

## Article

# Performance of a Continuous Dark–Photo Fermentation System to Produce Hydrogen from Simulated Sugar–Rich Processing Wastewater Under Suboptimal pH and Temperature Conditions

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## Abstract

The integration of coupled dark fermentation (DF) and photofermentation (PF) systems represents a promising approach for concurrent H<sub>2</sub> production and organic load reduction in carbohydrate-rich wastewater. However, their behavior under non-optimized, continuous industrial operation remains inadequately characterized, hindering practical implementation. This case study evaluated an integrated DF–PF system treating a synthetic sugar mixture mimicking acidic fruit and dairy processing wastewater. The system presented herein (5-L reactors) serves as an initial prototype to facilitate scaling to both pilot (40-L reactors) and, ultimately, industrial (600-L) scales using real effluents within the framework of a research project. The bioreactors were operated continuously with a 10 h hydraulic retention time and an organic loading rate of 2.5 g COD L<sup>−1</sup> d<sup>−1</sup>. The pH and temperature were monitored but intentionally left uncontrolled. The DF stage facilitated by hydrogen-producing bacteria achieved H<sub>2</sub> concentrations of up to 57% (*v/v*) and a maximum production rate of 177 mL H<sub>2</sub> L<sup>−1</sup> d<sup>−1</sup>; however, it demonstrated notable process instability due to the absence of controls. In contrast, the PF stage exhibited negligible H<sub>2</sub> production (1.8% *v/v*) attributable to the displacement of *Rhodospseudomonas* species by microbial competition, thereby functioning predominantly as a polishing step. Overall, the coupled system achieved an average COD removal of 34%, highlighting the functional differentiation between stages and identifying microbial competition as the primary constraint under suboptimal conditions.

**Keywords:** hydrogen production; dark fermentation; photofermentation; continuous system; wastewater treatment; microbial community dynamics



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## 1. Introduction

The utilization of H<sub>2</sub> has increased markedly across various sectors, ranging from industry to transportation [1]. This study explores the growing importance of H<sub>2</sub> as a clean alternative to fossil fuels. As a carbon-free energy carrier, its combustion with pure oxygen yields water as the sole byproduct; however, combustion in atmospheric air can also generate secondary nitrogen oxide (NO<sub>x</sub>) emissions [2]. H<sub>2</sub> is a colorless, odorless, non-toxic gas with a low ignition energy, making it a key candidate for achieving global

climate targets aimed at mitigating greenhouse gas emissions. Currently, only about 8% of global H<sub>2</sub> production is derived from natural resources [3], with an annual output of roughly 55 million metric tons growing at an estimated 6% per year [4]. Projections indicate that the global H<sub>2</sub> market will reach 1.6 trillion USD by 2050 [5].

Conventional H<sub>2</sub> production technologies are typically energy-intensive and carry significant environmental impacts. Thermochemical processes, such as steam methane reforming and coal gasification, are environmentally unsustainable in the long term due to substantial CO<sub>2</sub> emissions. This has driven growing interest in biological H<sub>2</sub> production methods, which offer a lower carbon footprint while enabling simultaneous waste stream treatment and bioenergy generation. Recent studies have highlighted the potential advantages of these bio-processes from a life cycle perspective [6]. Biological H<sub>2</sub> generation relies on anaerobic or facultative anaerobic microorganisms to convert biomass into H<sub>2</sub>, with process efficiency determined by the substrate degradation rate and the specific metabolic pathways involved [7,8].

Among biological approaches, dark fermentation (DF) and photofermentation (PF) have received significant attention [9]. PF is a light-dependent process in which photosynthetic bacteria utilize light energy to convert organic substrates into H<sub>2</sub> under anaerobic conditions. These bacteria can metabolize a wide range of substrates, including simple sugars, volatile fatty acids (VFAs), and industrial or agricultural waste streams [10]. Genera such as *Rhodobacter*, *Rhodobium*, *Rhodopseudomonas*, and *Rhodospirillum* are frequently employed in PF processes [11]. However, PF performance is highly sensitive to operational parameters such as pH, temperature, light intensity, and pressure; suboptimal conditions often result in reduced H<sub>2</sub> yields and limited organic load removal (OLR) [12].

In contrast, DF is a light-independent process carried out by facultative or strict anaerobic bacteria that convert organic matter into H<sub>2</sub> through acidogenic pathways. DF is closely linked to the acidogenic phase of anaerobic digestion and is recognized for its ability to degrade a wide range of substrates via acetate and butyrate pathways [13]. Numerous studies have documented the efficacy of microorganisms such as *Clostridium*, *Enterobacter*, and *Desulfovibrio* in H<sub>2</sub> production [8]. Despite its advantages, DF application is frequently limited by incomplete substrate oxidation and the accumulation of organic acids. This accumulation causes a pH drop and inhibits microbial activity, ultimately reducing H<sub>2</sub> production [14].

The integration of DF and PF in sequential systems has been proposed as an effective strategy to overcome the limitations of standalone processes [15–17]. In such coupled systems, short-chain organic acids produced during DF serve as carbon sources for PF bacteria, enabling additional H<sub>2</sub> production and reducing the effluent organic load. This synergistic interaction enhances overall process efficiency and resource recovery. However, real carbohydrate-rich wastewaters, such as those derived from fruit-processing industries, are characterized by fluctuations in composition, acidity and nutrient content, which can substantially affect microbial activity and process performance. Despite this, most studies reported in the literature have been conducted at the laboratory scale under tightly controlled conditions, limiting their applicability to real industrial systems. For instance, a coupled DF–PF system treating the organic fraction of municipal solid waste under controlled conditions (35 °C, magnetite catalyst addition) achieved H<sub>2</sub> yields of 4144 mL H<sub>2</sub> g<sup>−1</sup> volatile solids in 2.1-L biodigesters, highlighting its dependence on controlled parameters and additives [15]. Similarly, integrated systems utilizing lignocellulosic hydrolysates operated at a fixed temperature (35 ± 1 °C) and pH (6), achieved a maximum H<sub>2</sub> yield of 4.15 mol H<sub>2</sub> per mol sugar in small-scale reactors (150–525 mL) [16]. Furthermore, semi-continuous thermophilic systems demonstrated a 1.75-fold increase in H<sub>2</sub> production when DF effluents are used as PF substrates [17].

Despite these advances, scale-up introduces challenges related to mass transfer, light distribution, and process stability that are not adequately captured in laboratory-scale batch experiments. Uncontrolled pH and temperature conditions represent common operational realities in industrial facilities. In an 11-m<sup>3</sup> pilot-scale sequential DF–PF reactor, substantial variation in H<sub>2</sub> production was observed among reactor compartments, attributed to poor mixing, biomass washout, and other inhomogeneities associated with large-scale operation [18]. These findings indicate that seasonal fluctuations, variations in effluent pH, and disparities in microbial activity across temperature ranges substantially influence yields in large reactors. However, most studies in integrated systems are conducted under optimized temperature and pH levels, as well as fully sterile conditions, making it difficult to assess operational robustness for scale-up. Furthermore, the displacement of photofermentative *Rhodopseudomonas* species by microbial competitors has not been systematically evaluated under realistic continuous-flow conditions [8]. Consequently, the functional contribution of each stage in the integrated system, as well as overall process viability, remains inadequately understood under non-ideal operational conditions.

The objective of this study was to evaluate a continuous coupled DF–PF system treating a diluted synthetic substrate designed to mimic the carbohydrate-rich, acidic composition of fruit and dairy processing wastewater (excluding fats). The system was operated continuously at a hydraulic retention time (HRT) of 10 h without external pH or temperature control to investigate process behavior under suboptimal operational conditions. This was achieved through physicochemical monitoring and microbial community analysis based on 16S rRNA gene sequencing, building upon previous work on bacterial community identification in H<sub>2</sub>-producing systems [19]. This study provides novel insights by (i) identifying the primary stage responsible for H<sub>2</sub> production and (ii) evaluating the contribution of the PF stage to organic matter removal, while linking overall process performance to microbial community dynamics. By prioritizing process stability and functionality over maximized H<sub>2</sub> yield, this work offers a pragmatic evaluation of operational constraints under non-ideal continuous conditions. Engineered to assess inherent buffering capacity and resilience against typical industrial fluctuations, temperature regulation and pH stabilization were intentionally omitted to align with practical operational requirements rather than laboratory-scale precision.

This study constitutes the first of three stages in the Hi2BIO Project, conducted in collaboration with Ingeobras, a company specializing in wastewater treatment. The promising results obtained herein will serve as the foundation for scaling up to a pilot-plant level (40 L reactors), which in turn will inform the construction of an industrial-scale unit (300 L). In this final phase, following preliminary operational adjustments using synthetic matrices, the system is scheduled to treat real agro-industrial wastewater containing sugar residues and dairy fats characteristic of fruit juice and dairy processing effluents.

## 2. Materials and Methods

The experimental system comprised four identical, parallel processing lines. As illustrated in Figure 1, each line consisted of a cylindrical DF reactor connected in series to a cylindrical PF reactor, resulting in a total of four DF and four PF reactors. Each reactor possessed a total volume of 7 L and a working volume of 5 L. All four lines were operated simultaneously under identical nominal hydraulic and environmental conditions at the industrial facility of Ingeobras S.L. (Zaragoza, Spain). To simulate realistic operational conditions preferred for future large-scale applications, the system was run in continuous mode without active pH or temperature control. Ambient temperature at the facility ranged between 15 and 20 °C during the experimental period (winter–spring season), with no active heating or cooling applied. The reactors were fed at an average volumetric flow rate of

500 mL h<sup>-1</sup>, corresponding to a hydraulic retention time (HRT) of 10 h. Feeding was managed semi-continuously using 30-min on/off pump cycles to maintain the target average flow rate while minimizing hydraulic disturbances. Each reactor was inoculated separately and treated as an independent experimental unit. “Steady-state operation” was defined as the period following the initial acclimation phase, during which the DF effluent was continuously fed into the PF reactor, replacing the initial substrate. The overall monitoring period lasted 30 d. Measurements of VFA composition, biomass concentration as volatile suspended solids (VSS), and photosynthetically active radiation were intentionally omitted. This decision aligned with the host facility’s industrial monitoring constraints to enhance system transferability to large-scale operations. Consequently, results are interpreted at the system level rather than with stage-specific mechanistic resolution.

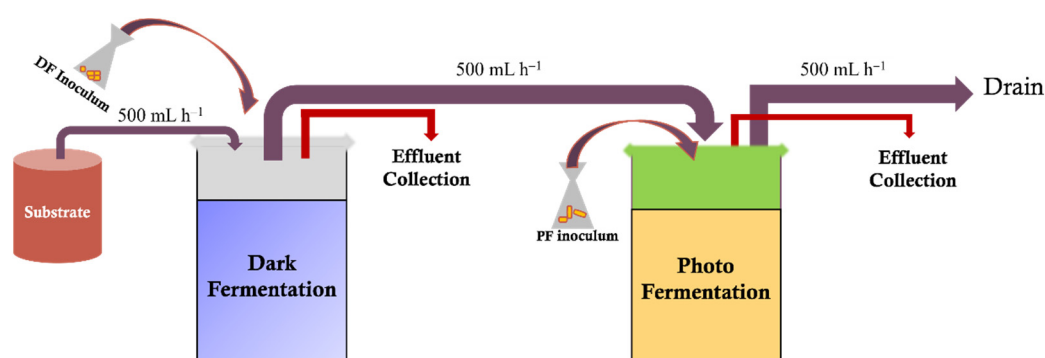


**Figure 1.** Schematic representation of the coupled DF-PF system setup in the industry facility operated in continuous mode.

The feed solution was composed of an analytical-grade sugar mixture containing sucrose (50%), fructose (20%), and lactose (30%) (Panreac Química S.L.U., Barcelona, Spain) at a concentration of 1 g L<sup>-1</sup>. This mixture was formulated to mimic wastewater from complex agri-food industries, specifically those operating processing lines for fruit juices and dairy derivatives. The initial chemical oxygen demand (COD) of the feed was approximately 1035 ± 339 mg L<sup>-1</sup>. This corresponds to an OLR of approximately 2.5 g COD L<sup>-1</sup> d<sup>-1</sup> within the reactor. This loading rate is compatible with the operational requirements of the PF stage, where elevated substrate concentrations are known to inhibit nitrogenase activity [20]. Nutrients were supplemented with 0.2% (*w/v*) orthophosphate and 0.5% (*w/v*) urea to provide buffering capacity and sufficient nitrogen for microbial growth. The characteristics of the influent are summarized in Table 1.

**Table 1.** Physicochemical characteristics of the influent used as substrate in the coupled DF-PF system.

| Parameter                       | Mean ± Standard Deviation |
|---------------------------------|---------------------------|
| COD (mg L <sup>-1</sup> )       | 1035 ± 339                |
| Phosphate (mg L <sup>-1</sup> ) | 20 ± 8                    |
| pH                              | 5.2 ± 0.6                 |
| TN (mg L <sup>-1</sup> )        | 35 ± 7                    |
| ORP (mV)                        | 1                         |

The DF reactor was inoculated with 5% (*v/v*) of a mixed microbial consortium (Bio-forZe MIX/MBR and AD-GAS, Bactemia, Barcelona, Spain), whereas the PF reactor received a 5% (*v/v*) inoculum of a photosynthetic bacterial culture (Photosynthesis Plus, Microbe Life Hydroponics, Island Park, NY, USA). Prior to starting continuous operation, both reactors were filled with the synthetic substrate. The PF reactor operated under alter-

nating light/dark cycles (30 min light/30 min dark) using 590 nm LED strip light sources delivering an average intensity of 5000 lx and a photosynthetic photon flux density of  $47.7 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Operating as a secondary polishing unit, the PF reactor continuously received the effluent from the DF stage. A continuous drain line was connected to the PF reactor to ensure steady effluent discharge, and the system was initially sparged with nitrogen to establish anaerobic conditions.

COD, total nitrogen (TN), and phosphate concentrations were measured using a multiparameter photometer (HI-83224-02, Hanna Instruments, Leighton Buzzard, UK). The pH and oxidation–reduction potential (ORP) were monitored using a combined pH/ORP electrode (HI-991003, Hanna Instruments, Leighton Buzzard, UK).

A total of eighty 100-mL gas samples were collected at intervals of 2 to 3 d using a gas-tight syringe (10 samples per reactor: 40 from DF and 40 from PF) from the 2-L headspace of each unit. The samples were analyzed using a micro–gas chromatograph (micro–GC, Agilent 3000A, Santa Clara, CA, USA). Chromatograms confirmed the presence of only three components:  $\text{H}_2$ ,  $\text{CO}_2$ , and  $\text{N}_2$ . Each sample was analyzed in duplicate, and measurements were averaged to obtain a single value per reactor for each sampling point. The gas volume generated over 24-h periods was determined using 0.5-L and 1-L single polypropylene fitting Tedlar<sup>®</sup> bags (SKC Ltd., Blandford Forum, UK) connected to the purge valve located at the top of each reactor. The valve was then opened to allow the gas accumulated over 24 h—manifested as overpressure—to vent into the bag until the system pressure stabilized. The 100-mL samples drawn for composition analysis were also accounted for in the cumulative volume determination. Bag volumes were measured based on Archimedes' principle using a glass thin-layer chromatography developing tank ( $27.0 \times 26.5 \times 7.0$  cm) slightly tilted and filled with distilled water. The volume was determined by measuring the displaced water mass with an electronic balance with a precision of 0.1 g. Finally, the  $\text{H}_2$  production rate (HPR), expressed as the volume of  $\text{H}_2$  per liter of working reactor volume per day ( $\text{mL H}_2 \text{ L}^{-1} \text{ d}^{-1}$ ) between consecutive sampling points, was calculated using Equation (1)

$$\text{HPR} = \frac{C_{\text{H}_2}}{100} \times \frac{V_t}{V_r \times t} \quad (1)$$

where  $C_{\text{H}_2}$  is the  $\text{H}_2$  concentration (%) determined by GC analysis,  $V_t$  is the cumulative gas volume collected between sampling events,  $V_r$  is the reactor working volume (5 L) and  $t$  is the time elapsed between consecutive sampling points. As detailed in the Results and Discussion section, the distribution of  $\text{H}_2$  concentrations was further characterized using boxplot and kernel density estimation analyses.

Microbial community analysis was performed on the samples collected from the reactors following a 2-week stabilization and subsequent 1-month operation. A total of eight samples were collected (four from DF and four from PF reactors). For each sample, 50 mL of effluent was filtered using a  $0.45\text{-}\mu\text{m}$  black membrane filter (MCE, MF-Millipore, Merck Millipore Ltd., Arklow, Ireland). Genomic DNA was extracted using a NZYtech Soil DNA kit (MB21802, NZYtech, Lisboa, Portugal) according to the manufacturer's protocol. Following extraction, double-stranded DNA concentrations were quantified using a Qubit 3.0 fluorometer with the Qubit Q32851 dsDNA High Sensitivity Assay Kit (both from Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was standardized to a fixed input of 10 ng per sample for PCR amplification. DNA absorbance and purity ratios ( $A_{260}/A_{280}$  and  $A_{260}/A_{230}$ ) were additionally evaluated in duplicate using a SPECTROstar Nano microplate spectrophotometer (BMG LABTECH, Ortenberg, Germany), with 2  $\mu\text{L}$  aliquots.

Amplification process was conducted using a 16S rRNA SQK-16S024 barcoding kit (Oxford Nanopore Technologies, Oxford, UK), which targets the 27F and 1492R priming

sites to generate near full-length amplicons of approximately 1500 bp spanning the V1 to V9 hypervariable regions. Following amplification, the barcoded PCR products were purified using AMPure XP A63880 paramagnetic beads (Beckman Coulter, Indianapolis, IN, USA). The purified amplicons were pooled and sequenced on the MinION platform (Oxford Nanopore Technologies, Oxford, UK). High-throughput sequence analysis was performed using EPI2ME software (version 23.05–02, Oxford Nanopore Technologies, Oxford, UK) aligned against the NCBI 16S reference database to identify bacterial taxa down to species level. For each sample, relative abundance was calculated as the percentage of quality-filtered reads assigned to each taxon.

Statistical analyses were performed using R (version 4.4.3, R Foundation for Statistical Computing, Vienna, Austria). The experimental design comprised four parallel, coupled DF–PF reactor trains, with ten repeated sampling points per reactor. Duplicate GC analyses of the same gas sample were treated as technical replicates and averaged, yielding 40 temporal observations for each reactor stage. Differences in H<sub>2</sub> concentrations between the DF and PF stages were evaluated using a paired Wilcoxon signed-rank test—rather than an independent two-sample test—to account for the fact that DF and PF headspace samples were collected from the same reactor train on the same sampling date. The paired sample size and exact test statistic are reported in Section 3.1. Furthermore, the repeated-measures correlation between pH and H<sub>2</sub> concentration in the DF stage was calculated following the methodology of Bakdash and Marusich [21], which partials out between-reactor variance to estimate the common within-reactor association. The resulting correlation coefficient, degrees of freedom, 95% confidence interval, exact *p*-value and sample size are represented alongside a scatterplot in Section 3.1. This result is interpreted considering that both pH decline and H<sub>2</sub> accumulation are expected co-products of acidogenic fermentation rather than independent variables.

### 3. Results and Discussion

#### 3.1. Reactor Performance

Continuous operation of the coupled DF–PF system at an HRT of 10 h revealed a distinct functional differentiation between the two stages: H<sub>2</sub> production occurred predominantly in the DF stage, whereas higher mass COD removal was achieved in the PF stage. The H<sub>2</sub> concentration in the headspace differed substantially between the two bioreactors throughout the experimental period. GC analysis revealed H<sub>2</sub> concentrations ranging from 0.44% to 57% (*v/v*), confirming the metabolic variability inherent to continuous operation under uncontrolled pH and temperature conditions. The HPR in the DF stage exhibited considerable fluctuation, ranging from nearly 0 to a peak value of 177 mL H<sub>2</sub> L<sup>−1</sup> d<sup>−1</sup>, reflecting periods of active acidogenic fermentation. However, the absence of a standalone DF control reactor precludes isolating the incremental contribution of the PF stage to overall system performance. Consequently, these findings are interpreted as system-level observations rather than definitive stage-specific attributions.

In contrast, the PF bioreactor exhibited consistently low headspace H<sub>2</sub> concentrations, averaging 1.8% ± 1.7% (*v/v*) and contributing negligibly to total H<sub>2</sub> production under the tested conditions. The mean HPR during positive accumulation periods was 5 mL H<sub>2</sub> L<sup>−1</sup> d<sup>−1</sup>, which was approximately 39-fold lower than the peak rate observed in the DF stage. These findings suggest that variations in substrate availability, redox conditions, and microbial competition could substantially influence metabolic pathways, leading to notable variability in fermentation products under uncontrolled pH and temperature conditions [7]. Therefore, the present results support the hypothesis that DF functions as the primary H<sub>2</sub>-producing stage in this two-stage system, whereas PF contributes negligibly to overall H<sub>2</sub> generation under the evaluated operational boundaries.

Headspace  $\text{CO}_2$  concentrations ranged between 4% and 25%, while  $\text{N}_2$  constituted the predominant component of the gas phase—in some cases exceeding 90%—as it was utilized both as a carrier gas and to maintain anaerobic conditions. The temporal evolution of gas composition in the DF and PF reactors is illustrated in Figure 2.

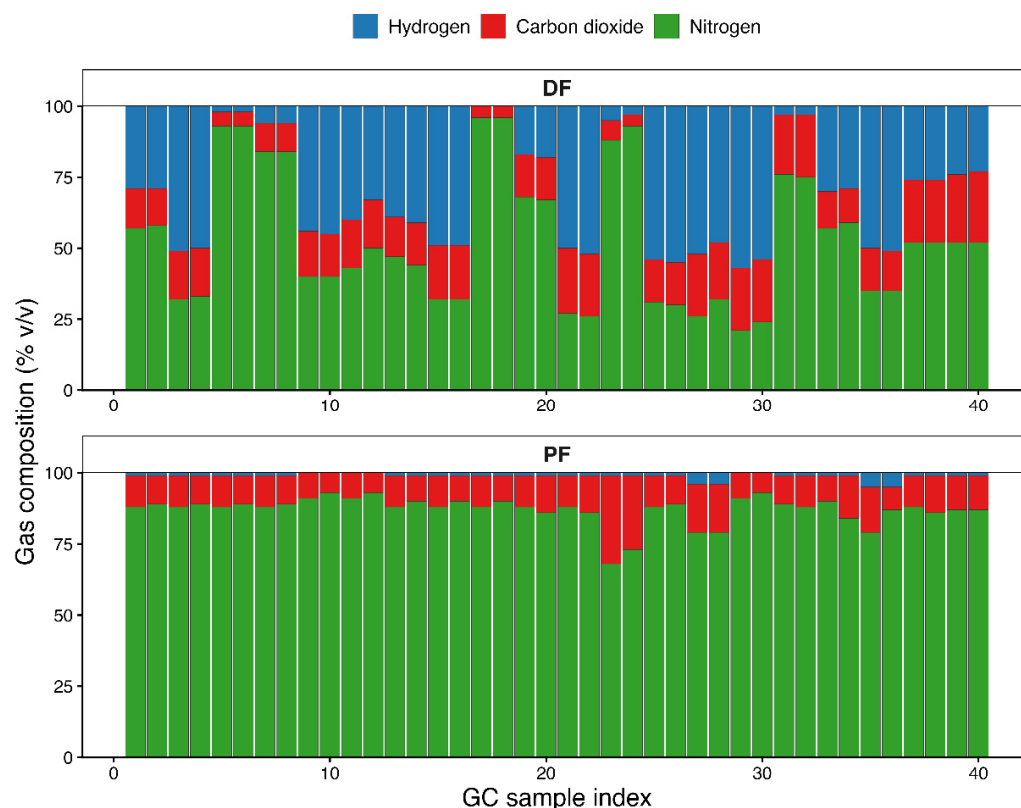


**Figure 2.** Temporal evolution of gas composition (%  $v/v$ ) in the DF and PF stages. Forty gas samples were analyzed per stage, comprising ten samples from each of four parallel reactor setups. Duplicate GC measurements were averaged before plotting. Each bar represents the relative fractions of  $\text{H}_2$ ,  $\text{CO}_2$ , and  $\text{N}_2$  in one gas sample.

Conversely, the PF reactor exhibited a more stable gas composition, dominated by  $\text{N}_2$ , followed by  $\text{CO}_2$  and characterized by low  $\text{H}_2$  fractions. This low  $\text{H}_2$  content is likely attributable to microbial competition and suboptimal environmental conditions, which can divert electron flow away from  $\text{H}_2$ -producing metabolic pathways. This comparison underscores the distinct performance dynamics and  $\text{H}_2$  generation potential inherent to each bioreactor stage.

The distribution of HPR in the DF stage was highly dispersed, with a coefficient of variation (CV) of 71.6% (Table 2). This pronounced variability reflects process instability during continuous operation in the absence of pH and temperature controls. The minimum pH recorded in DF was 2.5. Such severe acidity exerts an inhibitory effect on the  $\text{H}_2$ -producing bacteria, directly contributing to the elevated CV. Rather than representing routine dynamic behavior, this fluctuation highlights a fundamental operational constraint stemming from non-linear bacterial metabolism and interspecies substrate competition within the mixed consortium, a factor with direct implications for process reliability in any future scale-up scenarios. Although distinct phases of relatively stable production occurred during the experimental run, metabolic shifts between these phases ultimately led to the high overall CV. The descriptive statistics of the physicochemical parameters and  $\text{H}_2$  production are summarized in Table 2, while the temporal HPR profiles for both DF and PF across sampling points are depicted in Figure 3.

**Table 2.** Descriptive statistics of physicochemical parameters and gas-phase H<sub>2</sub> concentrations in the DF and PF stages.

|                                           | Mean   | St. Dev. | Minimum | 25%   | 50%    | 75%    | Maximum | CV%   |
|-------------------------------------------|--------|----------|---------|-------|--------|--------|---------|-------|
| COD_IN (mg L <sup>−1</sup> )              | 1034.6 | 339.5    | 380.0   | 790.0 | 1030.0 | 1290.0 | 1560.0  | 32.8  |
| N_IN (mg L <sup>−1</sup> )                | 35.14  | 10.32    | 20.0    | 29.8  | 34.0   | 40.4   | 58.4    | 29.4  |
| PO <sub>4</sub> _IN (mg L <sup>−1</sup> ) | 19.96  | 6.26     | 10.6    | 14.4  | 19.4   | 25.2   | 32.0    | 31.4  |
| pH_DF                                     | 4.23   | 0.68     | 2.65    | 3.85  | 4.3    | 4.71   | 5.57    | 16.1  |
| H <sub>2</sub> _DF_concentration (% v/v)  | 28.11  | 20.12    | 0.44    | 7.10  | 28.92  | 49.57  | 55.34   | 71.6  |
| H <sub>2</sub> _PF_concentration (% v/v)  | 1.77   | 1.78     | 0.66    | 0.82  | 0.87   | 1.82   | 4.79    | 100.6 |

Descriptive statistics for headspace H<sub>2</sub> concentration were calculated from 40 temporal observations per stage, comprising ten sampling points from each of four independent parallel reactors. For each sampling point, duplicate GC analyses were averaged prior to statistical calculations, with each reactor serving as an independent experimental unit.

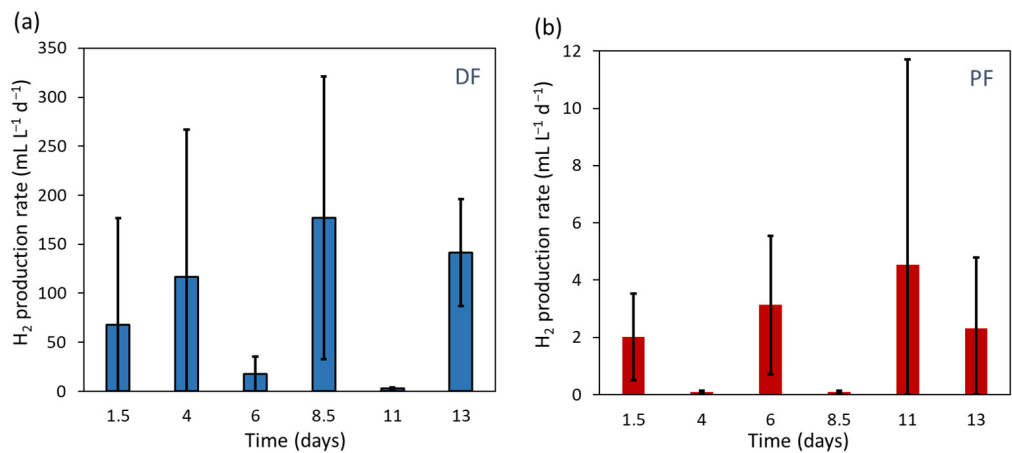


**Figure 3.** (a,b) HPRs (mean ± standard deviation) in DF and PF reactors across sampling points. The pronounced variability observed in the DF stage reflects metabolic shifts under uncontrolled operating conditions, whereas the PF stage shows consistently low H<sub>2</sub> production.

Headspace H<sub>2</sub> concentration distributions were analyzed using both boxplot (Figure 4) and kernel density estimation (Figure 5). The boxplot representation highlights a clear separation in performance between the DF and PF stages. The DF system exhibited a wider interquartile range and multiple high-value observations (>50% H<sub>2</sub>), indicative of dynamic metabolic fluctuations and favorable acidogenic phases associated with acetate and butyrate pathways. Conversely, the PF system displayed a narrow distribution centered around low H<sub>2</sub> concentrations, reflecting consistent low H<sub>2</sub> generation despite the presence of H<sub>2</sub>-producing bacteria. Skewness analysis indicated that H<sub>2</sub> concentration in the DF reactor was approximately symmetrical, suggesting transitions between low and high production states without a single dominant regime. This density profile reflects periodic shifts between metabolic phases rather than steady-state behavior. In contrast, the PF reactor exhibited slight positive skewness, indicating occasional moderate increases above its low baseline. The Shapiro–Wilk test confirmed that H<sub>2</sub> concentrations across all reactors deviated significantly from a normal distribution ( $p < 0.05$ ), justifying the use of non-parametric statistical methods. Because the DF and PF reactors operated in coupled series across four replicate trains, performance between the two stages was evaluated using a paired Wilcoxon signed-rank test rather than an independent rank-sum test. Headspace H<sub>2</sub> concentration was significantly higher in the DF stage than in the PF stage (paired Wilcoxon signed-rank test,  $V = 820$ ,  $n = 40$  paired observations,  $p < 0.001$ ), establishing DF as the primary H<sub>2</sub>-producing stage in the integrated system.

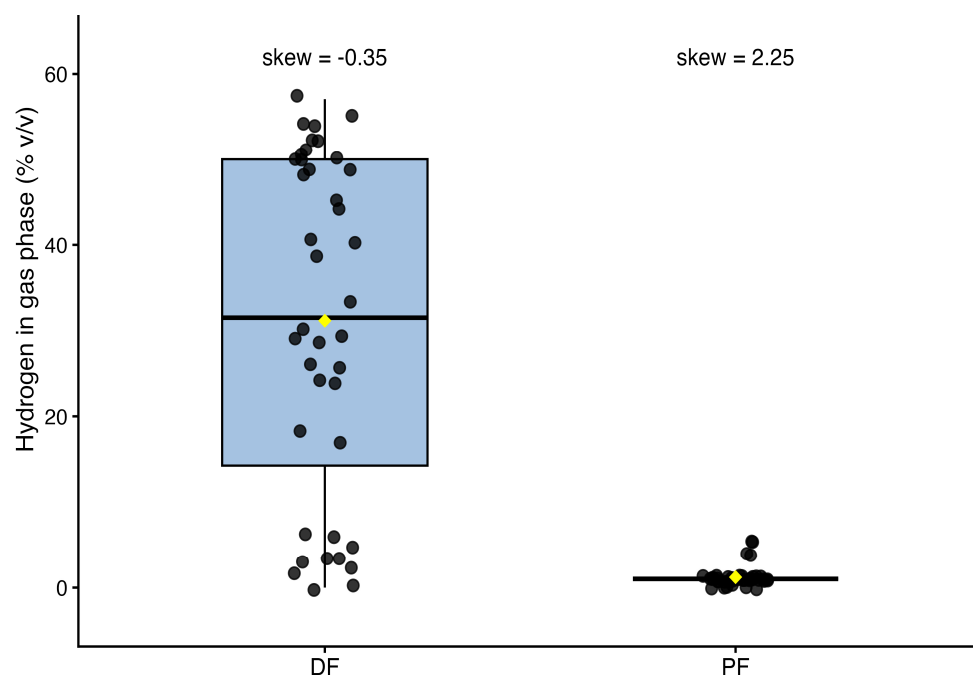


**Figure 4.** Distribution of headspace  $H_2$  concentrations in the DF and PF stages. Individual points represent repeated temporal observations across four parallel reactor trains after averaging technical duplicate GC analyses. Reactor–train identity is indicated by point symbols. Statistical comparison between stages was performed using a beta-mixed effects model to account for repeated observations within reactors.

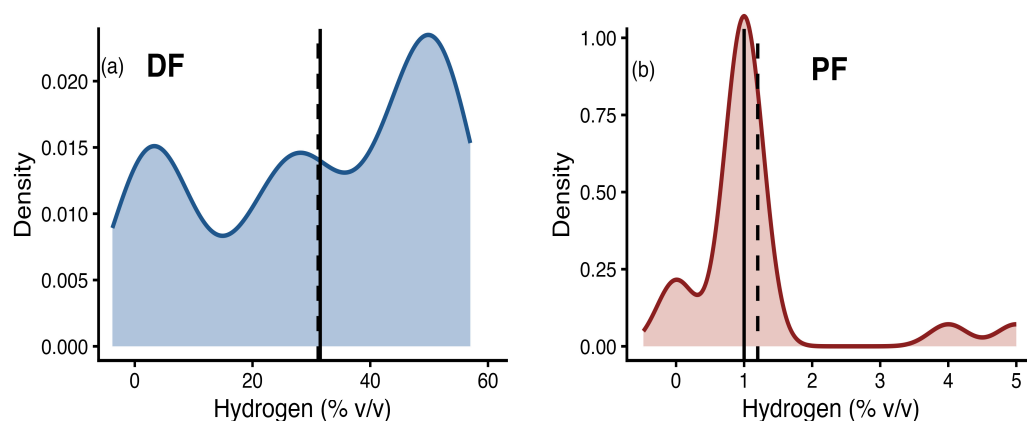


**Figure 5.** Kernel density distribution of  $H_2$  concentration (%  $v/v$ ) in (a) DF bioreactor and (b) PF bioreactor. Full line: median, dashed line: mean.

Kernel density estimates further support these observations. The DF distribution (Figure 5a) displays a broad profile with an extended right tail, indicating frequent high- $H_2$  production events. In contrast, the PF distribution (Figure 5b) is tightly clustered at low values, reflecting stable but minimal  $H_2$  generation. These findings are corroborated by the CV analysis. The marked distinction between the two distributions highlights the key role of DF in  $H_2$  generation, while confirming that PF makes a minor contribution under the tested conditions.

The initial COD of the effluent averaged  $1034.6 \text{ mg L}^{-1}$  (CV = 32.8%), indicating moderate variability in the OLR. In the DF reactor, COD decreased to  $850 \text{ mg L}^{-1}$ , corresponding to an average removal efficiency of 18% and a COD removal rate of  $0.44 \text{ g COD L}^{-1} \text{ d}^{-1}$ . In contrast, the PF reactor achieved an additional COD removal of approximately 20%, reducing the COD to  $684 \text{ mg L}^{-1}$  (removal rate:  $0.84 \text{ g COD L}^{-1} \text{ d}^{-1}$ ). Overall, the coupled

system achieved a total COD reduction of 34%, demonstrating the complementary role of PF as a secondary treatment stage. However, because the DF–PF system was integrated, the ~20% COD removal attributed to PF cannot be verified independently. This is due to intense competition between the species present in the effluent and the photofermentative bacteria, as well as the sustained metabolic activity of DF–derived species within the effluent. Since the objective of this study was to assess overall system activity rather than stage-specific operations, the additional COD reduction across the PF stage primarily indicates the continued transformation of residual organic matter. In sequential DF–PF systems, the PF influent commonly contains organic acids generated during DF, which serve as carbon sources and electron donors for photofermentative bacteria. Nevertheless, because individual VFAs and residual sugars were not quantified, the chemical identity of the COD removed during PF cannot be directly established. This behavior aligns with previous findings indicating that elevated substrate concentrations can shift bacterial metabolism toward VFA accumulation rather than H<sub>2</sub> generation [22]. Table 3 summarizes the substrate characteristics before and after each fermentation stage.

**Table 3.** Physicochemical characteristics of the feed, DF effluent/PF influent, and PF effluent (mean  $\pm$  standard deviation).

|                                       | Influent<br>(Mean $\pm$ St. Dev.) | DF<br>(Mean $\pm$ St. Dev.) | PF<br>(Mean $\pm$ St. Dev.) |
|---------------------------------------|-----------------------------------|-----------------------------|-----------------------------|
| COD (mg L <sup>−1</sup> )             | 1034.6 $\pm$ 339.5                | 850 $\pm$ 253.5             | 684 $\pm$ 334.2             |
| N (mg L <sup>−1</sup> )               | 35.14 $\pm$ 10.32                 | 35.3 $\pm$ 8.90             | 27.7 $\pm$ 9.20             |
| PO <sub>4</sub> (mg L <sup>−1</sup> ) | 19.96 $\pm$ 6.26                  | 18.6 $\pm$ 8.30             | 14.6 $\pm$ 6.20             |
| pH                                    | 5.2 $\pm$ 0.60                    | 4.23 $\pm$ 0.68             | 4.77 $\pm$ 0.56             |
| ORP (mV)                              | 1                                 | −200 $\pm$ 91               | −138 $\pm$ 89               |

The composition of the synthetic substrate may also have contributed to the marked difference between DF and PF performance. The feed consisted entirely of readily fermentable carbohydrates—such as fructose, lactose, and sucrose—which acidogenic microorganisms can utilize directly following initial uptake or hydrolysis. Consequently, substantial transformation of the available sugars likely occurred during DF, leaving the PF stage with an acidified effluent containing fewer readily fermentable sugars and a distinct spectrum of soluble metabolites. Crucially, the 18% decrease in COD across DF does not imply that only 18% of the sugars were consumed, as the conversion of sugars into soluble organic acids and alcohols retains a considerable portion of the initial COD within the liquid phase.

The composition of the DF effluent is critical for subsequent PF performance. Previous integrated DF–PF studies have shown that rapid sugar conversion during DF can generate organic acids faster than photofermentative bacteria can consume them. When the metabolic rates of the two stages are unbalanced, this mismatch leads to acid accumulation and a severe pH drop [23]. Furthermore, the specific profile and relative proportions of acetate, butyrate, propionate, and lactate in DF effluents strongly influence photofermentative growth and H<sub>2</sub> production [24]. Therefore, the prior depletion of simple sugars during DF and the resulting metabolite profile offer a plausible explanation for the low PF H<sub>2</sub> production observed in this study. However, because residual sugars and individual fermentation metabolites were not quantified, this interpretation remains a hypothesis rather than a verified mechanism.

Comparing these results with those previously reported DF–PF systems indicates that our performance reflects a trade-off between H<sub>2</sub> generation and operational realism. Higher HPRs are routinely achieved in tightly controlled conditions, such as optimized temperature, fixed pH, or the use of addition of chemical supplements [15–17]. For in-

stance, a recent review of integrated  $H_2$  systems highlighted theoretical or highly optimized HPR values between 1500 and 3500 kg  $H_2$   $d^{-1}$  for food waste-based processes [25]. Similarly, batch studies treating wheat wastewater reached HPRs of approximately 700 mL  $H_2$   $L^{-1}$  in DF and 680 mL  $H_2$   $L^{-1}$  in PF under controlled temperature (30 °C) and pH conditions [26], whereas systems treating cheese whey and fruit/vegetable waste achieved up to 95% COD removal [24]. Furthermore, two-stage batch systems operated under thermophilic or pH-optimized conditions have yielded HPRs of 27.3 mmol  $H_2$   $L^{-1}$   $d^{-1}$  in DF and 9.33 mmol  $H_2$   $L^{-1}$   $d^{-1}$  in PF [27]. A more directly comparable benchmark for our continuous system is a pilot-scale sequential DF–PF trial (11  $m^3$  total: 3  $m^3$  DF, 8  $m^3$  PF) fed with hydrolyzed corn stover. Operated under controlled conditions (DF at pH 4.5, 35 °C; PF at pH 7.0, 30 °C), that system achieved DF and PF rates of 7.5  $m^3$   $H_2$   $m^{-3}$   $d^{-1}$  (7500 mL  $H_2$   $L^{-1}$   $d^{-1}$ ) and 4.7  $m^3$   $m^{-3}$   $d^{-1}$  (4700 mL  $H_2$   $L^{-1}$   $d^{-1}$ ), respectively [18]. In contrast, the present study recorded maximum HPRs of 177 mL  $H_2$   $L^{-1}$   $d^{-1}$  in DF and 5 mL  $H_2$   $L^{-1}$   $d^{-1}$  in PF. This marked difference underscores that volumetric  $H_2$  productivity under the unmonitored regime used here is constrained primarily by the absence of strict temperature and pH control, rather than by reactor scale.

In contrast, the present study was conducted in continuous mode, in a 5 L working volume and without external temperature or pH control. While these conditions more closely resemble industrial operation—aligning with the ultimate goal of scaling up to pilot (40 L) and industrial levels (600 L)—they inherently constrain process optimization. Under these unmonitored conditions,  $H_2$  generation was predominantly associated with the DF stage, whereas the PF stage exhibited a low HPR while contributing to the OLR. This functional differentiation is particularly evident under suboptimal operating regimes, where overall process stability and robustness are prioritized over maximizing HPR.

The application of metabolic modeling to carbohydrate conversion provides valuable insights into the efficiency of  $H_2$  generation. Based on electron equivalency, 1 g COD is equivalent to 1 g  $O_2$ , or 0.125 mol of electrons. Since the reduction of protons to  $H_2$  requires two electrons per molecule, the theoretical yield is 0.0625 mol  $H_2$   $g^{-1}$  COD removed. At STP, this corresponds to approximately 1.40 L  $H_2$   $g^{-1}$  COD removed. However, this theoretical maximum is unattainable due to thermodynamic constraints, metabolic competition, and incomplete substrate conversion. Based on the COD removed in each stage (DF: 18%, PF: 20%) and an initial COD of 1035 mg  $L^{-1}$ , the maximum theoretical  $H_2$  yields are 1.29 L  $H_2$   $g^{-1}$  COD removed for DF and 1.16 L  $H_2$   $g^{-1}$  COD removed for PF. These bounds represent ideal thermodynamic conditions, assuming complete substrate oxidation and electron transfer to protons. Furthermore, biological pathways impose additional constraints. The Thauer limit dictates a maximum stoichiometric yield of 4 mol  $H_2$   $mol^{-1}$  glucose via the acetate and butyrate pathways, corresponding to approximately 0.47 L  $H_2$   $g^{-1}$  COD for glucose-equivalent substrates. In this study, the maximum observed HPRs—177 mL  $H_2$   $L^{-1}$   $d^{-1}$  in DF and 5 mL  $H_2$   $L^{-1}$   $d^{-1}$  in PF—represent only a modest (DF) to minimal (PF) fraction of these theoretical limits. This gap underscores the combined effects of thermodynamic losses, microbial competition, and incomplete substrate conversion, inherent to the unoptimized continuous operation employed.

The initial ORP in the DF bioreactor was 1 mV, while the pH was monitored but not externally controlled. Over time, the DF reactor maintained strongly reducing conditions with an average ORP of  $-200 \pm 91$  mV, which are conducive to strict anaerobic bacteria and [Fe–Fe]–hydrogenase activity [28]. In contrast, the PF reactor sustained moderately reducing conditions ( $-138 \pm 89$  mV). Negative ORP values have been shown to promote the proliferation of anaerobic  $H_2$ –producing bacteria [29]. Typically, facultative anaerobes predominate initially until sufficiently low ORP conditions allow strict anaerobes to thrive. In the DF reactor, the sustained negative ORP values confirmed the presence of a stable

anaerobic environment, thereby facilitating efficient electron transfer for H<sub>2</sub> production. Conversely, although anaerobic conditions were maintained in the PF reactor, the less reducing environment likely favored non-H<sub>2</sub>-producing species, ultimately contributing to a lower HPR.

Moreover, pH is of critical importance in continuous H<sub>2</sub> production. In DF systems, acidic conditions inhibit methanogens while favoring H<sub>2</sub>-producing bacteria. Conversely, PF systems typically require neutral to slightly alkaline conditions (around pH 7.5) for optimal activity [30], although the exact optimal pH depends on the inoculum and substrate [31]. In this study, the influent pH decreased from  $5.2 \pm 0.6$  to  $4.23 \pm 0.68$  in the DF reactor, reflecting active acidogenic metabolism and favorable conditions for H<sub>2</sub> generation. However, the reactor repeated-measures correlation between pH and H<sub>2</sub> concentrations in DF was weak and not statistically significant ( $r = -0.30$ ,  $p = 0.198$ ). This result does not support a direct causal link between pH and H<sub>2</sub> concentration; rather, it indicates that both the pH drop and the headspace H<sub>2</sub> accumulation are concurrent co-products of acidogenic fermentation, rather than one variable driving the other. In contrast, the pH in the PF reactor exhibited a marginal increase to  $4.8 \pm 0.6$ , attributable to the partial consumption of VFAs. Nevertheless, this remained well below the optimal pH range for PF bacteria, likely constraining the observed HPR. Notwithstanding this limitation, the PF stage contributed significantly to organic matter removal, which may play a key role in microbial competition dynamics and substrate utilization.

Nutrient dynamics were also monitored, as phosphate and nitrogen are essential for microbial growth and metabolism [32]. A progressive reduction in phosphate concentration was observed across both reactors, indicating its role in supporting overall microbial activity rather than directly driving H<sub>2</sub> production. In contrast, TN exhibited a dual effect: while insufficient TN concentrations can restrict microbial growth and reduce H<sub>2</sub> production, elevated levels may alter metabolic pathways and divert carbon toward alternative byproducts [33]. In the DF bioreactor, TN decreased slightly from  $35 \pm 7$  to  $33 \pm 1$  mg L<sup>-1</sup>, reflecting minimal consumption and negligible inhibition of hydrogenase activity. Conversely, the PF reactor exhibited a more pronounced TN reduction, dropping to  $27 \pm 3$  mg L<sup>-1</sup>. This enhanced nitrogen assimilation likely suppressed the expression of the nitrogenase enzyme complex—responsible for photofermentative H<sub>2</sub> generation—thereby contributing to the low HPR observed in the PF stage [34].

A key limitation of this process evaluation is the lack of specific measurements for residual sugars, VFAs, and alcohols in the DF and PF effluents. While COD quantifies the aggregate oxidation equivalent of organic matter, it cannot distinguish whether the remaining COD represents unconsumed sugars, acetate, butyrate, lactate, ethanol, or other metabolites. Consequently, the individual impacts of substrate depletion, metabolite composition, and product inhibition on PF H<sub>2</sub> production could not be decoupled. Future continuous DF–PF studies should combine COD monitoring with chromatographic profiling of sugars and fermentation products at the feed, DF outlet, and PF outlet.

### 3.2. Bacterial Community Composition

The relative abundance of the microbial community provides a mechanistic basis for interpreting the distinct H<sub>2</sub> production performances of the DF and PF stages. Reactor sample analysis revealed a total of 26 bacterial species, as shown in Table 4. The microbial communities in both reactors are described below in terms of their relative abundance and functional roles in H<sub>2</sub> production and OLR reduction (Figure 6). In the DF bioreactor, H<sub>2</sub>-producing bacteria collectively accounted for 80.7% of the total community, driven by the dominance of strict and facultative anaerobic taxa. The most abundant species included *Raoultella ornithinolytica*, *Clostridium saccharobutylicum*, *Clostridium saccharoperbuty-*

*lacetonicum*, *Raoultella planticola*, *Klebsiella aerogenes*, *Clostridium chromiireducens*, *Klebsiella pasteurii*, *Pseudomonas extremaustralis*, *Clostridium beijerinckii*, *Pseudomonas veronii*, *Clostridium puniceum*, and *Citrobacter murlinae*. The presence of *Clostridium* species is commonly associated with high H<sub>2</sub> yields, as these microorganisms utilize metabolic pathways involving pyruvate formate-lyase and [Fe-Fe] hydrogenase systems (Table 4). Furthermore, they are well adapted to reduced environments characterized by negative ORP and acidic pH, as observed in this study.

**Table 4.** Bacterial species identified in the inocula of the DF and PF reactors, including metabolic classification and associated H<sub>2</sub> production pathways or functional roles.

| Bacteria                                      | Class                                                            | Reported H <sub>2</sub> -Related Pathway or Potential Ecological Role                                                                          |
|-----------------------------------------------|------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Raoultella ornithinolytica</i>             | Facultative anaerobe                                             | Pyruvate formate-lyase [35]                                                                                                                    |
| <i>Clostridium saccharobutylicum</i>          | Strict anaerobe                                                  | Butyrate-acetate fermentation via [FeFe]-hydrogenase [36]                                                                                      |
| <i>Clostridium saccharoperbutylacetonicum</i> | Strict anaerobe                                                  | Solventogenic phase, formate hydrogenlyase and hydrogenase [37]                                                                                |
| <i>Huaxiibacter chinensis</i>                 | Aerobe                                                           | Indirectly helps in maintaining a reduced environment by consuming oxygen-aerobic respiration (electron transport chain) [38]                  |
| <i>Raoultella planticola</i>                  | Facultative anaerobe                                             | Formate H <sub>2</sub> -lyase [39]                                                                                                             |
| <i>Janthinobacterium rivuli</i>               | Aerobe                                                           | Indirectly helps in maintaining a reduced environment by consuming oxygen-aerobic respiration [38,40]                                          |
| <i>Klebsiella aerogenes</i>                   | Facultative anaerobe                                             | Formate H <sub>2</sub> -lyase system [41]                                                                                                      |
| <i>Lactococcus lactis</i>                     | Facultative anaerobe                                             | Inhibitor of H <sub>2</sub> production pathway or may produce simple substrates for H <sub>2</sub> producers in a competitive environment [42] |
| <i>Clostridium chromiireducens</i>            | Strict anaerobe                                                  | Metal-reducing <i>Clostridium</i> [43]                                                                                                         |
| <i>Klebsiella pasteurii</i>                   | Facultative anaerobe                                             | Mixed-acid fermentation H <sub>2</sub> [44]                                                                                                    |
| <i>Leuconostoc mesenteroides</i>              | Facultative anaerobe                                             | Lactate-driven pathways hydrolyze the complex organic matter present into lactate to be used by H <sub>2</sub> producers [45]                  |
| <i>Pseudomonas extremaustralis</i>            | Aerobe                                                           | [FeFe] hydrogenase pathway, when co-cultured with algae for H <sub>2</sub> [46,47]                                                             |
| <i>Clostridium beijerinckii</i>               | Strict anaerobe                                                  | Butanol-acetone pathway [48]                                                                                                                   |
| <i>Pseudomonas veronii</i>                    | Aerobe                                                           | <i>Pseudomonas</i> can be used as a co-culture with algae for H <sub>2</sub> [47]                                                              |
| <i>Clostridium puniceum</i>                   | Strict anaerobe                                                  | Anaerobic fermentation [49]                                                                                                                    |
| <i>Comamonas testosteroni</i>                 | Aerobe but strains with nitrate reductases-facultative anaerobes | Facultative anaerobiosis via Nar-type nitrate reductases [50]                                                                                  |
| <i>Janthinobacterium lividum</i>              | Aerobe                                                           | Indirectly favoring anaerobic H <sub>2</sub> producers by removing O <sub>2</sub> - Aerobic respiration [51]                                   |
| <i>Pseudomonas fildesensis</i>                | Aerobe                                                           | [FeFe] hydrogenase pathway, when co-cultured with algae for H <sub>2</sub> [47]                                                                |
| <i>Clostridium acidisoli</i>                  | Strict anaerobe                                                  | Acid-tolerant fermenter with hydrogenase activity [52]                                                                                         |

Table 4. Cont.

| Bacteria                          | Class                | Reported H <sub>2</sub> -Related Pathway or Potential Ecological Role                                                                                     |
|-----------------------------------|----------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Lactococcus cremoris</i>       | Facultative anaerobe | Inhibitor of H <sub>2</sub> production pathway or indirectly helps by producing substrates for H <sub>2</sub> producers in a competitive environment [53] |
| <i>Citrobacter murlinae</i>       | Facultative anaerobe | Not widely studied by Genus, it is well known for mixed-acid fermentation to produce H <sub>2</sub> [54]                                                  |
| <i>Duganella levis</i>            | Aerobe               | Indirectly favoring anaerobic H <sub>2</sub> producers by removing O <sub>2</sub> —aerobic respiration [55]                                               |
| <i>Lactococcus chungangensis</i>  | Facultative anaerobe | Lactate-driven pathways hydrolyze the complex organic matter present into lactate to be used by H <sub>2</sub> producers [56]                             |
| <i>Novosphingobium capsulatum</i> | Aerobe               | May indirectly favor anaerobic H <sub>2</sub> producers by removing O <sub>2</sub> —aerobic respiration [57]                                              |
| <i>Rhodopseudomonas faecalis</i>  | Facultative anaerobe | Photoheterotrophic pathway using nitrogenase enzyme for H <sub>2</sub> production [58]                                                                    |
| <i>Rhodopseudomonas telluris</i>  | Facultative anaerobe | Photoheterotrophic pathway using nitrogenase enzyme for H <sub>2</sub> production [8]                                                                     |

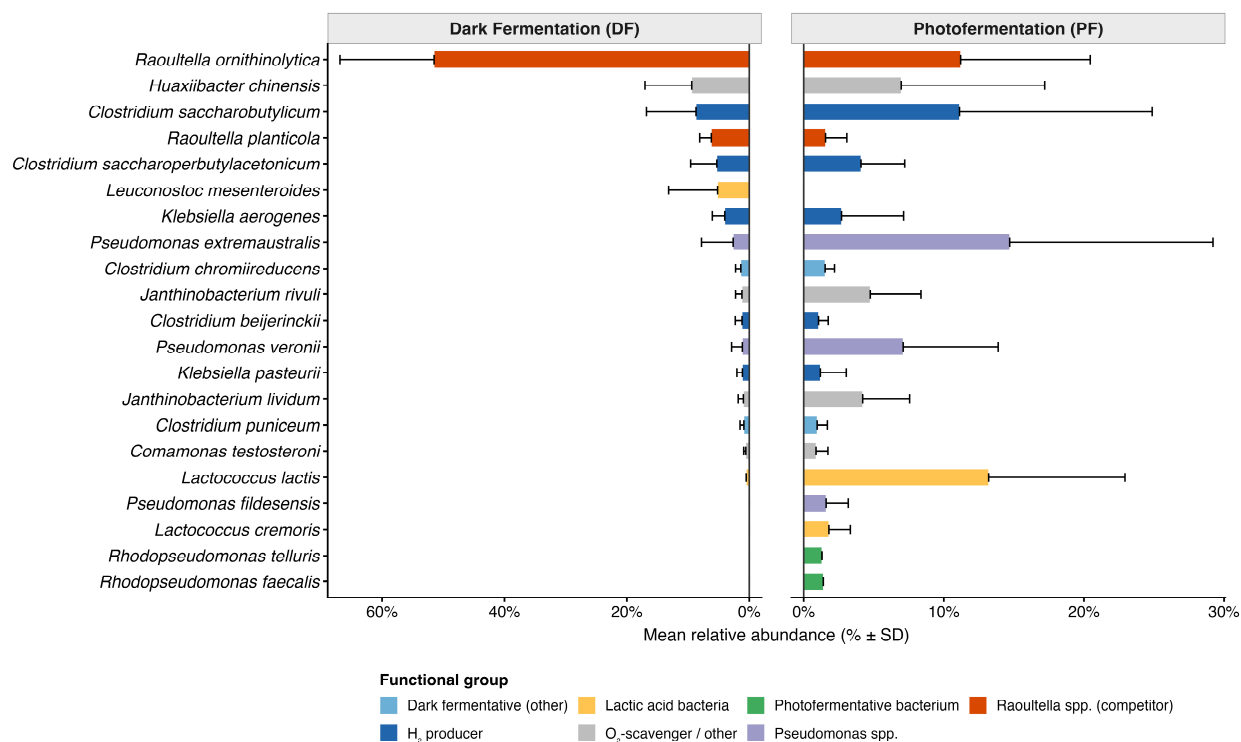


Figure 6. Mean relative abundance (%) ± standard deviation (SD) of bacterial species identified in the DF and PF reactors. Error bars represent the standard deviation across samples. Functional roles are assigned from Table 4.

Furthermore, oxygen-tolerant species, including *Huaxiibacter chinensis*, *Janthinobacterium rivuli*, *Janthinobacterium lividum*, *Comamonas testosteroni*, and *Duganella levis*, were also detected. These organisms likely competed with anaerobic H<sub>2</sub>-producing bacteria for available substrate. Nonetheless, the overall dominance of strict and facultative anaerobes

maintained strongly reducing conditions, supporting continuous NADH regeneration and sustained organic carbon oxidation [59]. Among the dominant taxa in the DF reactor were *Clostridium saccharobutylicum* (8.7%) and *Clostridium saccharoperbutylacetonicum* (5.3%). *Raoultella planticola* (6.2%), *Klebsiella aerogenes* (4.0%), and *Raoultella ornithinolytica* (51.5%). The predominance of *Raoultella ornithinolytica* is particularly noteworthy, as *Clostridium* species are more commonly reported as the primary H<sub>2</sub>-producing genus in DF systems. This shift likely reflects the continuous, unmonitored operating conditions and the carbohydrate-rich substrate supplied. Aerobic, oxygen-scavenging species (e.g., *Huaxiibacter chinensis* at 9.4% and *Janthinobacterium* species at 1.7%) collectively accounted for 15.6% of the DF community. They likely helped maintain the strongly reducing conditions observed (average ORP:  $-200 \pm 91$  mV) by consuming residual dissolved oxygen, thereby protecting strict anaerobes [60]. Beyond oxygen scavenging, *Pseudomonas extremaustralis* (2.6%) and *Pseudomonas veronii* (1.1%) compete with *Clostridium* species for carbohydrates. *Pseudomonas* strains can ferment glucose and fructose via mixed-acid pathways, diverting carbon flux away from the butyrate-acetate pathways responsible for H<sub>2</sub> generation [47]. Consequently, the co-existence of *Clostridium* and *Pseudomonas* reflects a balance between an indirect syntrophic benefit (oxygen scavenging enabling strict anaerobic metabolism) and direct substrate competition that partially limits HPR. The interaction between *Clostridium* and *Lactococcus* in the DF reactor also exhibited a dual character. At its low abundance observed in the DF stage (0.5%), *Lactococcus lactis* likely acted as a substrate pre-processor rather than a competitor. Lactic acid produced from residual lactose hydrolysis can be cross-fed to *Clostridium* species through the electron-transferring flavoprotein-lactate dehydrogenase (EtfAB-ldh) complex, converting lactate and acetate to butyrate, H<sub>2</sub> and CO<sub>2</sub> [45]. This syntrophic interaction supplements rather than diminishes H<sub>2</sub> production at low cell densities, which aligns with the DF stage achieving H<sub>2</sub> concentrations up to 57% (v/v). At this low relative abundance, *Lactococcus* did not cross the threshold where competitive substrate diversion outweighs the benefits of syntrophic cross-feeding [42].

In the PF bioreactor, a marked shift in community composition was observed relative to the DF stage, characterized by a reduction in the relative abundance of H<sub>2</sub>-producing taxa to 40.0% and a corresponding increase in competing and inhibitory populations. Photofermentative H<sub>2</sub>-producing bacteria, including *Rhodopseudomonas faecalis* (1.4%) and *Rhodopseudomonas telluris* (1.3%), accounted for a combined mean relative abundance of only 2.7% (Figure 6). This exceptionally low abundance indicates that PF species were severely suppressed by substrate competition and environmental constraints. Furthermore, this depletion aligns with the negligible H<sub>2</sub> concentrations observed in the PF stage, suggesting that the limited HPR was driven primarily by the competitive exclusion of *Rhodopseudomonas* species rather than by the suboptimal environmental parameters alone. Interestingly, the PF reactor retained several H<sub>2</sub>-producing bacteria typically associated with DF, including *Clostridium saccharobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium beijerinckii*, *Clostridium puniceum*, *Clostridium chromiireducens*, *Clostridium acidisoli*, *Klebsiella aerogenes*, *Klebsiella pasteurii*, *Raoultella ornithinolytica*, and *Raoultella planticola*. This finding suggests that the DF effluent acted simultaneously as a substrate source and a microbial inoculum for the PF stage. Ultimately, the combined effects of suboptimal pH, enhanced nitrogen assimilation, and moderately reducing conditions in the PF reactor (average ORP:  $-138 \pm 89$  mV) favored the proliferation of competing and inhibitory taxa over active photofermenters.

The mean relative abundance of lactic acid bacteria (LAB) in the PF community reached 15.0% (*Lactococcus lactis*, 13.2%, *Lactococcus cremoris*, 1.8%). In comparison, the DF stage exhibited a LAB abundance of only 0.5%, substantiating the hypothesis that the operating conditions in the second stage favored LAB enrichment. Similarly, *Pseudomonas* species—

comprising *Pseudomonas extremaustralis* (14.7%), *Pseudomonas veronii* (7.1%), *Pseudomonas fildesensis* (1.6%)—collectively accounted for 22.8% of the PF community, indicating a distinct competitive advantage under these environmental conditions. Together, these trends confirm that the PF environment promoted the proliferation of non-photofermentative organisms at the expense of *Rhodopseudomonas* species, thereby limiting nitrogenase-mediated  $H_2$  production.

The competitive dynamics in the PF reactor can be explained more precisely at the metabolic level. *Pseudomonas* species are aerotolerant facultative organisms capable of utilizing acetate and butyrate, the principal VFAs produced during DF and the primary carbon sources for photofermentative  $H_2$  production by *Rhodopseudomonas* [47]. Their enrichment in the PF reactor represents a direct diversion of organic acids away from the nitrogenase-mediated photofermentative pathway, independently of the suboptimal pH and temperature conditions discussed in Section 3.1. Simultaneously, *Clostridium* species carried over from the DF stage continued to consume residual sugars and generate additional organic acids in PF. This activity further acidified the environment and produced metabolites that accumulated faster than the sparse population could consume them [22]. Furthermore, the marked enrichment of *Lactococcus lactis* and *Lactococcus cremoris* introduced a third competitive axis. At the high relative abundances observed in PF, *Lactococcus* shifts from a syntrophic cross-feeding partner to a dominant competitor. It diverts sucrose and residual lactose via homolactic fermentation and, critically, synthesizes nisin-class bacteriocins under acidic conditions. These antimicrobial peptides inhibit *Clostridium* species and destabilize the broader hydrogenogenic community [61]. The three-way interaction among *Clostridium*, *Pseudomonas* and *Lactococcus* in the PF reactor thus constitutes a cascading suppression mechanism of  $H_2$  production: *Pseudomonas* consumes organic acid substrates intended for *Rhodopseudomonas*, *Clostridium* contributes to acidification that disfavors PF growth, and *Lactococcus* reinforces competitive exclusion through both substrate diversion and bacteriocin-mediated inhibition. Additionally, *Lactococcus* redirects pyruvate toward lactate rather than formate, reducing the overall efficiency of  $H_2$  generation. Nevertheless, a fraction of the metabolic byproducts generated during this shift may still be utilized by residual PF bacteria for downstream  $H_2$  production [42].

Optimized DF systems treating sugar-based substrates have been reported to attain  $H_2$  concentrations of up to 63% (v/v) [62]. In the present study, a maximum  $H_2$  fraction of 57% (v/v) was achieved in the DF reactor, with an HPR of  $177 \text{ mL } H_2 \text{ L}^{-1} \text{ d}^{-1}$  based on total working volume. This indicates robust acidogenic activity despite the absence of external temperature and pH control. In contrast, while PF systems can achieve  $H_2$  concentrations of up to 38% (v/v) when treating starch-rich substrates under controlled pH and nitrogen conditions [61], the PF reactor in this study exhibited significantly lower  $H_2$  levels (averaging 1.8% (v/v) and an HPR of  $5 \text{ mL } H_2 \text{ L}^{-1} \text{ d}^{-1}$ ). Microbial community analysis provides a mechanistic explanation for this disparity. *Rhodopseudomonas* species, the primary  $H_2$  producers, accounted for only 2.7% of the PF community. Conversely, *Lactococcus* (15.0%) and *Pseudomonas* species (22.8%) were markedly enriched compared to the DF stage. The competitive displacement of *Rhodopseudomonas* species under acidic pH and moderately reducing conditions is consistent with reported sensitivities of purple non-sulphur bacteria to environmental stress [30,34], providing a quantitative basis for the low PF productivity observed under unmonitored conditions.

The combined analysis of microbial community structure and physicochemical parameters provides key insights into the operational mechanics of the coupled DF–PF system. These results demonstrate that  $H_2$  generation relies strongly on the dominance of *Clostridium* and *Klebsiella* species, the suppression of lactic acid bacteria, acidic conditions (pH 4–5), and strongly reducing environments (ORP  $\approx -200 \text{ mV}$ ) [7]. Under these conditions, carbo-

hydrate metabolism yields organic acids, alcohols, and gases via pathways involving the conversion of pyruvate into acetyl-CoA. This process releases CO<sub>2</sub> and transfers electrons to ferredoxin, which are subsequently utilized for proton reduction to generate molecular H<sub>2</sub> through acetate and butyrate pathways [7]. In contrast, the PF reactor exhibited greater nutrient competition, contributing to enhanced COD removal and moderate phosphate assimilation, thereby confirming its role as a secondary polishing stage. However, the enrichment of lactic acid bacteria alongside this competition diverted metabolic pathways towards lactic acid synthesis and bacteriocin release, both of which are known to inhibit hydrogenogenic activity [61].

From an environmental perspective, these findings underscore the merits of the coupled DF–PF system over single-stage configurations. Integrating both stages enables simultaneous H<sub>2</sub> production and OLR, with the DF stage achieving H<sub>2</sub> concentrations of up to 57% (v/v) and the overall system attaining 34% COD removal. This demonstrates the potential of the coupled setup as a sustainable approach for energy recovery and wastewater treatment. Furthermore, it offers a realistic baseline for designing large-scale systems required by this industry under continuous, unmonitored operation using 5 L reactors and a synthetic substrate.

#### 4. Conclusions

This study demonstrates the operational feasibility of a continuous, coupled dark fermentation–photofermentation (DF–PF) system (5 L working volume) for biohydrogen (H<sub>2</sub>) production from sugar-rich substrate simulating acidic fruit and dairy processing wastewater under high organic loading rates (OLR) without external pH or temperature control. A clear functional differentiation emerged between the two stages: (i) DF functioned as the primary H<sub>2</sub>-producing unit, achieving peak H<sub>2</sub>-concentrations of 57% (v/v) and a maximum HPR of 177 mL H<sub>2</sub> L<sup>−1</sup> d<sup>−1</sup>, driven by the dominance of *Clostridium* species under acidic and strongly reducing conditions; (ii) PF served primarily as a polishing stage, contributing to an overall system COD removal of 34% but its H<sub>2</sub> yield was severely limited.

Microbial community analysis provided a mechanistic explanation for this performance disparity: primary photofermentative producers (*Rhodospseudomonas* species) were competitively displaced (declining to 2.7% abundance) by non-photofermentative organisms, notably *Lactococcus* and *Pseudomonas* species, under suboptimal pH and moderately reducing conditions. Ultimately, these findings establish that under unmonitored, industrially representative constraints, controlling competitive microbial dynamics is a critical process parameter to ensure effective scalability for integrated wastewater treatment and energy recovery.

Building upon these insights, the results obtained herein enabled a successful scale-up to pilot-plant level (40 L reactors). This pilot phase has been completed, with preliminary analysis demonstrating high fidelity in replicating the bench-scale findings. As the final milestone of the Hi2BIO project, conducted in collaboration with Ingeobras, initial trials are currently underway using 300 L reactors with a synthetic matrix. Following the operational optimization, the ultimate stage will evaluate system performance treating real agro-industrial wastewater containing both sugars and fats, representative of juice and dairy processing effluents.

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