

Article

Voltage-Driven Regulation of Metabolic Flux and Biohydrogen Production in a Dynamic Membrane Bioreactor Coupled with Electro-Fermentation

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Abstract

Dynamic membrane bioreactors (DMBRs) are promising systems for continuous biohydrogen production because they enable effective biomass retention under short hydraulic retention time (HRT) conditions. In this study, a dynamic membrane bioreactor coupled with electro-fermentation (DMBR-EF) was operated for 59 days to investigate the effect of applied voltage on biohydrogen production and metabolic flux regulation. The reactor was sequentially operated at 0 (no applied voltage), 0.2, 0.4, 0.6, 0.8, and 1.0 V using glucose as a model substrate. The highest hydrogen production rate (HPR) and hydrogen yield (HY) were achieved at 0.2 V, reaching 15.35 ± 0.48 L H₂/L/d and 1.54 ± 0.05 mol H₂/mol glucose_{added}, respectively, which were 33.71% and 33.91% higher than those of the 0 V control. At 0.2 V, residual glucose and effluent volatile suspended solids (VSS) were minimized, while butyric acid (HBu) formation was enhanced and lactic acid (HLA) accumulation was suppressed. In contrast, voltages above 0.4 V reduced hydrogen recovery by shifting metabolic flux toward HLa, propionic acid (HPr), formic acid (HFO), and homoacetogenic pathways. Microbial analysis showed that *Clostridium* dominated under all conditions, but voltage application selectively altered the relative abundance and metabolic output of *Clostridium*-related amplicon sequence variants (ASVs). These results indicate that mild electrochemical stimulation at 0.2 V effectively enhances continuous biohydrogen production by promoting butyric acid-type fermentation, suppressing lactic acid accumulation, and reducing hydrogen loss through competing metabolic pathways in DMBR-EF systems.



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Keywords: biohydrogen production; dynamic membrane bioreactor; electro-fermentation; applied voltage; metabolic flux; *Clostridium*

1. Introduction

The increasing depletion of fossil fuels and the growing concern over greenhouse gas emissions have accelerated the demand for sustainable and carbon-neutral energy

carriers [1,2]. Hydrogen has attracted considerable attention as a clean energy carrier because its combustion produces only water and it has a high gravimetric energy density [3]. However, most hydrogen is still produced from fossil fuel-based processes such as steam methane reforming and coal gasification, which are associated with substantial CO₂ emissions [4]. Therefore, the development of environmentally sustainable hydrogen production technologies is essential for establishing future renewable energy systems.

Biological hydrogen production has been proposed as a promising alternative to conventional thermochemical and electrochemical hydrogen production methods [5]. Among various biological routes, including biophotolysis, photo-fermentation, dark fermentation, and microbial electrolysis cells, dark fermentation is considered one of the most practical approaches because it can produce hydrogen at relatively high rates without light energy and can utilize various organic wastes and wastewaters as substrates [6–8]. In dark fermentation, glucose is converted into pyruvate through glycolysis, and hydrogen is produced through acidogenic pathways, mainly associated with acetic acid (HAc) and H₂ formation [9,10]. Theoretically, acetic acid-type fermentation can produce up to 4 mol H₂/mol glucose, whereas butyric acid-type fermentation can produce up to 2 mol H₂/mol glucose [11]. However, actual hydrogen yields are generally much lower than theoretical values because a substantial portion of reducing equivalents is diverted to soluble metabolites such as H₂, ethanol, H₂, and other reduced by-products [9,12,13]. In addition, hydrogen-consuming pathways such as homoacetogenesis can further reduce actual hydrogen recovery [14].

For continuous dark fermentation, reactor configuration is a key factor affecting hydrogen production performance because high-rate operation requires efficient biomass retention under short HRT conditions [15,16]. Dynamic membrane bioreactors (DMBRs) have gained attention as a promising platform for continuous biohydrogen production. In DMBRs, suspended solids and microbial flocs form a dynamic membrane layer on a low-cost support material, allowing effective solid–liquid separation without the use of conventional microfiltration or ultrafiltration membranes [17–19]. This configuration enables the decoupling of HRT and solids retention time (SRT), thereby maintaining a high biomass concentration even under short HRT operation [20,21]. As a result, DMBRs can improve biohydrogen production performance and process stability while reducing membrane-related costs and energy demand [15,19,22,23].

Electro-fermentation has also emerged as an effective strategy to regulate fermentative metabolism by applying external electrical stimulation. In electro-fermentation systems, electrodes can function as electron sinks or electron sources, thereby influencing extracellular and intracellular redox conditions [24]. Previous studies have suggested that electrochemical stimulation may influence intracellular redox balance, including the NADH/NAD⁺ ratio, thereby affecting metabolic flux toward different fermentation products. However, the extent of this regulation depends on reactor configuration, operating conditions, and microbial community [25–29]. When electro-fermentation is combined with dark fermentation, relatively low applied voltages (0.2–0.4 V) may promote hydrogen-producing pathways by improving electron distribution and suppressing unfavorable reduced by-product formation [26,30,31]. However, higher applied voltages (>0.4 V) have been reported to disturb microbial metabolism and reduce hydrogen production efficiency [32–34]. Therefore, identifying an appropriate applied voltage is critical for improving hydrogen production efficiency in electro-fermentation-based systems [26,35].

Although both DMBR and electro-fermentation have shown potential for enhancing biohydrogen production, limited information is available on the integration of these two strategies in a continuous dark fermentation system [36]. In particular, the effect of applied voltage on hydrogen production performance, substrate utilization, soluble metabo-

lite distribution, and hydrogen-consuming pathways in a DMBR coupled with electro-fermentation has not been fully elucidated. Understanding these relationships is important because hydrogen production is not determined solely by substrate consumption but also by how carbon and reducing equivalents are distributed among hydrogen-producing, non-hydrogen-producing, and hydrogen-consuming pathways [37,38].

Therefore, this study developed a dynamic membrane bioreactor coupled with electro-fermentation (DMBR-EF) for continuous biohydrogen production from glucose as a model organic substrate. The reactor was operated under sequentially increased applied voltages of 0 (no applied voltage), 0.2, 0.4, 0.6, 0.8, and 1.0 V. The specific objectives of this study were to: (i) evaluate the effects of applied voltage on hydrogen production rate and hydrogen yield; (ii) assess substrate utilization and biomass washout through residual glucose and VSS concentrations; (iii) investigate shifts in fermentation pathways based on soluble metabolite distribution and COD (Chemical Oxygen demand) balance; and (iv) quantify the relationship between theoretical hydrogen yield, measured hydrogen yield, and homoacetogenic flux. This study provides insight into the role of voltage-driven metabolic regulation in DMBR-EF systems and identifies an optimal voltage range for high-rate continuous biohydrogen production.

2. Materials and Methods

2.1. Substrate and Inoculum

Anaerobic sludge collected from a wastewater treatment plant in Bucheon, Republic of Korea, was used as the inoculum. The characteristics of the inoculum were as follows: total solids (TS), 40.19 ± 0.06 g/L; volatile solids (VS), 21.02 ± 0.10 g/L; fixed solids (FS), 19.17 ± 0.16 g/L; total suspended solids (TSS), 38.20 ± 0.72 g/L; volatile suspended solids (VSS), 20.98 ± 0.35 g/L; and fixed suspended solids (FSS), 17.22 ± 0.37 g/L. To suppress methanogenic activity, the inoculum was heat-treated in a water bath (DAIHAN Scientific Co., Ltd., Wonju, Republic of Korea) at 90 °C for 60 min. Glucose (stemko Pure Chemical Co., Ltd., Pyeongtaek, Republic of Korea) was used as the carbon source at a concentration of 20 g/L. The substrate was supplemented with the following nutrients: NH_4HCO_3 (stemko Pure Chemical Co., Ltd., Pyeongtaek, Republic of Korea), 3 g/L; KH_2PO_4 (Daejung Chemicals & Metals Co., Ltd., Siheung, Republic of Korea), 0.125 g/L; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Daejung Chemicals & Metals Co., Ltd., Siheung, Republic of Korea), 0.1 g/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Daejung Chemicals & Metals Co., Ltd., Siheung, Republic of Korea), 0.025 g/L; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Duksan General Science Co., Ltd., Seoul, Republic of Korea), 0.005 g/L; $\text{CoCl}_2 \cdot 5\text{H}_2\text{O}$ (Duksan General Science Co., Ltd., Seoul, Republic of Korea), 0.001 g/L; and $\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$ (stemko Pure Chemical Co., Ltd., Pyeongtaek, Republic of Korea), 0.015 g/L [39]. During continuous operation, the NaHCO_3 (stemko Pure Chemical Co., Ltd., Pyeongtaek, Republic of Korea) dosage was adjusted as needed to maintain the reactor pH within the range of 5.5–6.5. Depending on the amount of organic acids produced under each operating condition, the NaHCO_3 concentration ranged from 4 to 8 g/L [40].

2.2. Operation of the Dynamic Membrane Bioreactor Coupled with Electro-Fermentation Reactor

The configuration of the dynamic membrane bioreactor coupled with electro-fermentation (DMBR-EF) is shown in Figure 1 and Figure S1. The DMBR-EF system consisted of a continuous stirred tank reactor (CSTR) and a dynamic membrane (DM) module. An anode and cathode were integrated into the DM module to establish dark fermentation and electro-fermentation conditions. A cylindrical titanium was used as the anode, with a surface area of 701.04 cm². A 400 µm stainless-steel mesh wound around a cylindrical support was used as the cathode, with a surface area of 254.40 cm². The distance between the two electrodes was maintained at 2 cm. External voltages of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 V were sequentially

applied using a DC power supply (Series 2230-30-3, Keithley Instruments, Cleveland, OH, USA). Under the applied voltage conditions, electrochemical reactions may occur at both electrodes. Possible anodic reactions include the oxidation of water or reduced intermediates, releasing electrons to the external circuit. At the cathode, reduction reactions involving protons or other electron acceptors may occur depending on the local electrochemical conditions. However, because individual electrode potentials and current were not monitored in the present study, these reactions are presented only as possible electrochemical processes and were not experimentally verified.

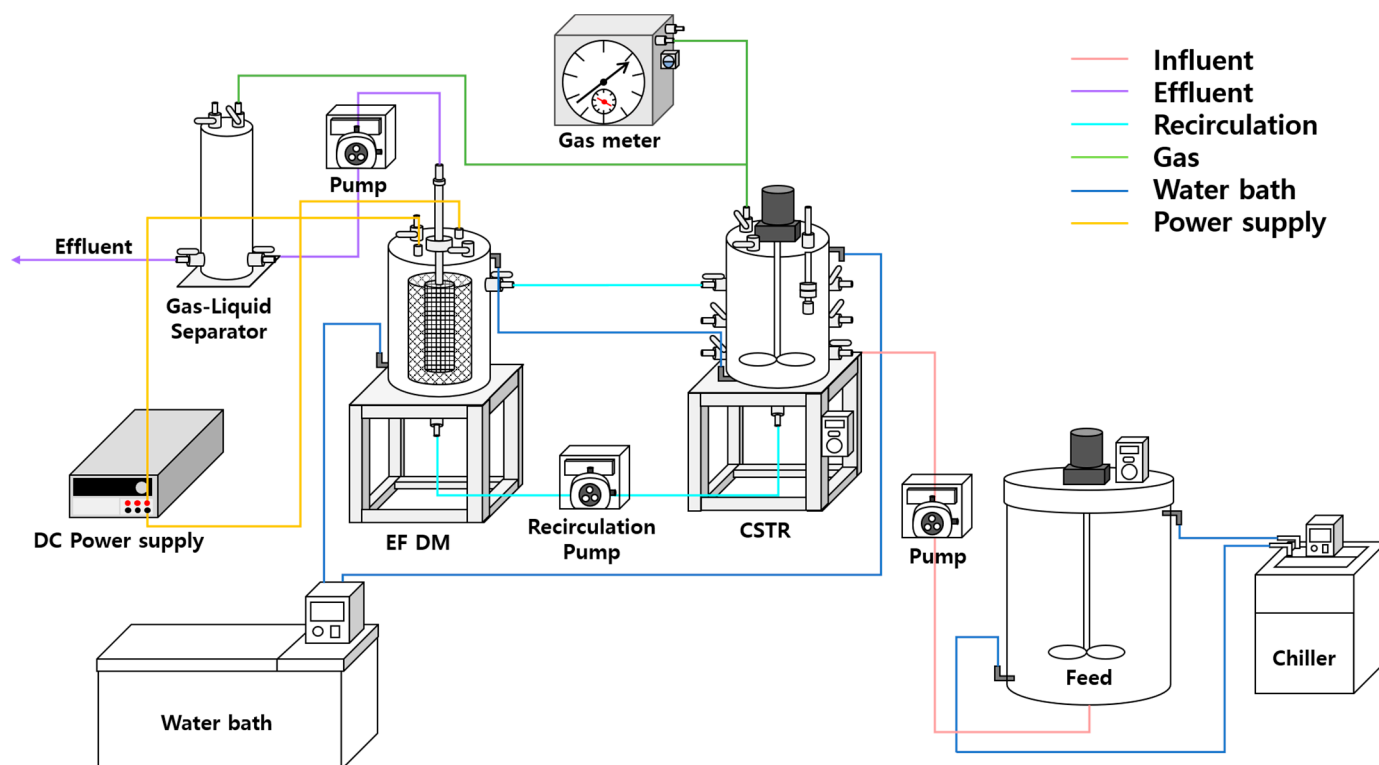


Figure 1. Schematic diagram of the dynamic membrane bioreactor coupled with electro-fermentation (DMBR-EF) system.

The total working volume of the reactor was 5.5 L. Heat-treated inoculum was added at 10% of the total working volume, and the remaining 90% was filled with the substrate medium. To establish anaerobic conditions, the reactor was purged with high-purity nitrogen gas (99.999%) for 3 min. The reactor was initially operated in batch mode for approximately 24 h until the pH decreased to around 5.5, after which it was switched to continuous operation. The substrate was maintained at 4 °C using a chiller (DAIHAN Scientific Co., Ltd., Wonju, Republic of Korea) and continuously supplied to the reactor using a peristaltic pump (Labscitech, Inc., Corona, CA, USA). The HRT was maintained at 6 h. During operation, the mixed liquor was continuously recirculated between the CSTR and the DM module. When the liquid level in the CSTR increased, a water-level sensor (YM Tech, Daejeon, Republic of Korea) activated effluent discharge through the DM module. The effluent was then discharged through a gas–liquid separator (YM Tech, Daejeon, Republic of Korea), maintaining the working volume at 5.5 ± 1 L. The reactor temperature was maintained at 37 ± 1 °C using a water bath.

2.3. Analytical Methods

The pH of the reactor was continuously monitored using a pH meter (Orion Versa Star Pro Benchtop pH Meter, Thermo Fisher Scientific, Waltham, MA, USA). The volume

of biogas produced during operation was measured using a wet gas meter (W-NK-0.5BE, Shinagawa Corporation, Tokyo, Japan) and corrected to standard temperature and pressure (STP). Hydrogen content in the biogas was analyzed using gas chromatography (GC; ACME 6100, Young Lin Instruments, Anyang, Republic of Korea) equipped with a thermal conductivity detector (TCD). For each analysis, a 150 μ L gas sample was injected into the GC-TCD. Hydrogen was quantified using a standard calibration curve (Equation (1)), where y is the H_2 peak area, and x is the injected hydrogen volume (μ L). The hydrogen fraction was then calculated from the peak area according to Equation (2). The hydrogen production rate (HPR) was subsequently determined by multiplying the total biogas production rate measured by the wet gas meter by the hydrogen fraction determined by GC (Equation (3)).

$$y = 53.007x - 73.027 \quad (1)$$

$$\text{Hydrogen fraction}(\%) = \frac{(H_2 \text{ peak area} + 73.027) / 53.007}{150} \times 100 \quad (2)$$

$$\text{HPR} = \text{Biogas production rate} \times \text{Hydrogen fraction} \quad (3)$$

Organic acids and residual glucose concentrations were analyzed using high-performance liquid chromatography (HPLC; Waters Corp., Milford, MA, USA). Before analysis, samples were centrifuged at 13,000 rpm using a SUPRA 30K centrifuge with an A250-6N rotor (DAIHAN Scientific Co., Ltd., Wonju, Republic of Korea), and the supernatant was sequentially filtered through 0.45 μ m and 0.20 μ m membrane filters (LKLABKOREA Inc., Namyangju, Republic of Korea). Organic acids were quantified using a UV detector at 210 nm with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA), whereas residual glucose was quantified using a refractive index detector with an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA). The solid contents of the effluent, including TSS and VSS, were analyzed according to the Standard Methods of the American Public Health Association (APHA) [41].

2.4. Microbial Analysis

To evaluate the microbial community composition under different applied voltages, samples were centrifuged at 13,000 rpm, and DNA was extracted from the collected biomass using a DNA extraction kit (SG-DN-SO-L, SmartGene, Daejeon, Republic of Korea). The V4 region of the bacterial 16S rRNA gene was amplified using barcode-tagged primers. For bacterial amplification, the fusion primers 515F (5'-GTGCCAGCMGCC GCGGTAA-3') and 806R (5'-GGACTACHVGGGTWT CTAAT-3') were used [42], and sequencing was carried out on an Illumina iSeq 100 system (Illumina, San Diego, CA, USA) [43]. The relative abundance of microbial communities was visualized using QIIME2 software version 2024.10. Genera and species with relative abundances below 2% of the total microbial community were grouped as "others."

2.5. Statistical Analyses

Alpha-diversity indices, including Shannon [44], Simpson [45], and Chao1 [46,47], were calculated to compare bacterial richness and evenness among voltage conditions using R based on formulas as follows (Equations (4)–(6)):

$$H_{\text{shannon}} = -\sum_{i=1}^{S_{\text{obs}}} \left(\frac{n_i}{N} \cdot \ln \frac{n_i}{N} \right) \quad (4)$$

$$D_{\text{simpson}} = \frac{\sum_{i=1}^{S_{\text{obs}}} n_i(n_i - 1)}{N(N - 1)} \quad (5)$$

$$S_{chao} = S_{obs} + \frac{n_1(n_1 - 1)}{2(n_2 + 1)} \quad (6)$$

Beta-diversity was evaluated using non-metric multidimensional scaling (NMDS) based on ASV-level community profiles using Bray–Curtis dissimilarities using R software ver. 4.6.2 with the vegan package, version 2.6-2 [48].

Pearson's correlation analysis was conducted to examine relationships among major ASVs, applied voltage, hydrogen production performance, soluble fermentation products, and residual glucose using PAST software ver. 3.06. Correlations with p -values below 0.1 were used for network visualization to identify potential associations between microbial community shifts and process performance using Cytoscape software ver. 3.10.4.

3. Results and Discussion

3.1. Biohydrogen Production Performance Under Different Applied Voltages

Continuous biohydrogen production using the DMBR-EF system was conducted for 59 days. To compare conventional dark fermentation with electro-fermentation, the reactor was first operated at 0 V as the control condition for 9 days. The applied voltage was then sequentially increased to 0.2, 0.4, 0.6, 0.8, and 1.0 V, and each voltage condition was maintained for approximately 10 days. The HPR and HY were evaluated under each condition. The temporal variations in HPR and HY during reactor operation are shown in Figure 2, and the average hydrogen production performance under each applied voltage is summarized in Table 1. The applied voltage clearly affected the hydrogen production performance of the DMBR-EF system. Under the control condition of 0 V, the HPR and HY were 11.48 ± 0.38 L H₂/L/d and 1.15 ± 0.04 mol H₂/mol glucose_{added}, respectively. In contrast, at 0.2 V, the HPR and HY increased to 15.35 ± 0.48 L H₂/L/d and 1.54 ± 0.05 mol H₂/mol glucose_{added}, respectively, which were the highest values observed in this study. Compared with the 0 V control, the HPR and HY increased by 33.71% and 33.91%, respectively. These results indicate that low-voltage electrochemical stimulation effectively enhanced hydrogen production during dark fermentation in the DMBR system.

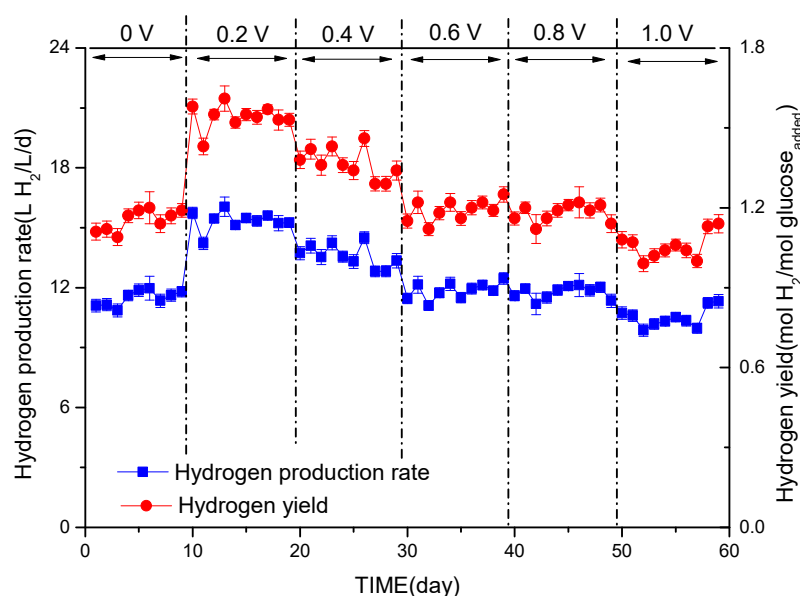


Figure 2. Temporal variations in hydrogen production rate (HPR) and hydrogen yield (HY) during continuous operation of the DMBR-EF system under different applied voltages.

Table 1. Average hydrogen production performance and effluent VSS concentration under different applied voltages.

	Applied Voltage					
	0 V	0.2 V	0.4 V	0.6 V	0.8 V	1.0 V
Hydrogen production rate (L H ₂ /L/d)	11.48 ± 0.38	15.35 ± 0.48	13.59 ± 0.56	11.85 ± 0.41	11.75 ± 0.33	10.51 ± 0.49
Hydrogen yield (mol H ₂ /mol glucose _{added})	1.15 ± 0.04	1.54 ± 0.05	1.37 ± 0.06	1.19 ± 0.04	1.18 ± 0.03	1.06 ± 0.05
Hydrogen yield (mol H ₂ /mol glucose _{consumed})	1.39 ± 0.11	1.60 ± 0.05	1.48 ± 0.06	1.33 ± 0.05	1.30 ± 0.04	1.16 ± 0.06
Volatile suspended solids (g VSS/L) in EF	1.09 ± 0.18	0.67 ± 0.08	1.02 ± 0.24	1.04 ± 0.06	1.03 ± 0.12	1.01 ± 0.09

However, further increases in applied voltage did not result in additional improvement. When the applied voltage was increased above 0.4 V, both HPR and HY gradually decreased. At 0.4 V, the HPR and HY decreased to 13.59 ± 0.56 L H₂/L/d and 1.37 ± 0.06 mol H₂/mol glucose_{added}, respectively. At 0.6 and 0.8 V, the HPR further decreased to 11.85 ± 0.41 and 11.75 ± 0.33 L H₂/L/d, respectively. Notably, at 1.0 V, the HPR and HY were 10.51 ± 0.49 L H₂/L/d and 1.06 ± 0.05 mol H₂/mol glucose_{added}, respectively, which were even lower than those of the 0 V control. These results suggest that voltage applications can enhance hydrogen production only within an appropriate voltage range, whereas excessive voltage may inhibit fermentative metabolism.

The HY values calculated based on glucose added and glucose consumed were also compared to evaluate substrate utilization efficiency. Under all conditions, the HY based on glucose consumed was higher than that based on glucose added, indicating that part of the supplied glucose remained unconsumed in the effluent. At 0.2 V, the HY values based on glucose added and glucose consumed were 1.54 ± 0.05 mol H₂/mol glucose_{added} and 1.60 ± 0.05 mol H₂/mol glucose_{consumed}, respectively, showing the smallest difference between the two values. This indicates that most of the supplied glucose was consumed at 0.2 V and that the consumed substrate was efficiently utilized for hydrogen production. In contrast, at 0 V, the HY based on glucose consumed was 1.39 ± 0.11 mol H₂/mol glucose_{consumed}, which was higher than those observed at 0.6 and 0.8 V. This suggests that, under relatively high-voltage conditions, the consumed glucose was not efficiently converted into hydrogen but was instead redirected toward HLa, HPr, or other reduced by-product formation pathways. Therefore, hydrogen production performance cannot be explained solely by substrate consumption; rather, the distribution of consumed substrate among different metabolic pathways is a key factor determining hydrogen recovery.

The VSS concentration in the effluent also varied depending on the applied voltage. At 0.2 V, the effluent VSS concentration was the lowest at 0.67 ± 0.08 g VSS/L, whereas the other voltage conditions showed values of approximately 1.0 g VSS/L. This suggests that microbial metabolism at 0.2 V may have been directed more toward hydrogen production rather than biomass synthesis [26,49]. Because the DMBR-EF system used in this study was operated as a single-chamber reactor containing both an anode and a cathode, the individual contributions of anodic electron withdrawal and cathodic microenvironmental changes could not be independently distinguished. Therefore, the enhanced hydrogen production observed at 0.2 V should not be attributed to a specific electrode reaction. Rather, the improved HPR and HY at 0.2 V likely resulted from the overall electro-fermentative environment established within the reactor, which may have collectively influenced extracellular electron transfer, local redox conditions, and fermentative metabolism [24,30,50]. The concurrent increase in HPR and HY, enhancement of butyric acid production, suppression of lactic acid accumulation, and reduction in residual glucose suggest that low-voltage

electrochemical stimulation favored hydrogen-producing metabolism under the present reactor configuration. However, further studies using two-chamber reactors or electrode-specific potential control are required to distinguish the individual roles of anodic and cathodic processes. Overall, 0.2 V was identified as the optimal applied voltage for hydrogen production in the DMBR-EF system, whereas voltages above 0.4 V appeared to disturb metabolic balance and reduce hydrogen production performance. Although internal resistance and Joule heating may influence bio-electrochemical systems, the reactor temperature was maintained at 37 ± 1 °C throughout the experiment. Therefore, the enhanced hydrogen production observed at 0.2 V is more likely attributable to voltage-dependent metabolic regulation than to thermal effects.

3.2. Soluble Fermentation By-Products and COD Balance

To interpret the differences in hydrogen production performance at the metabolic level, soluble fermentation by-products, residual glucose, effluent VSS, and produced hydrogen were converted into COD equivalents, and the COD balance was evaluated under each applied voltage. The COD equivalent of produced hydrogen was calculated using the theoretical oxygen demand of hydrogen (8 g COD/g H₂). The concentrations of soluble fermentation by-products, residual glucose, produced hydrogen, effluent VSS, and the corresponding COD balance are summarized in Table 2. The major soluble fermentation products analyzed were HLa, HFo, HAc, HPr, and HBU, which were used as indicators of changes in dark fermentation pathways. HBU was the dominant fermentation by-product under all applied voltage conditions, followed by HAc. This indicates that butyric acid-type and acetic acid-type fermentation were the main metabolic pathways in the DMBR-EF system throughout the operation [21,51]. In glucose fermentation, the acetic acid-producing pathway can theoretically generate up to 4 mol H₂/mol glucose, whereas the butyric acid-producing pathway can generate up to 2 mol H₂/mol glucose [11,33]. Therefore, the formation of HAc and HBU can be regarded as favorable metabolic routes directly associated with hydrogen production.

Table 2. Soluble fermentation by-products, residual glucose, produced hydrogen, effluent VSS and COD balance under different applied voltages.

Applied Voltage	Soluble By-Products Concentration in Effluent (g COD/L)					Residual Glucose (g COD/L)	Produced H ₂ (g COD/L)	Effluent VSS (g COD/L)	COD Balance (g COD/L)
	HLa	HFo	HAc	HPr	HBU				
0 V	2.94 ± 0.99	0.12 ± 0.12	3.06 ± 0.32	0.03 ± 0.03	7.45 ± 0.95	3.57 ± 0.92	2.05 ± 0.07	1.55 ± 0.26	20.78 (97.08%)
0.2 V	1.12 ± 0.21	0.00 ± 0.00	3.88 ± 0.25	0.01 ± 0.02	10.47 ± 0.81	0.72 ± 0.31	2.74 ± 0.09	0.95 ± 0.11	19.90 (92.97%)
0.4 V	1.95 ± 0.35	0.00 ± 0.01	3.45 ± 0.35	0.03 ± 0.02	7.33 ± 0.59	1.67 ± 0.28	2.43 ± 0.10	1.45 ± 0.34	18.31 (85.57%)
0.6 V	2.18 ± 0.30	0.00 ± 0.00	3.53 ± 0.25	0.04 ± 0.02	6.92 ± 0.49	2.19 ± 0.21	2.12 ± 0.07	1.48 ± 0.61	18.47 (86.29%)
0.8 V	2.12 ± 0.20	0.01 ± 0.01	3.70 ± 0.27	0.08 ± 0.06	7.65 ± 0.86	1.93 ± 0.21	2.10 ± 0.06	1.47 ± 0.17	19.05 (89.01%)
1.0 V	2.33 ± 0.26	0.09 ± 0.08	3.50 ± 0.32	0.69 ± 0.18	7.54 ± 0.66	1.90 ± 0.40	1.88 ± 0.09	1.43 ± 0.13	19.36 (90.45%)

At 0.2 V, the HBU concentration reached 10.47 ± 0.81 g COD/L, which was the highest among all conditions, and HAc also remained relatively high at 3.88 ± 0.25 g COD/L. In contrast, HLa decreased markedly to 1.12 ± 0.21 g COD/L compared with 2.94 ± 0.99 g COD/L at 0 V. This indicates that the application of 0.2 V promoted HBU formation while suppressing HLa accumulation. Since HLa production does not directly generate hydrogen [33], the reduced HLa accumulation observed at 0.2 V was accompanied by enhanced hydrogen production and HBU formation. Although this metabolic shift is consistent with electro-fermentative regulation reported in previous studies [27,52], the present study did not measure reactor ORP (Oxidation-reduction potential) or intracellular NADH/NAD⁺ ratios. Therefore, changes in intracellular redox balance cannot be directly confirmed and require further investigation. This result is consistent with the highest HPR and HY observed at 0.2 V. In contrast, HLa increased again when the applied

voltage exceeded 0.4 V. The HLa concentrations at 0.4, 0.6, 0.8, and 1.0 V were 1.95 ± 0.35 , 2.18 ± 0.30 , 2.12 ± 0.20 , and 2.33 ± 0.26 g COD/L, respectively. This suggests that part of the metabolic flux shifted back toward HLa production as the voltage increased. Because HLa is not directly linked to hydrogen generation, its accumulation under high-voltage conditions was likely one of the major causes of the decreased HY.

HPr was detected at very low concentrations of 0.01–0.04 g COD/L under 0–0.6 V conditions but increased to 0.08 ± 0.06 g COD/L at 0.8 V and sharply increased to 0.69 ± 0.18 g COD/L at 1.0 V. HPr formation is generally considered unfavorable for hydrogen production because it consumes hydrogen or reducing equivalents [33]. Therefore, the accumulation of HPr at 1.0 V likely contributed directly to the decrease in hydrogen production performance. HFo was also negligible under most conditions but increased to 0.09 ± 0.08 g COD/L at 1.0 V. Although HFo can serve as an intermediate in hydrogen production through the HFo hydrogen lyase (FHL) system [10], the reversibility of this reaction ($\text{HCOO}^- + \text{H}_2\text{O} \rightleftharpoons \text{H}_2 + \text{HCO}_3^-$) indicates that HFo can also be synthesized from H_2 and CO_2 , thereby consuming hydrogen under certain conditions [53]. Therefore, its concurrent increase with HPr under high-voltage conditions may indicate metabolic imbalance.

The COD balance ranged from 85.57% to 97.08%, indicating that the measured hydrogen, soluble by-products, effluent VSS, and residual glucose reasonably accounted for the influent COD [54,55]. At 0.2 V, the produced hydrogen was the highest at 2.74 ± 0.09 g COD/L, while residual glucose was the lowest at 0.72 ± 0.31 g COD/L. This indicates that glucose was effectively consumed and converted mainly into hydrogen and butyric acid-dominant fermentation products under this condition. In contrast, the residual glucose concentration was highest at 0 V, reaching 3.57 ± 0.92 g COD/L, suggesting that substrate utilization was relatively limited in the absence of applied voltage. At 1.0 V, produced hydrogen decreased to 1.88 ± 0.09 g COD/L, while HPr and HFo increased. These results indicate that excessive voltage did not simply suppress substrate utilization but redirected the consumed substrate toward metabolic pathways unfavorable for hydrogen production. Consequently, the reduced hydrogen recovery at 1.0 V was associated with the diminished contribution of butyric acid-type fermentation and the increased formation of lactic acid-, propionic acid-, and formic acid-related products. Furthermore, these observations demonstrate that voltage-dependent changes in hydrogen production were closely accompanied by corresponding shifts in metabolite distribution, indicating that higher applied voltages progressively redirected metabolic flux toward competing fermentation pathways.

3.3. Theoretical Hydrogen Yield and Homoacetogenic Flux

To further quantify the effect of applied voltage on hydrogen production, measured hydrogen yield, theoretical hydrogen yield, and homoacetogenic flux were compared. The theoretical hydrogen yield was calculated by considering hydrogen generation from HAc and HBu formation and subtracting hydrogen consumption associated with HPr and HFo formation [56], as shown in Equation (7). The homoacetogenic flux was estimated from the gap between measured HY and theoretical HY [57], as shown in Equation (8).

$$\begin{aligned} \text{Theoretical hydrogen yield} & \left(\frac{\text{mol H}_2}{\text{mol glucose}_{\text{consumed}}} \right) \\ &= \left(\frac{2 \text{ mol H}_2}{\text{mol butyric acid}} \times \frac{\text{mol butyric acid}}{\text{mol glucose}_{\text{consumed}}} \right) \\ &+ \left(\frac{2 \text{ mol H}_2}{\text{mol acetic acid}} \times \frac{\text{mol acetic acid}}{\text{mol glucose}_{\text{consumed}}} \right) \\ &- \left[\left(\frac{\text{mol H}_2}{\text{mol propionic acid}} \times \frac{\text{mol propionic acid}}{\text{mol glucose}_{\text{consumed}}} \right) \right. \\ &\quad \left. + \left(\frac{\text{mol H}_2}{\text{mol formic acid}} \times \frac{\text{mol formic acid}}{\text{mol glucose}_{\text{consumed}}} \right) \right] \end{aligned} \quad (7)$$

$$\text{Homoacetogenic flux} \left(\frac{\text{mol acetic acid}}{\text{mol glucose}_{\text{consumed}}} \right) = \frac{\text{Theoretical hydrogen yield} \left(\frac{\text{mol H}_2}{\text{mol glucose}_{\text{consumed}}} \right) - \text{Measured hydrogen yield} \left(\frac{\text{mol H}_2}{\text{mol glucose}_{\text{consumed}}} \right)}{4 \left(\frac{\text{mol H}_2}{\text{mol acetate}} \right)} \quad (8)$$

This approach allowed the relationship between metabolite distribution and actual hydrogen recovery to be evaluated under each voltage condition. The distributions of fermentation products, theoretical hydrogen yield, measured hydrogen yield, and homoacetogenic flux under different applied voltages are presented in Figure 3. Under all conditions, the theoretical HY was higher than the measured HY, indicating that the hydrogen production potential estimated from the metabolite profile was greater than the actual hydrogen recovered. This suggests that part of the produced hydrogen may have been consumed through homoacetogenesis or other hydrogen-consuming pathways. Homoacetogenesis, in which H_2 and CO_2 are converted into HAc, can reduce actual hydrogen recovery in dark fermentation systems [14]. It should be noted that the difference between the theoretical and measured hydrogen yields may also include contributions from other hydrogen-consuming processes, such as microbial assimilation, anodic hydrogen oxidation, or other unidentified hydrogen sinks. Therefore, the homoacetogenic flux estimated in the present study should be interpreted as an indirect indicator of hydrogen loss rather than a direct measurement of homoacetogenic activity.

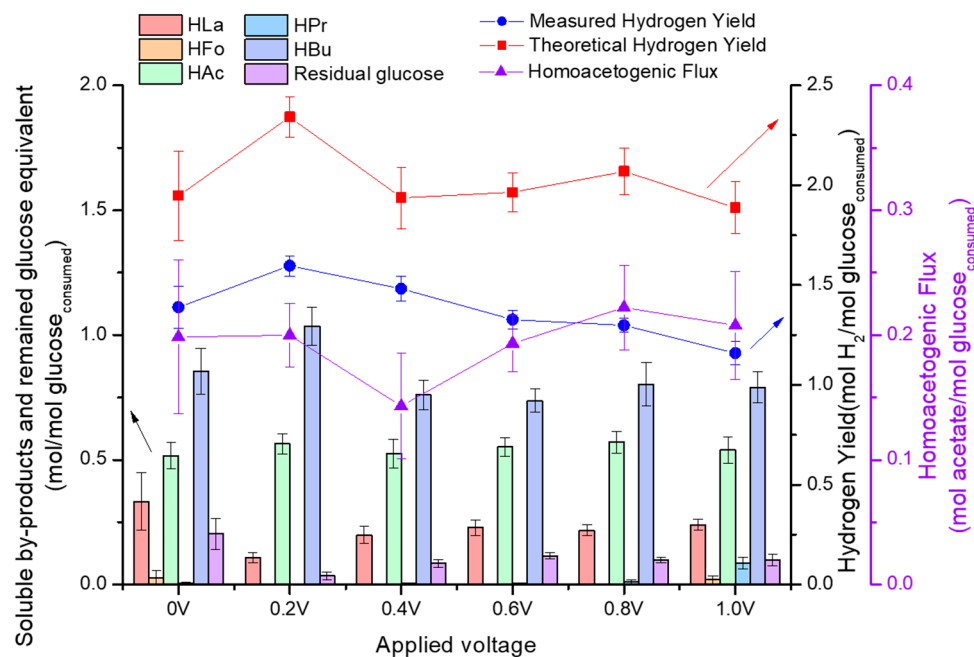


Figure 3. Relationship between fermentation product distribution, theoretical hydrogen yield, measured hydrogen yield, and homoacetogenic flux under different applied voltages.

At 0.2 V, the theoretical HY reached the highest value of 2.34 ± 0.10 mol H_2 /mol glucose_{consumed}, and the measured HY was also the highest among all conditions. Under this condition, HBu was 1.03 ± 0.08 mol/mol glucose_{consumed}, which was the highest value observed, while residual glucose was the lowest at 0.04 ± 0.02 mol/mol glucose_{consumed}. These results indicate that glucose utilization was most active at 0.2 V and that the consumed substrate was effectively converted through butyric acid-type fermentation, which is favorable for hydrogen production. In addition, HLa at 0.2 V was 0.11 ± 0.02 mol/mol glucose_{consumed}, which was substantially lower than the value of 0.33 ± 0.11 mol/mol glucose_{consumed} observed at 0 V. Since HLa formation does not directly accompany hydrogen production, the suppression of

HLa accumulation at 0.2 V likely played an important role in improving hydrogen production. Thus, mild electrochemical stimulation at 0.2 V enhanced measured HY by improving glucose utilization, suppressing non-hydrogen-producing HLa formation, and strengthening butyric acid-type fermentation.

At 0.4 and 0.6 V, the theoretical HY decreased to 1.94 ± 0.15 and 1.96 ± 0.10 mol H₂/mol glucose_{consumed}, respectively, and the measured HY also decreased compared with 0.2 V. At these voltages, HBu decreased to 0.76 ± 0.06 and 0.74 ± 0.05 mol/mol glucose_{consumed}, respectively, while HLa increased to 0.20 ± 0.03 and 0.23 ± 0.03 mol/mol glucose_{consumed}, respectively. This indicates that, when the applied voltage exceeded 0.2 V, the dominance of butyric acid-type fermentation weakened, and part of the carbon flux was redirected toward HLa formation. At 0.8 V, the theoretical HY was 2.07 ± 0.12 mol H₂/mol glucose_{consumed}, which was slightly higher than those at 0.4 and 0.6 V, but the measured HY remained low at 1.30 ± 0.04 mol H₂/mol glucose_{consumed}. Under this condition, HAc reached the highest value of 0.57 ± 0.04 mol/mol glucose_{consumed}, and homoacetogenic flux also increased to 0.22 ± 0.03 mol acetic acid/mol glucose_{consumed}. This suggests that HAc formation at 0.8 V may not have resulted solely from the hydrogen-producing HAc pathway but may also have been partly associated with homoacetogenesis, which consumes H₂ and CO₂. Therefore, the discrepancy between the relatively high theoretical HY and the low measured HY at 0.8 V can be explained by hydrogen loss through homoacetogenic activity.

At 1.0 V, the measured HY was the lowest, while the theoretical HY also decreased to 1.89 ± 0.13 mol H₂/mol glucose_{consumed}. HLa remained high at 0.24 ± 0.02 mol/mol glucose_{consumed}, and HPr and HFo increased to 0.09 ± 0.02 and 0.02 ± 0.02 mol/mol glucose_{consumed}, respectively. In particular, HPr formation is unfavorable for hydrogen production because it consumes reducing equivalents. Thus, HPr accumulation at 1.0 V may have contributed to the reduction in hydrogen production, although other mechanisms, including possible electrochemical hydrogen evolution followed by biological hydrogen consumption, cannot be excluded. In addition, homoacetogenic flux remained high at 0.21 ± 0.04 mol acetic acid/mol glucose_{consumed}, indicating that part of the produced hydrogen may have been consumed for HAc formation under high-voltage conditions. An alternative explanation for the reduced hydrogen recovery at 1.0 V is the possible occurrence of electrochemical hydrogen evolution at the cathode, followed by biological hydrogen consumption through homoacetogenesis or other hydrogen-consuming pathways [58,59]. However, in the present study, only the overall cell voltage was controlled, whereas individual electrode potentials and current were not monitored. Therefore, it could not be confirmed whether the cathode potential reached the hydrogen evolution region under the applied voltage of 1.0 V. Consequently, the occurrence of electrochemical hydrogen evolution and its contribution to hydrogen recovery remain speculative and require further electrochemical characterization in future studies.

Taken together, the enhanced hydrogen production at 0.2 V was not simply due to voltage application itself, but rather to the combined effects of improved glucose utilization, enhanced butyric acid-type fermentation, suppressed HLa and HPr formation, and relatively low diversion of metabolic flux toward non-hydrogen-producing pathways. In contrast, at applied voltages above 0.4 V, excessive electrochemical stimulation dispersed fermentative carbon flux toward HLa, HPr, HFo, and homoacetogenic pathways, thereby reducing actual hydrogen recovery. These results demonstrate that applied voltage is a critical operational parameter for DMBR-EF-based continuous biohydrogen production, and that exceeding the optimal voltage range can induce metabolic disturbance and hydrogen-consuming pathways. Because the applied voltage was sequentially increased from 0 to 1.0 V in the present study, the reversibility of voltage-induced metabolic responses could not be evaluated. Future studies should investigate reverse voltage operation (e.g., from

1.0 V back to 0.2 V) and recovery experiments after high-voltage exposure to determine whether the observed metabolic shifts are reversible or associated with the accumulation of electrochemical side reactions or their products.

3.4. Microbial Community Analysis

In the HTS (High-throughput sequencing) analysis, a total of 96,982 bacterial reads were obtained and resolved into 135 ASVs. The taxonomic affiliations of major bacterial ASVs ($\geq 1\%$ of the total reads in at least one bacterial library) are presented in Table S1. In all conditions, the bacterial community structure was dominated by *Clostridium* (82.8–92.8%), which is hydrogen producing bacteria with various types of acids from sugars [60] (Figure 4). The microbial community was dominated by *Clostridium* at both the genus and ASV levels. The major ASVs, including ASV1–ASV15, were assigned to *Clostridium*, indicating that this genus was the primary microbial group involved in hydrogen production and organic acid formation in the DMBR-EF system. Nevertheless, the relative abundance of individual *Clostridium*-related ASVs differed among voltage conditions, suggesting that voltage application selectively affected the dominance of specific ASVs within the *Clostridium*-dominated community. The NMDS ordination (stress = 0.00001) showed that the ASV-level community structure shifted with voltage application, although the separation was not strictly proportional to the 0.2 V voltage interval (Figure 5A). The 0 V and 0.6 V conditions were positioned closely, and the 0.4 V and 0.8 V conditions also showed similar community profiles. By contrast, the 0.2 V and 1.0 V conditions were relatively separated from the other voltage conditions, suggesting that these two conditions induced distinct microbial responses within the *Clostridium*-dominated community.

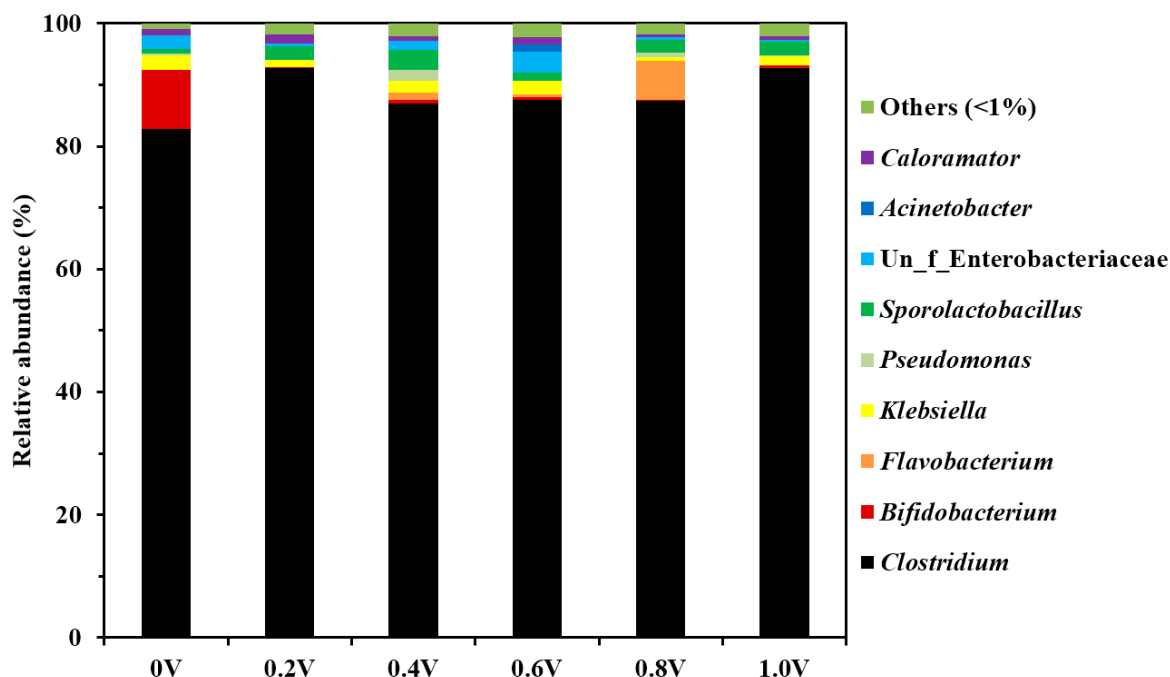


Figure 4. Genus-level bacterial community composition at steady state across experimental phases. *Un_f_Enterobacteriaceae* indicates an unclassified taxon assigned to the family *Enterobacteriaceae*, but not resolved to the genus level.

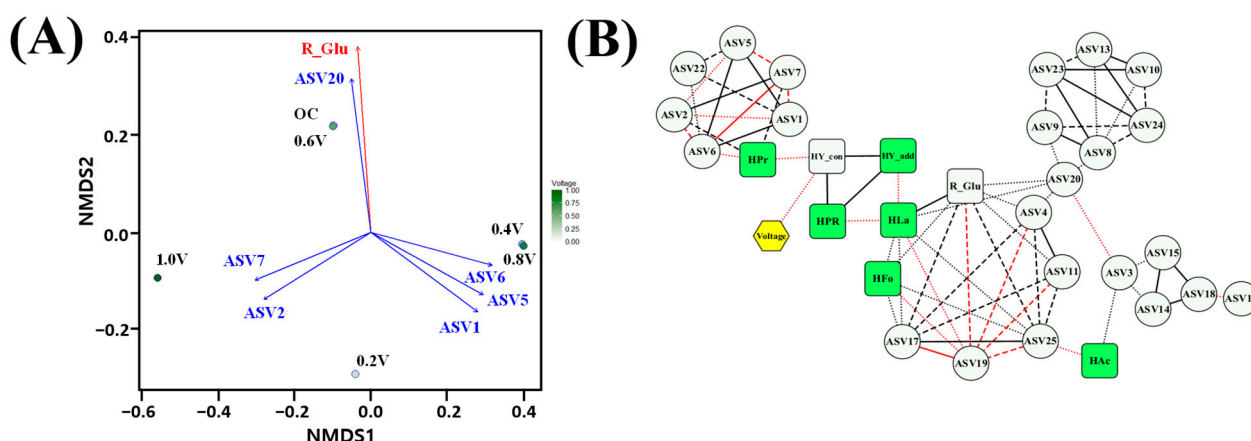


Figure 5. Comprehensive analyses of bacterial community structure and its relatedness with operational and performance parameters: **(A)** NMDS triplot showing variation in bacterial community structure across experimental phases. Each point is labeled with its corresponding voltage level. Arrows indicate the direction and magnitude of correlations between ordination axes and selected variables (permutation test, $p < 0.1$). **(B)** Correlation network ($p < 0.1$) among the major populations ($\geq 1\%$ relative ASV abundance in at least one bacterial library), properties of additives, and process parameters. White circles, green squares, and yellow hexagons represent bacterial ASV, performance parameters, and voltage level, respectively. Black and red edges represent positive and negative correlations, respectively. Solid, dashed, and dotted lines represent p -value under 0.01, 0.05, and 0.1, respectively. HAc, acetic acid; HPr, propionic acid; HFo, formic acid; HLa, lactic acid; HPR, hydrogen production rate; HY_Con, hydrogen yield based on glucose consumed; HY_Add, hydrogen yield based on glucose added; R_Glu, Removed glucose fraction.

The distinct community structure observed at 0.2 V corresponded to the highest hydrogen production rate and hydrogen yield, together with enhanced acetic acid/butyric acid-type fermentation and reduced HLa accumulation. This suggests that low-voltage electrochemical stimulation at 0.2 V provided a favorable electro-fermentative condition for hydrogen-producing acidogenesis. Similar observations were reported that a relatively low applied voltage (0.2–0.4 V) could shift fermentation metabolism toward HAc or both HAc and HBu production, supporting the possibility that mild electrical stimulation can enhance hydrogen-producing metabolic routes [29,61]. Therefore, the improved hydrogen production at 0.2 V was likely associated with the combined effects of favorable *Clostridium*-related ASV composition and enhanced acetic acid/butyric acid-type metabolic activity. In contrast, hydrogen production performance declined when the applied voltage was increased above 0.2 V, indicating that stronger electrochemical stimulation began to perturb the redox balance and metabolic activity of the *Clostridium*-dominated community [62,63]. This performance deterioration was accompanied by increased HLa formation from 0.4 V, and further accumulation of HPr and HFo at 1.0 V. Therefore, the decrease in hydrogen production at 0.4–1.0 V was unlikely to be caused by the disappearance or replacement of *Clostridium*. Rather, increasing voltage likely altered the relative dominance and metabolic output of *Clostridium*-related ASVs, progressively redirecting carbon and electron flux from hydrogen-producing acetic acid/butyric acid-type fermentation toward non-hydrogen-producing lactic acid-, propionic acid-, and formic acid-related pathways. In particular, the strong separation of the 1.0 V community in the NMDS plot may reflect severe voltage-induced stress, which resulted in a marked restructuring of the dominant *Clostridium*-related ASV composition and the lowest hydrogen production performance.

The alpha diversity results further supported the effect of voltage application, particularly for the distinct community structure observed at 1.0 V (Table 3). The 1.0 V condition showed the lowest Shannon, Simpson, and Chao1 indices among all voltage conditions,

indicating that excessive voltage reduced both microbial richness and evenness. This suggests that the strong separation of the 1.0 V community in the NMDS plot was associated with severe voltage-induced selective pressure, which simplified the microbial community and promoted the dominance of specific *Clostridium*-related ASVs. In contrast, the alpha diversity indices at 0.2 V were comparable to those under the 0 V condition, indicating that the distinct NMDS position of 0.2 V was not caused by a major increase or decrease in overall microbial diversity. Rather, the 0.2 V community likely differed from the other conditions mainly through changes in the relative abundance of dominant *Clostridium*-related ASVs while maintaining similar richness. Therefore, the 0.2 V condition may represent a favorable restructuring of the *Clostridium*-dominated community, whereas the 1.0 V condition reflects severe voltage-induced community disturbance.

Table 3. Alpha-diversity of microbial community structure in each condition.

Condition	Shannon	Simpson	Chao1
0 V	2.59	0.88	48
0.2 V	2.43	0.86	48
0.4 V	2.47	0.84	47
0.6 V	2.82	0.91	48
0.8 V	2.39	0.84	50
1.0 V	2.07	0.75	40

Correlation-based network analysis further supported the voltage-dependent restructuring of the *Clostridium*-dominated community (Figure 5B). ASV1, ASV5, and ASV6 formed a strongly positive cluster, with significant correlations between ASV1 and ASV5 ($r = 0.996, p < 0.01$), ASV5 and ASV6 ($r = 0.985, p < 0.01$), and ASV1 and ASV6 ($r = 0.978, p < 0.01$). In contrast, ASV2 and ASV7 formed another positive cluster ($r = 0.987, p < 0.01$), which showed negative relationships with the ASV1/ASV5/ASV6 cluster, including ASV6 versus ASV7 ($r = -0.941, p < 0.01$), ASV5 versus ASV7 ($r = -0.870, p < 0.05$), and ASV1 versus ASV7 ($r = -0.854, p < 0.05$). These contrasting ASV clusters indicate that voltage application shifted the relative dominance among major *Clostridium*-related ASVs rather than causing a replacement of *Clostridium* by other taxa.

The network also showed that HLa was strongly and positively correlated with residual glucose ($r = 0.971, p < 0.01$), suggesting that HLa accumulation was associated with less favorable substrate conversion. This is consistent with previous reports that a metabolic shift toward HLa fermentation can reflect metabolic limitations that lower substrate conversion efficiency [64,65]. In addition, HPR and HY based on glucose added showed negative correlations with HLa at a marginal level ($r = -0.790, p < 0.1$). These relationships are consistent with the observed decline in hydrogen production above 0.2 V, where HLa formation increased and acetic acid/butyric acid-type hydrogen-producing fermentation became less dominant. Therefore, the network analysis supports the interpretation that increasing voltage altered the internal structure and metabolic output of the *Clostridium*-dominated community, redirecting carbon and electron flux away from hydrogen-producing acetic acid/butyric acid-type fermentation and toward lactic acid-related non-hydrogen-producing metabolism.

4. Conclusions

This study demonstrated that applied voltage plays a decisive role in regulating continuous biohydrogen production and fermentative metabolic flux in a DMBR-EF system. Among the tested voltage conditions, 0.2 V was identified as the optimal applied voltage, achieving the highest HPR of 15.35 ± 0.48 L H₂/L/d and HY

of 1.54 ± 0.05 mol H₂/mol glucose_{added}. The enhanced performance at 0.2 V was associated with improved glucose utilization, the lowest effluent VSS concentration, increased H₂ formation, and suppressed HLa accumulation, indicating that mild electrochemical stimulation promoted hydrogen-producing metabolic pathways. In contrast, voltages above 0.4 V reduced hydrogen production efficiency by weakening butyric acid-type fermentation and promoting HLa formation, while 1.0 V further increased HPr, H₂Fo, and homoacetogenic flux, leading to the lowest hydrogen recovery. Microbial community analysis showed that *Clostridium* remained dominant throughout the operation; however, voltage application selectively altered the relative abundance and metabolic output of *Clostridium*-related ASVs. These findings indicate that the beneficial effect of electro-fermentation is highly voltage-dependent. Because electrode potentials were not monitored, the possible contribution of electrochemical hydrogen evolution at high applied voltages could not be evaluated and should be investigated in future studies. Overall, the DMBR-EF system is a promising platform for high-rate continuous biohydrogen production, but careful voltage control is essential to enhance hydrogen-producing metabolism while avoiding metabolic disturbance and hydrogen-consuming pathways. Because the operating current was not monitored in the present study, the electrical energy consumption of the DMBR-EF system could not be quantified. Future studies should include current measurements to evaluate the energy efficiency and practical feasibility of electro-fermentation under different applied voltages.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/hydrogen7030102/s1>, Figure S1: Photograph of the dynamic membrane bioreactor coupled with electro-fermentation (DMBR-EF) system used for the continuous biohydrogen production experiments. Table S1: Relative abundances and taxonomic affiliations of major bacterial ASVs ($\geq 1\%$ of total bacterial reads in at least one sample).

Author Contributions: E.C.: Visualization, Investigation, Data curation. G.-B.K.: Writing—original draft, Methodology, Formal analysis. G.C.: Formal analysis. J.-H.J.: Writing—review and editing, Conceptualization, Supervision. E.C. and G.-B.K. contributed equally to this work and are considered co-first authors. All authors have read and agreed to the published version of the manuscript.

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