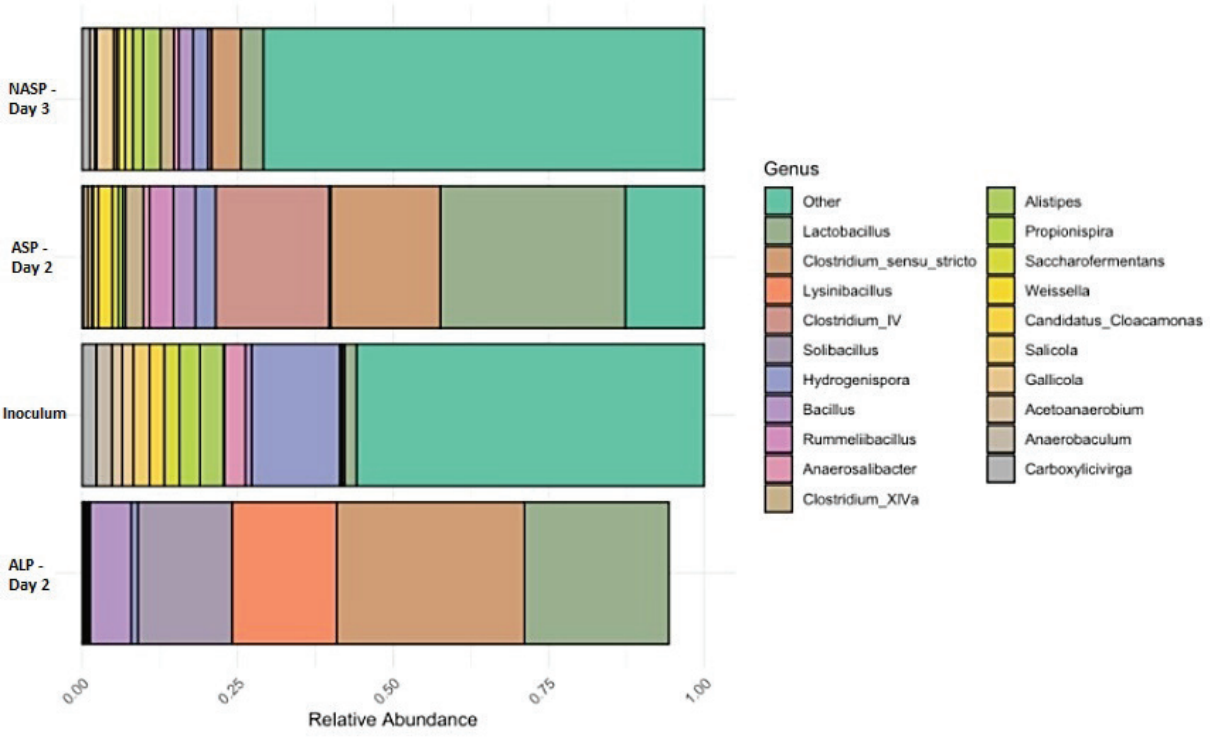

Figure 5. The relative abundance graph of genera. The bar graph presents the top 20 bacterial genera observed in all samples. Genera detected in less abundance than the top 20 are grouped as "Others".

It is thought that the high H_2 production in the ASP - Day 2 and ALP - Day 2 reactor samples could be due to the action of *Clostridium* IV and *Clostridium* sensu stricto genera. In the literature, the *Clostridium* group, known for its high efficiency in H_2 production is referred to as *Clostridium* cluster I. [29]. Zagrodnik et al., [30] reported the results of H_2 production from crude cellulose by dark fermentation. It was found that the dominant species from *Clostridium* sensu stricto 1 group in the reactor where 2.14 L H_2/L H_2 production was detected with mixed culture. Yang and Wang [31] investigated in detail the changes in the microbial profile during the dark fermentation process using high-throughput pyrosequencing. In the study, the genera *Clostridium* sensu stricto 1, *Paraclostridium*, *Romboutsia,* and *Paeniclostridium*, which were almost absent in the inoculum, became dominant after 6 h of fermentation. They reported that the genus *Clostridium* sensu stricto 1 played a key role in H_2 production.

According to the results of the NGS analysis, high amounts of *Lactobacillus* genus was detected in the DFs. The inoculum used in the DFs was taken from a fermenter in a milk production plant. *Lactobacillus* genus is a group of microorganisms commonly found in dairy products. Therefore, considering that the inoculum used in the study was obtained from a dairy fermenter, *Lactobacillus* genus is expected to be present at a certain population density. Higher H_2 production was observed in heat pre-treated ASP and ALP despite the presence of the *Lactobacillus* genus. The abundance of the *Lactobacillus* genus increased from 1.91% in the inoculum to 29.75% in ASP - Day 2, 3.68% in NASP - Day 3 and 23.20% in ALP - Day 2. On ASP - Day 2 and ALP - Day 2, the abundance of the genus *Lactobacillus* and the genus *Clostridium* increased as DF increased H_2 production. On NASP - Day 3, the abundance of these two genera increased over time. However, this increase was not as significant as on other days. One study reported that thermal pretreatment of the substrate/inoculum between 50°C and 90°C inhibited lactic acid bacteria (LAB) growth [32]. However, another study showed that inoculum/substrate pretreatment did not always inhibit lactic acid bacteria growth. Although sunflower stalk hydrolysate was pretreated with combined heat and acid prior to H_2 production, this treatment was not effective in suppressing (LAB) [33].

It is well known that the *Clostridium* group is the best H_2 producer in dark fermentation. But for the *Lactobacillus*/LAB group, the situation is a bit more complicated. It is also known that the LAB generally consists of nonsporulating aerotolerant anaerobic bacteria. How LAB could survive in the heat pre-treated environment and how LAB influences H_2 production in an environment dominated by Clostridial cells can be considered issues that need to be elucidated. In the literature, two opposing views regarding LAB have been presented in studies or reviews where H_2 production is performed by mixed culture in dark fermentation. The first view is that the presence of LAB inhibits H_2 production by suppressing H_2-producing microorganisms through substrate competition or the release of competing molecules such as bacteriocin. Secondly, the presence of LAB has been shown to stabilize the pH and provide anaerobic conditions by consuming oxygen in the environment. Furthermore, some *Clostridium* species can consume lactate, the main metabolic product of LAB, and promote H_2 production in butyrate conversion [4]. Specifically, *C. butyricum*, *C. beijerinckii*, *C. felsineum*, and *C. pasteurianum* yielded the highest H_2 production in pure cultures or co-cultures when lactate was the dominant metabolite in starch fermentation. Conversely, one of the significant disadvantages in H_2 production is the decrease in pH in the medium due to the synthesis of organic molecules such as lactic, acetic and propionic acids by LAB [34]. Gomes et al. [35] used heat-treated cassava meal wastewater as a substrate and noted that the initial pH of 6.50 dropped to a final value between 3.17 and 4.27, which may have been due to inhibition of hydrogen production by LABs due to lactic acid and bacteriocin production. Conversely, Perez-Rangel et al. [36] observed the positive outcome of a synergistic interaction involving cross-feeding of lactate and acetate in a system composed of *Clostridium* and LABs. In this study, based on findings in the literature, the pH was maintained at 5.5 to reduce acidification triggered by LAB activity. This regulatory approach likely averted the accumulation of excessive lactic acid, thereby enabling *Clostridium* species to utilize lactate without the presence of inhibitory conditions.

LAB are reported to contribute to global H_2 production through different mechanisms [37]. Ziara et al. [38] obtained the highest hydrogen yield of 0.85 mol H_2/mol lactate from lactate wastewater at 45°C and initial pH of 8.5, with the genera *Clostridium*, *Sporanaerobacter* and *Pseudomonas* dominating in the reactor. Ordonez-Frias et al. [39] emphasized that there was a positive relationship between *Lactobacillus* and *Clostridium* genera in the study in which they realized hydrogen production with a yield of 3.2 L H_2/L-day by lactate fermentation. In this study, it was observed that the presence of LAB in ASP-Day 2 and ALP-Day 2 coincided with the increased H_2 production. This suggests a possible syntrophic interaction where lactate produced by LAB acts as an electron donor for *Clostridium* species, facilitating H_2 production.

In another study, it was determined that the Clostridia and LAB ratios were approximately 2:1 and 1:2, respectively, in the medium that yielded maximum H_2 from a mixed culture with sugar beet molasses. However, the dominance of either species led to a decrease in H_2 production [40]. In this study, the ratios of Clostridia and *Lactobacillus* were determined as follows: ASP - day 2:1:1 (equal ratio, high H_2 production), NASP - day 3:7:1 (Clostridia dominant, low H_2 production) and ALP - day 2:1:2 (*Lactobacillus* dominant, low H_2 production). The result shows that the balance between *Lactobacillus* and *Clostridium* is critical for H_2 production. The 1:1 ratio suggests that lactate produced by LAB is efficiently utilized by *Clostridium* species, preventing pH

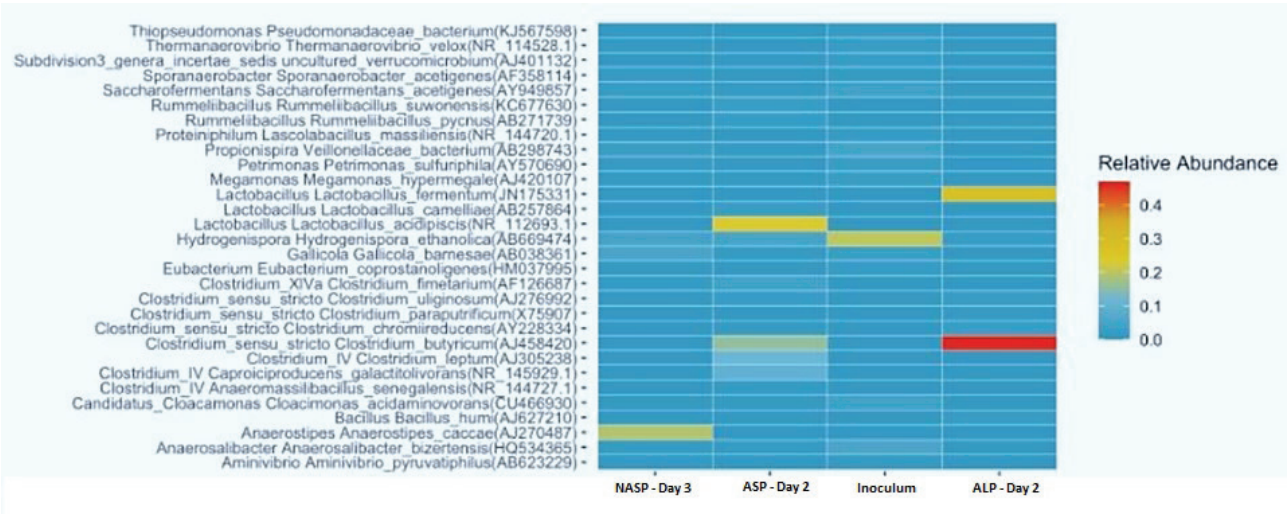

Figure 6. Species relative abundance graph. A bar graph presenting the top 30 bacterial species observed in all samples.

drop by promoting electron transfer and ensuring optimal metabolic interactions. In contrast, in cases where LAB are over-dominated (e.g. ALP - 1:2 ratio on day 2), excess lactate accumulation may inhibit H_2 production.

NGS analysis identified a total of 2475 species across all samples. The top 30 species with the highest abundance are shown in Figure 6. In the inoculum sample, *Hydrogenispora ethanolica* (AB669474) from the genus *Hydrogenispora* was the most abundant species at 20%. In the ASP - Day 2 reactor sample, *L. acidipiscis* (NR 112693.1) represented 23.96%, *C. butyricum* (AJ458420) 15.72%, and *C. leptum* (AJ305238) 11.29% of the microbial profile. In the NASP - Day 3 reactor sample, *Anaerostipes caccae* (AJ270487) from the genus *Anaerostipes* was the most abundant species with 19.19%. Finally, in the ALP - Day 2 reactor sample, *C. butyricum* (AJ458420) accounted for 46.93% and *L. fermentum* (JN175331) for 28.28% of the microbial profile.

It was observed that *C. butyricum* was generally responsible for H_2 production in ASP and ALP reactors and coexisted with *Lactobacillus.* LAB can produce lactic acid during homo- or heterofermentative metabolism [41]. While homofermentative bacteria produce only lactic acid as the primary by-product of glucose fermentation, heterofermentative bacteria can produce other compounds such as ethanol, propionic acid, acetic acid, and CO_2 in addition to lactic acid [42]. Park et al. [43] reported an increase in H_2 production efficiency from 1.57 mol H_2/mol glucose to 1.84 mol H_2/mol glucose by co-culturing *Sporolactobacillus vineae* and *C. butyricum*, contributing to the understanding of the synergistic mechanisms of both strains, and found that *C. butyricum* constituted 90% of the microbial composition. They could not explain the difference in H_2 production with *S. vineae* alone. However, they reported that although *S. vineae* alone did not produce butyrate, it increased the expression of genes related to lactate, butyrate, and H_2 pathways when cultured together with *C. butyricum* [43]. In another study examining the effects of 249 selected LAB strains on the growth of *C. butyricum*, it was reported that of these 24 strains did not inhibit growth [44]. In this study, the authors observed that among these 24 strains, 4, specifically *L. brevis* JL16 and *L. parabucheri* MH44, doubled the growth of *C. butyricum*.

C. butyricum is an anaerobic acidogenic bacterium and is efficient at producing H_2 from organic substrates, especially carbohydrates [45]. It can produce lactate, butyrate, acetate, ethanol and CO_2 as the main by-products of glucose utilization. The conversion of pyruvate to lactate, butyrate, and ethanol involves the oxidation of NADH [46]. The results of NGS analysis and gene characterization performed in this study show that H_2 production of high amounts of *C. butyricum* occurs via the butyrate pathway. The presence of *Lactobacillus* in reactors with high H_2 production suggests that the presence of *Lactobacillus* further promotes H_2 production by *C. butyricum* using lactate. This suggests that synergistic effects play an important role in H_2 production.

Characterization and Analysis Results of *hydA* Gene

Based on the species level percentile results of the NGS analyses, *C. butyricum* was highly abundant in the samples from the ASP - Day 2 and the ALP - Day 2 reactors. Therefore, the presence of the *hydA* gene of *C. butyricum* was analyzed by conventional PCR in the samples of inoculum, ASP – Day 2, NASP – Day 3, and ALP – Day 2 samples (Fig. 7). As a result of PCR analysis, inoculum shows that the $hydA_{cbutyricum}$ is absent. A negative result was obtained in the NASP – Day 3 reactor sample. The $hydA_{cbutyricum}$ was detected in the samples from the ASP – Day 2 and ALP – Day 2. H_2 was produced at the highest rates in these two reactor samples containing the *hydA* gene of *C. butyricum*.

The phylogenetic tree of the $hydA_{cbutyricum}$ detected in ASP - Day 2 and ALP - Day 2 reactor samples shows their

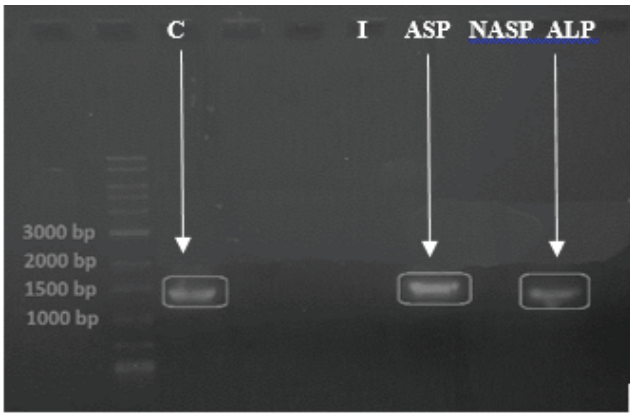

Figure 7. The gel electrophoresis image shows the results of PCR analysis using primers specific for the *hydA* gene. The first band C was amplified from a pure *C. butyricum* culture as a positive control.

closeness and distance in the 100%-70% similarity range. In these reactor samples, the $hydA_{cbutyricum}$ is 100% similar and 96% to the hydrogenase gene of *C. butyricum* E4 strain BONT E BL5262. Other close similarities include *C. saccharobutyricum*, *Clostridium* sp., and the *hydA* gene of *C. chromiireducens*. *C. baratii* strains Sullivan and *C. vincentii* are closely related to the *hydA* gene of the *C. paraputrificum* and are located on the same branch. In addition, these organisms are very similar to the *hydA* gene of *C. butyricum*. *C. oryzae* and *C. acidisoli* are on the same branch as *C. pasteurianum* in the phylogenetic tree (Fig. 8).

Figure 9 shows the similarities and differences between the *hydA* gene amino acid sequences of ASP - Day 2 and ALP - Day 2 reactor samples with other *Clostridium* species. As a result of the alignment of multiple amino acid sequences, the reactor samples were found to be 100% similar to each other and to $hydA_{cbutyricum}$.

The $hydA_{cbutyricum}$ genes of these reactor samples show 81.5%, 79.5%, 78%, and 75.7% similarity to the *hydA* genes of *C. saccharobutylicum, C. chromiireducens, C. baratii,* and *C. paraputrificum*, respectively. It is observed that *C. butyricum* has amino acid sequences almost in common with the conserved region of the *hydA* gene and with *C. saccharobutylicum* and *C. paraputrificum* species. However, it shows shifts in the amino acid sequences of *C. chromiireducens* and *C. baratii*.

The *hydA* gene can have different substitutions at different conserved sites in *Clostridium* species. Therefore,

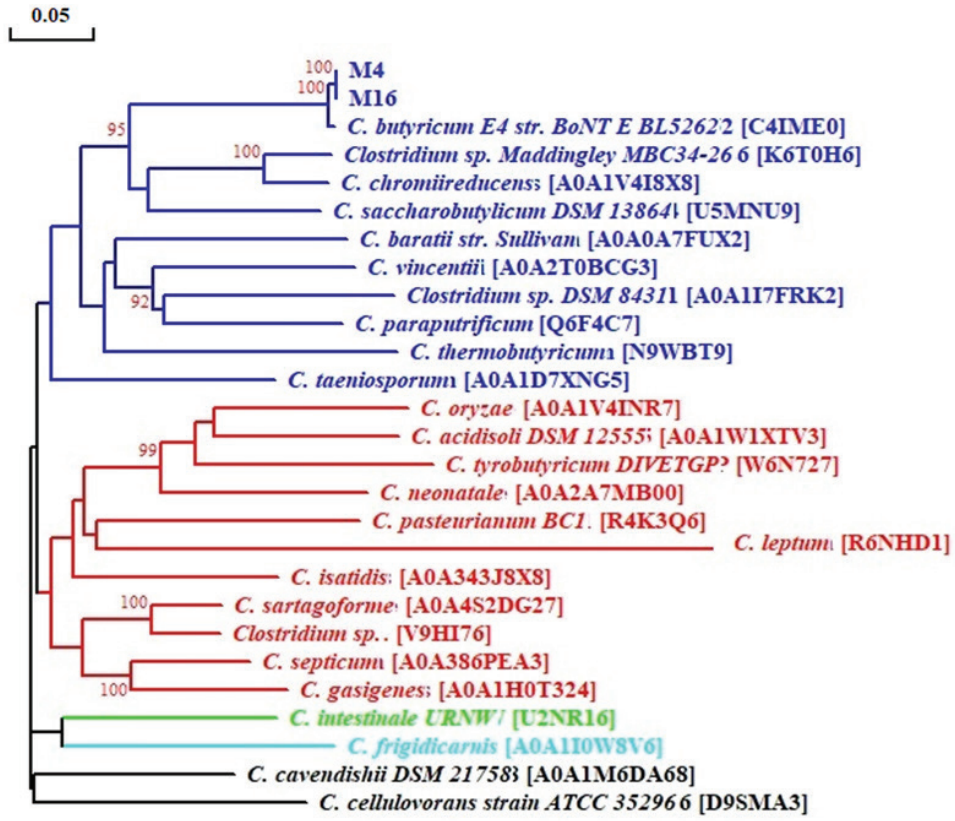

Figure 8. Phylogenetic tree constructed from amino acid sequences showing similarities and relationships of the $hydA_{cbutyricum}$ in the reactors samples of ASP – Day 2 and ALP – Day 2.

a separate primer set was designed for the species-specific *hydA* gene region. However, it has been reported in the literature that a single universal primer pair targeting conserved regions is usually used to study *hydA* diversity in environmental samples. In this case, differential binding may occur in mixed cultures [20]. For example, Boyd et al. [47] reported using a universal primer set of approximately 500 bp to identify hydrogenase diversity in the microbial community. As a result, they found that hydrogenase sequences were generally similar to Firmicutes, but not closely similar to *Clostridium* sp. Using a similar approach, Schmidt et al. reported that they designed a universal *hydA* primer pair targeting highly conserved regions to detect all hydrogenase diversity in environmental samples. However, it was emphasized that different *hydA* genes were amplified in the study, confirming the presence of novel hydrogenases. Based on studies in the literature, a large diversity of *hydA* can be found within conserved regions. However, it is unclear whether this diversity has been fully explored. Some studies have examined this gene only in pure cultures and assessed phylogenetic relationships using partial sequences. For example, Zhao et al. [19] evaluated the phylogenetic relationships of the *hydA* gene from *C. guangxiense* ZGM211T and *C. neuense* G1T strains with the partial sequences of this gene. They concluded that these strains were highly related to each other and represented new *Clostridium* species. In another study, ten pairs of different *hydA* cluster-specific degenerate primers were designed and used to detect the diversity of *hydA*. Thus, various hydrogenase genes were discovered in H_2-producing species *C. sartagoforme*, *C. felsineum*, *C. roseum*, and *C. pasteurianum* [20].

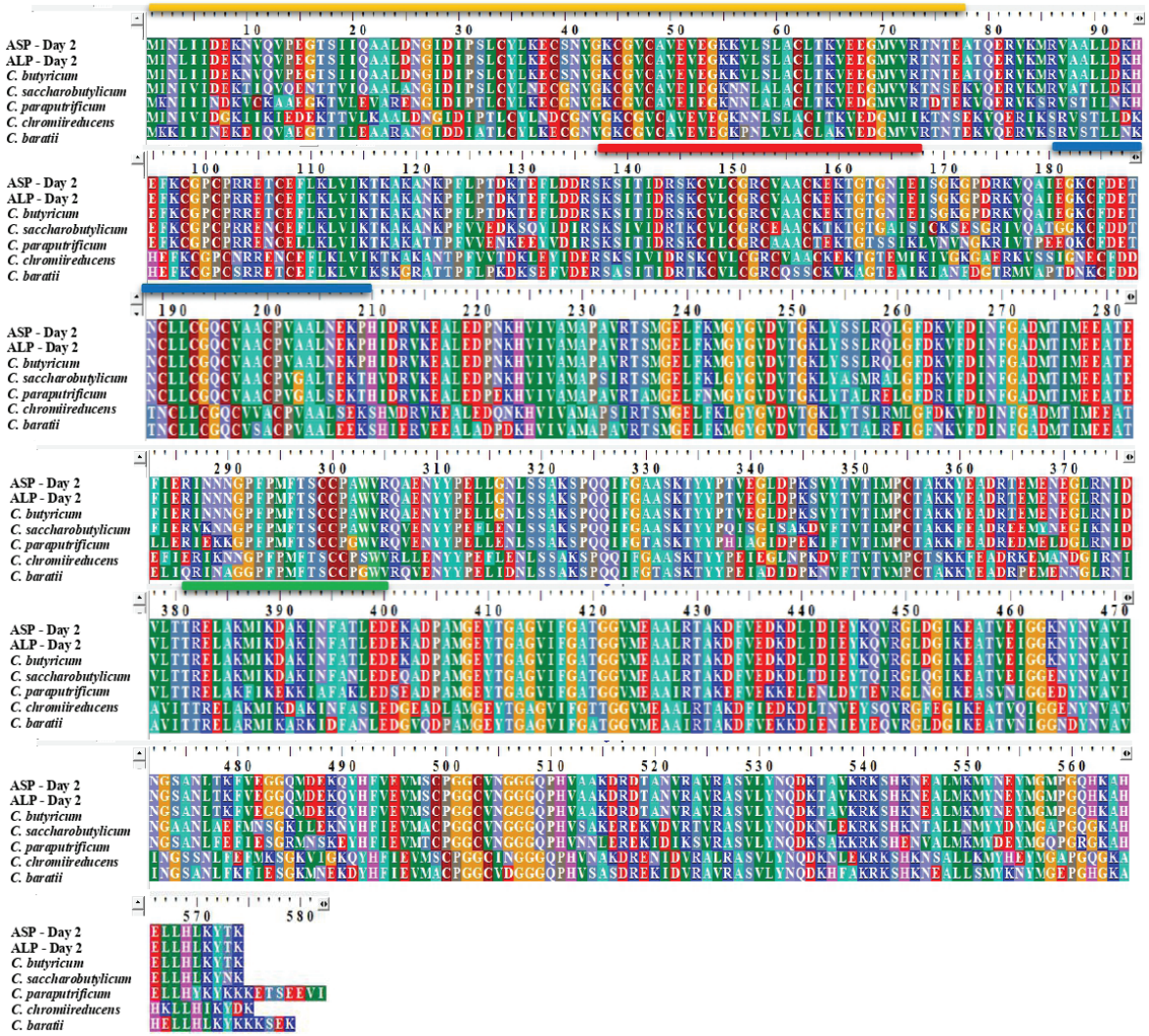

Figure 9. Multiple alignment of the amino acid sequences of the $hydA_{cbutyricum}$ with closely related *Clostridium* species. The $hydA_{cbutyricum}$ was found to contain 3 distinct domains: the 2Fe-2S ferredoxin type cluster marked with a yellow bar at positions 1 – 120, the 4Fe-4S ferredoxin type cluster marked with a red bar between 138-167, and the blue bar at positions 181-210. The 4Fe-4S ferredoxin type cluster marked with a bar, the L1 motif indicated by the green bar at positions 381 – 401.

The efficient functioning of the *C. butyricum* hydrogenase contributed to the high H_2 yields in the ASP and ALP reactors. Identifying the similarities between the *hydA* gene of *C. butyricum* and *hydA*s of other *Clostridium* species will provide an important basis for future studies aimed at increasing H_2 yields. Understanding these similarities may help to better understand the genetic and biochemical mechanisms involved in H_2 production and to develop strategies to improve the efficiency of H_2 production. Therefore, an in-depth study of the structural and functional characteristics of the hydrogenase genes of *C. butyricum* and other H_2-producing organisms could be an important step towards the sustainable production of biofuels.

CONCLUSION

Hydrogen is a by-product of the metabolic pathways of many microorganisms with a wide taxonomic distribution. In the rapidly advancing field of microbial biotechnology, the identification and quantification of potential hydrogen-producing microorganisms is crucial for optimizing hydrogen yield. This study presents a comprehensive microbial profile analysis of biohydrogen production from FVWs under mesophilic conditions via dark fermentation in three different reactor trials. In reactor samples where maximum H_2 production occurred, *Clostridium* and *Lactobacillus* genera were observed to be dominant, and *C. butyricum* was identified as a significant hydrogen-producing *Clostridium* species.

The study also revealed the significant effect of heat-pretreatment on microbial communities. An increase in hydrogen-producing bacteria was observed in reactors inoculated with heat-pretreated FVWs. However, despite pretreatment, *Lactobacillus* species remained dominant in the system. This finding highlights the need for further research into the role of *Lactobacillus* in hydrogen fermentation systems.

Characterization of the *hydA* gene, which plays a crucial role in biohydrogen production by dark fermentation, has contributed a novel perspective. Unlike the general primer sets used in previous studies in the literature, species-specific primers targeting the *hydA* gene of *C. butyricum* were designed in this study. This will allow for a more precise analysis of hydrogenase gene distribution in mixed cultures, offering a new and effective perspective for studies using mixed cultures. The successful amplification of the *hydA* gene of [FeFe]-hydrogenase and the evaluation of its phylogenetic relationships using species-specific primers demonstrates its potential as a molecular tool for identifying different enzyme forms.

A better understanding of species-specific *hydA* metabolism is crucial for developing efficient hydrogen production systems. Furthermore, newly characterized *hydA* genes or genetically engineered variants may have potential applications as electrocatalysts in biofuel cells or as biosensors for hydrogen detection. Future studies focusing on genetic modifications and microbial interactions could further enhance the efficiency of biohydrogen systems and expand their applicability in sustainable energy technologies.

ABBREVIATIONS

NGS	Next Generation Sequencing
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
NCBI	The National Center for Biotechnology Information
UniProt	The Universal Protein Resource
DF	Dry Fermenter
hydA	[FeFe]- hydrogenase
hydAcbutyricum	[FeFe]- hydrogenase of *Clostridium butyricum*
LAB	Lactic Acid Bacteria
ASP – Day 2	Autoclaved Small Particles – Dry Fermenter Sample of Day 2
NASP – Day 3	Non-Autoclaved Small Particles – Dry Fermenter Sample of Day 3
ALP – Day 2	Autoclaved Large Particles – Dry Fermenter Sample of Day 2
FVWs	Fruit and Vegetable Wastes

ACKNOWLEDGMENTS

The authors wish to thank the project TUBITAK-MAG-215 M 314 from the Scientific and Technological Research Council of Turkey. The authors gratefully acknowledge the YÖK 100/2000 Doctoral Scholarship Programs from the Council of Higher Education and TÜBİTAK Priority Areas Doctoral Scholarship Programs from the Scientific and Technological Research Council of Turkey, which provided significant financial support in the realization of this study.

AUTHORSHIP CONTRIBUTIONS

Cansu Vural: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Caner Vural:** Methodology and Writing – original draft. **Nuri Azbar:** Project administration and Writing – review & editing. **Güven Ozdemir:** Methodology, Supervision, Writing – review & editing.

DATA AVAILABILITY

Data will be made available on request.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS

There are no ethical issues with the publication of this manuscript.

STATEMENT ON THE USE OF ARTIFICIAL INTELLIGENCE

Artificial intelligence was not used in the preparation of the article.

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