Universidad de Valladolid

Escuela de Doctorado Universidad de Valladolid

PROGRAMA DE DOCTORADO EN
INGENIERÍA QUÍMICA Y AMBIENTAL

TESIS DOCTORAL:

Integral Valorisation of Food Waste through the Recovery of Biohydrogen & Methane

Presentada por **Leonardo José Martínez Mendoza**
para optar al grado de Doctor
por la Universidad de Valladolid

Dirigida por:

Dr. Raúl Muñoz Torre
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“I have not failed. I have just found

10,000 ways that will not work.”

– *Thomas Alba Edison*

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RESUMEN

La creciente generación global de residuos de frutas y verduras (RFV) representa un desafío ambiental, económico y social significativo. Debido a su alto contenido de humedad y azúcares, estos residuos son altamente susceptibles a la descomposición microbiana y a procesos de acidificación incontrolada si no se gestionan de forma adecuada, lo que dificulta su tratamiento. En la Unión Europea, se estima una producción de aproximadamente 21 kg per cápita al año de RFV inevitables a lo largo de la cadena agroalimentaria. A pesar de su riqueza en materia orgánica biodegradable, estos residuos suelen estar infrautilizados, siendo comúnmente depositados en vertederos o incinerados, lo que contribuye a la emisión de gases de efecto invernadero y a la pérdida de recursos valiosos. En este contexto, y en concordancia con la Estrategia de Bioeconomía Circular y los Objetivos de Desarrollo Sostenible al 2030, la valorización integrada de los RFV para la obtención de bioenergía renovable y productos de valor añadido se posiciona como una prioridad estratégica para una gestión sostenible de los residuos.

En este marco, la fermentación oscura (FO) y la digestión anaerobia (DA) emergen como procesos biotecnológicos clave para la conversión de RFV en hidrógeno (H_2) y metano (CH_4), respectivamente. La FO es particularmente prometedora debido a su bajo requerimiento energético y su capacidad para generar H_2 en condiciones anaerobias. Sin embargo, su aplicación a escala industrial enfrenta obstáculos importantes, como la inestabilidad operativa en sistemas continuos, la acumulación de metabolitos inhibidores como el ácido láctico (HLac) y una baja reproducibilidad. Estos problemas se ven agravados por la alta biodegradabilidad y el contenido en

carbohidratos de los RFV, que aceleran la acidificación del medio y comprometen la eficiencia del proceso. Una alternativa emergente es la fermentación oscura impulsada por lactato (FOIL), en la cual el HLac es aprovechado como intermediario fermentable en lugar de considerarse un inhibidor, lo que permite una producción de H_2 más estable y eficiente. No obstante, los efectos de parámetros operativos como el pH, los sólidos totales (ST), la concentración de biomasa, el tiempo de retención hidráulica (TRH) y la carga orgánica (CO) sobre el rendimiento de la FOIL aún no se comprenden completamente. La integración de la FOIL con la DA en sistemas de dos etapas puede generar sinergias significativas, al facilitar la especialización metabólica, mejorar la eficiencia de acidificación del sustrato y aumentar la producción de CH_4 mediante la conversión completa del HLac durante la fase metanogénica. Dada la limitada madurez tecnológica de la LDDF y la escasa exploración de su integración con la AD, es esencial investigar y comparar las ventajas y mejoras que puede presentar este enfoque con respecto a la operación tradicional, así como evaluar el rendimiento de los sistemas en dos etapas bajo condiciones controladas.

Esta tesis aborda estas brechas científicas y tecnológicas mediante la evaluación sistemática del efecto del pH, ST, concentración de biomasa, TRH, CO cómo parámetros clave en sistemas FOIL operados en modo discontinuo y continuo. En condiciones mesófilas, los ensayos discontinuos mostraron que la producción de H_2 se optimiza a pH neutro (7,0), con bajos sólidos totales (5%) y alta concentración de inóculo (1800 mg VSS/L), alcanzando un rendimiento de 49,5 NmL H_2/g $VS_{alimentado}$ y una productividad máxima de 976,4 mL H_2/L-h. Estas condiciones redujeron la acumulación de HLac y favorecieron la coproducción de acetato y butirato, evidenciando un delicado equilibrio entre las rutas metabólicas y la necesidad de un control preciso de las condiciones operativas.

En sistemas en continuo, la reducción progresiva del TRH de 24 a 6 horas (correspondiente a una CO de 47–188 g VS/L-d) reveló un TRH óptimo de 9 horas, en el que se alcanzó una tasa de producción de H_2 sin precedentes de 11,8 NL H_2/L-d y un rendimiento de 95,6 NmL/g $VS_{alimentado}$. Estos resultados destacan la importancia crítica del tiempo de residencia y consolidan el papel del HLac como impulsor clave en la producción de H_2.

Con el fin de ampliar la valorización energética, se compararon la DA convencional en una sola etapa y un sistema de dos etapas con generación previa de HLac. Este último, conformado por una fase acidogénica inicial seguida de una etapa metanogénica, superó al sistema tradicional con un aumento del 32% en la productividad de CH_4 (959 NmL CH_4/L-d) y un 36% en el rendimiento de CH_4 (398 NmL CH_4/g $VS_{alimentado}$). Si bien ambos sistemas mostraron perfiles similares de contenido y estabilidad de CH_4, la configuración en dos etapas logró una conversión más eficiente del sustrato y una mayor especialización microbiana. La fase acidogénica estuvo dominada por Lactobacillus, mientras que en la fase metanogénica prevalecieron Methanobacterium y Methanothrix, lo que sugiere que la separación de fases promueve un funcionamiento microbiano más eficiente.

Asimismo, se abordó la problemática de la reproducibilidad en procesos de FO, un aspecto crucial para su escalado. Utilizando reactores paralelos operados de forma idéntica durante seis fases experimentales, se logró una productividad de H_2 consistente (6,7 ± 0,7 NL H_2/L-d), un contenido medio de H_2 del 65 ± 5% y perfiles estables de ácidos orgánicos, validando la viabilidad y reproducibilidad de la FOIL bajo condiciones controladas. Estrategias como la bio-aumentación y la suplementación de nutrientes solo generaron mejoras temporales, lo que resalta la necesidad de enfoques de optimización adaptativa a largo plazo para mitigar la variabilidad biológica.

En conjunto, esta investigación aporta conocimiento relevante para la optimización e integración de procesos de FO y DA en la valorización de RFV. Los resultados demuestran que, con un ajuste adecuado, la FOIL puede consolidarse como una plataforma confiable para la producción de H_2, y que los sistemas de DA en dos etapas basados en HLac ofrecen una alternativa superior para la generación de CH_4. Desde una perspectiva integral, la combinación de FOIL y DA permite transformar residuos orgánicos en dos biocombustibles de alto valor como el H_2 y CH_4, promoviendo así la recuperación de recursos, la economía circular y la mitigación del cambio climático. Los hallazgos de esta tesis sientan las bases para el diseño de biorrefinerías robustas, escalables y sostenibles que contribuyan a enfrentar los desafíos ambientales y energéticos actuales mediante el aprovechamiento eficiente de residuos orgánicos.

ABSTRACT

The growing global generation of fruit and vegetable waste (FVW) poses a significant environmental, economic, social problem. The high moisture and sugar content in FVW, which promote a rapid microbial decomposition and uncontrolled acidification when unmanaged, entails significant technical challenges. In the European Union alone, approximately 21 kg of unavoidable FVW is generated per capita annually across the agri-food chain. This biomass, though rich in biodegradable organic matter, is frequently underutilized (disposed of in landfills or incinerated) contributing to greenhouse gas emissions and the loss of valuable resources. In alignment with the Circular Bioeconomy Strategy and the 2030 Sustainable Development Goals, the integrated valorisation of FVW into renewable bioenergy and value-added products is a strategic priority for achieving sustainable waste management.

Dark fermentation (DF) and anaerobic digestion (AD) are two key biological processes capable of converting FVW into hydrogen (H_2) and methane (CH_4), respectively. DF is particularly attractive for its low energy demand and ability to produce H_2 under anaerobic conditions. However, DF scale-up to commercial scale remains limited by several bottlenecks namely, operational instability under continuous conditions, accumulation of inhibitory metabolites such as lactic acid (HLac), and low reproducibility. These issues are further exacerbated by the high biodegradability and carbohydrate content of FVW, which accelerate acidification and system inhibition. A promising alternative is lactate-driven dark fermentation (LDDF), in which HLac is no longer viewed as a mere inhibitor but as a fermentable intermediate, enabling more robust and energetically favourable H_2 production. Despite its potential, the effects of operational parameters such as pH, total solids (TS), biomass concentration, hydraulic

retention time (HRT), and organic loading rate (OLR) on LDDF performance remain poorly understood. On the other hand, integrating HLac fermentation with AD in a two-stage configuration provides synergistic benefits by enhancing substrate solubilization and acidification efficiency, promoting metabolic specialization, and significantly increasing CH_4 production through the complete conversion of HLac during the methanogenic phase. Given the limited technological maturity of LDDF and the scarce exploration of its integration with AD, it is essential to investigate and compare the advantages and improvements this approach may offer over traditional operation, as well as to evaluate the performance of two-stage systems under controlled conditions.

This thesis addresses these scientific and technological gaps by systematically evaluating the influence of pH, TS, biomass concentration, HRT, OLR as key operational parameters in batch and continuous LDDF systems. Results from mesophilic batch experiments demonstrated that H_2 production was optimized at neutral pH (7.0), low TS (5%), and high inoculum concentrations (1800 mg VSS/L), yielding 49.5 NmL H_2/g VS_{FED} and reaching maximum volumetric H_2 productivities of 976.4 mL H_2/L-h. These conditions minimized HLac accumulation and favoured acetate and butyrate co-production. Such metabolic shifts highlight the delicate balance between fermentative pathways and the importance of precise operational control. In continuous LDDF systems, the stepwise reduction of HRT from 24 to 6 hours (with corresponding OLRs of 47–188 g VS/L-d) revealed an optimal HRT of 9 hours, at which an unprecedented H_2 production rate of 11.8 NL H_2/L-d and a yield of 95.6 NmL/g VS_{FED} were achieved. These results confirmed that LDDF performance is strongly dependent on residence time, and underscored HLac's central role as a driver of H_2 production.

To extend the valorisation chain and enhance energy recovery, a comparative evaluation between conventional single-stage AD and a lactate-type two-stage AD configuration was performed. The two-stage system, which included an initial acidogenic phase focused on HLac generation followed by a methanogenic reactor, outperformed the single-stage setup by achieving a 32% increase in CH_4 productivity (959 NmL CH_4/L-d) and a 36% increase in CH_4 yield (398 NmL CH_4/g VS_{FED}). Both systems showed comparable CH_4 content and stability, yet the two-stage process enabled better substrate conversion and more defined microbial specialization. The acidogenic phase was dominated by *Lactobacillus*, while *Methanobacterium* and *Methanothrix* were prevalent in the methanogenic stage, suggesting that phase separation facilitated optimized microbial community performance.

The thesis also tackled the challenge of process reproducibility in DF, which is crucial for scaling bioprocesses. A parallel reactor setup operating three identical continuous systems over six operational phases demonstrated consistent H_2 productivity (6.7 ± 0.7 NL H_2/L-d), H_2 content (65 ± 5%), and organic acid profiles, validating the reproducibility of LDDF under controlled conditions. Process enhancement strategies such as bioaugmentation and nutrient supplementation produced only temporary improvements, reinforcing the need for longer-term, adaptive optimization strategies to mitigate biological variability.

Collectively, this work advances the understanding of how to optimize and integrate DF and AD technologies for the valorisation of FVW. It demonstrates that LDDF, when fine-tuned, can serve as a reliable H_2 production platform, and that two-stage AD systems leveraging HLac metabolism offer superior CH_4 yields over conventional setups. From a systems perspective, the integration of LDDF and two-phase AD enables the conversion of food waste into two valuable biofuels (H_2 and CH_4) supporting resource recovery, circular economy principles, and greenhouse gas mitigation. The findings of this research provided valuable insights towards the development of robust, scalable, and sustainable biorefinery models capable of addressing both environmental and energy challenges through organic waste valorisation.

LIST OF PUBLICATIONS

The current thesis includes the following publications, all of which have been published or submitted to international peer-reviewed journals indexed in the ISI Web of Knowledge.

1. **Martínez-Mendoza, L.J.**, Lebrero, R., Muñoz, R., García-Depraect, O., 2022. Influence of key operational parameters on biohydrogen production from fruit and vegetable waste via lactate-driven dark fermentation, Bioresource Technology. 364, 128070. https://doi.org/10.1016/j.biortech.2022.128070

2. **Martínez-Mendoza, L. J.**, García-Depraect, O., Muñoz, R.,2023. Unlocking the high-rate continuous performance of fermentative hydrogen bioproduction from fruit and vegetable residues by modulating hydraulic retention time, Bioresource Technology, 373, 128716. https://doi.org/10.1016/j.biortech.2023.128716

3. **Martínez-Mendoza, L. J.**, Muñoz, R., García-Depraect, O., 2024. Enhanced methane production from food waste: A systematic comparison between conventional single-stage and lactate-based two-stage anaerobic digestion processes, Biomass and Bioenergy, 188, 107312, https://doi.org/10.1016/j.biombioe.2024.107312

4. **Martínez-Mendoza, L. J.**, Muñoz, R., García-Depraect, O., 2025. Continuous fermentative biohydrogen production from fruit-vegetable waste: A parallel approach to asses process reproducibility, Fermentation, 11, 9, https://doi.org/10.3390/fermentation11090545

CONTRIBUTION TO THE PAPERS INCLUDED IN THE THESIS

1. Throughout the course of this work, I assumed primary responsibility for the conceptual design, commissioning, and operational management of the experimental system. The interpretation of experimental data and the preparation of the resulting manuscript were conducted under the academic supervision of Dr. Octavio García Depraect and Dr. Raúl Muñoz.

2. This research was carried out under the guidance of Dr. Octavio García Depraect, with whom I collaborated closely in the design, implementation, and operation of the experimental setup. The subsequent data analysis and manuscript preparation were performed jointly with Dr. Octavio García Depraect and Dr. Raúl Muñoz.

3.Under the supervision of Dr. Raúl Muñoz, I was responsible for the design and operation of the experimental system. The subsequent evaluation of results and manuscript drafting were undertaken in collaboration with both Dr. Raúl Muñoz and Dr. Octavio García Depraect.

4. In this study, I independently led the design, configuration, and operational oversight of the experimental platform, under the academic supervision of Dr. Octavio García Depraect. The interpretation of the findings and the preparation of the manuscript were carried out collaboratively with Dr. Octavio García Depraect and Dr. Raúl Muñoz.

Chapter 1

Introduction

1.1 Organic Waste Valorization: A Key Step Towards a Circular Bioeconomy

Organic waste management represents a critical global challenge with profound environmental, social, and economic implications. Each year, approximately 1.3 billion tons of food are wasted globally, with fruits and vegetables accounting for nearly 50% of that figure (Piwowarek et al., 2023; Díaz et al., 2017). This waste not only reflects inefficiencies in the food supply chain but also contributes significantly to greenhouse gas (GHG) emissions, resource depletion, and financial losses. Addressing Fruit-Vegetable Waste (FVW) is therefore essential for achieving a sustainable, equitable, and circular food system. From an environmental point of view, the decomposition of organic waste such as FVW in landfills leads to the release of methane (CH_4), a GHG approximately 25 times more potent than carbon dioxide (CO_2) (Khalid et al., 2011). Organic waste has become the largest component of municipal solid waste globally (Díaz et al., 2017), and its mismanagement results in soil and water contamination, as well as avoidable GHG emissions from transportation and treatment (Wikandari et al., 2014). These impacts underscore the urgent need to integrate FVW management into climate change mitigation strategies, as well as resource recovery roadmaps (Stoknes et al., 2016).

From a social point of view, the paradox of widespread food waste (FW) alongside global hunger is stark. Indeed, an estimated 1.3 billion people could be fed with the calories lost in FW (Piwowarek et al., 2023). The loss of nutrient-rich FVW particularly exacerbates food insecurity and nutritional deficiencies, especially in vulnerable populations (De Laurentiis et al., 2018; De Moraes et al., 2022). Reducing FVW is thus not merely a logistical issue, it is a moral imperative linked to health and social equity. Economically, the costs of FVW are staggering. In addition to the economic value of the wasted products, there are associated costs in terms of land use, energy, water, and labor (Okoro et al., 2022). Tens of billions of dollars are lost annually due to inefficiencies in the production, distribution, and disposal of FVW (Piwowarek et al., 2023). However, a better FVW management (through valorization strategies such as composting and anaerobic digestion (AD)) can generate renewable energy, improve soil fertility, and reduce dependence on synthetic inputs (Jiang et al., 2012; Gómez-Romero et al., 2014). The valorization of FVW aligns with the principles of the circular economy (Fig. 1.1), aiming at converting waste into value-added products such as biofuels, biodegradable plastics, bioactive compounds, animal feed, and organic fertilizers (Bayram et al., 2021; Bas-Bellver et al., 2020; Sagar et al., 2018). Economically, this transformation fosters the development of green industries and job creation, while environmentally, it contributes to GHG mitigation and improved resource efficiency (Cassani and Gómez-Zavaglia, 2022; Duque-Acevedo et al., 2020; Zulkifli et al., 2023; Błaszczyk et al., 2024).

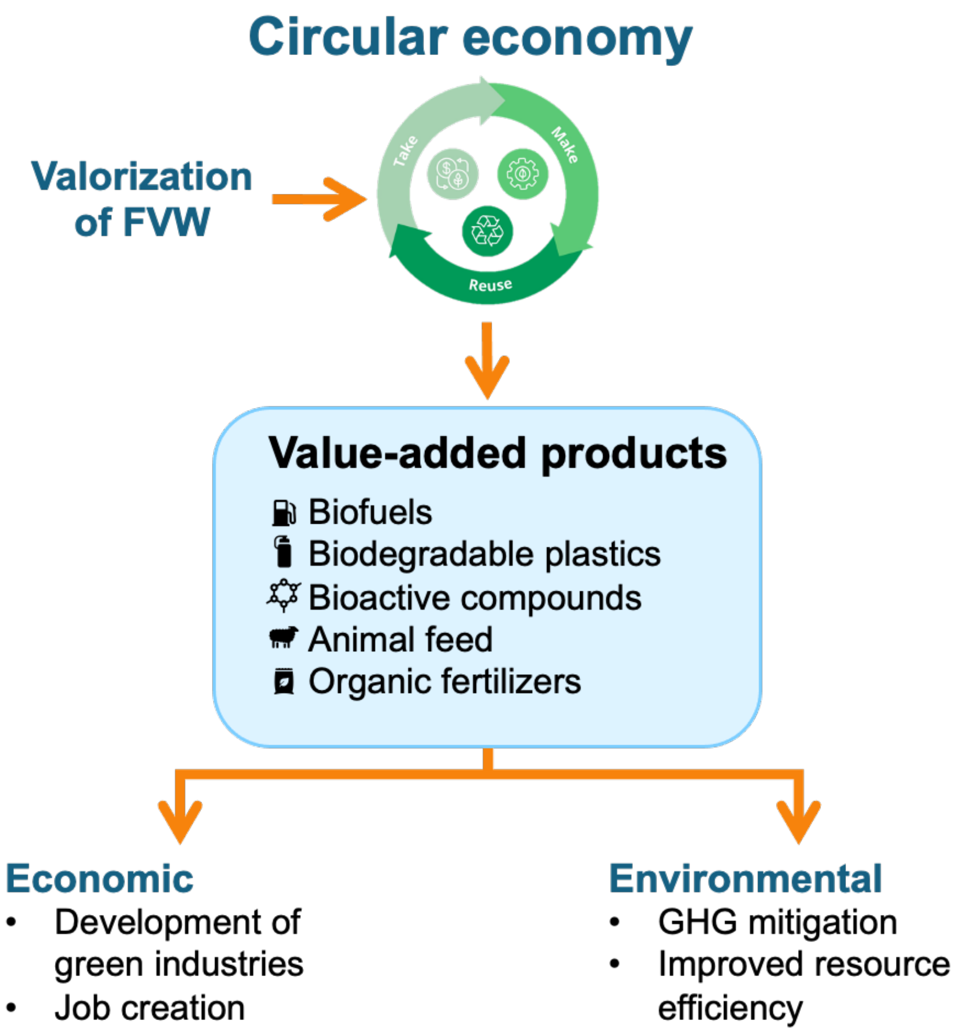

Figure 1.1 Overview of circular valorization of fruit and vegetable waste (FVW).

In addition to its intrinsic value, FVW valorization supports the achievement of several United Nations Sustainable Development Goals (SDG). It contributes to SDG 12 (Responsible Consumption and Production) by reducing food loss and waste, and promoting resource efficiency (Looveren et al., 2023). It supports SDG 13 (Climate Action) through biogas production and CH_4 emission reductions (Mazareli et al., 2016; Kıran et al., 2014). From an economic point of view, it drives SDG 8 (Decent Work and Economic Growth) through the creation of bioeconomy-related jobs (Gómez-Romero et al., 2014; Shokrollahi et al., 2024). Socially, it enhances SDG 2 (Zero Hunger) and SDG 1 (No Poverty) by increasing agricultural productivity and providing economic opportunities in low-income communities (Stoknes et al., 2016; Tsapekos et al., 2018).

From a technological perspective, FVW valorisation stimulates innovation in bioprocessing, nutrient recycling, and resource recovery models such as biorefineries (Ebrahimian et al., 2022; Papa et al., 2020). These advancements enable closed-loop systems that minimize environmental footprints and maximize economic returns (Foggia and Beccarello, 2020; Vanierschot et al., 2023). Moreover, the extraction of bioactive compounds from FVW opens new markets in food, cosmetics, and pharmaceuticals sectors (Yaashikaa et al., 2022). Lastly, FVW valorization fosters a culture of sustainability through education and public awareness (Wilson et al., 2015; Liikanen et al., 2016). It encourages more responsible consumption, supports research and academic engagement, and empowers communities to take part in transformative environmental solutions. In conclusion, FVW is not merely a byproduct of inefficiency but a resource of immense potential. Its proper management and valorisation by means of physicochemical and biological treatments are key to addressing global challenges related to climate change, food insecurity, economic resilience, and sustainable development. Moving forward, interdisciplinary efforts and integrated policies will be essential to harness the full potential of FVW in building a more circular and sustainable future.

1.2 Physicochemical Treatments for Organic Waste Valorization

Physicochemical treatments represent powerful tools to transform organic waste into valuable resources. These technologies not only help reducing environmental burdens but also open the door to innovative solutions in energy recovery, sustainable agriculture, and material reuse, making them central to circular economy strategies.

1.2.1 Hydrothermal Carbonization

Hydrothermal carbonization (HTC) (Fig. 1.2) stands out for its ability to convert wet biomass into energy-dense hydrochar without prior drying, offering both energy savings and reduced environmental pollution (Lucian et al., 2018; Nobre et al., 2021; Mannarino et al., 2022; Su et al., 2023; Javid et al., 2022). Studies such as Suárez et al. (2020), who evaluated the feasibility of applying HTC to transform industrial apple waste into value-added products, achieving a retention of 80–93% of the carbon and 82–96% of the original biomass energy content in the hydrochar. This demonstrates that the process is highly efficient in preserving the energy value of the waste.

Further highlight its role in zero-waste strategies, particularly when integrated with AD for combined energy recovery (Rubia et al., 2018). This technology is currently at a Technology Readiness Level (TRL) of 5–6, indicating that it has been validated in relevant environments and is approaching demonstration at pilot scale. However, key technical limitations remain, including the high energy demand for water handling, scaling challenges, and the need for efficient separation and recovery of valuable compounds from the liquid phase. Addressing these issues is essential to advance towards full-scale industrial implementation.

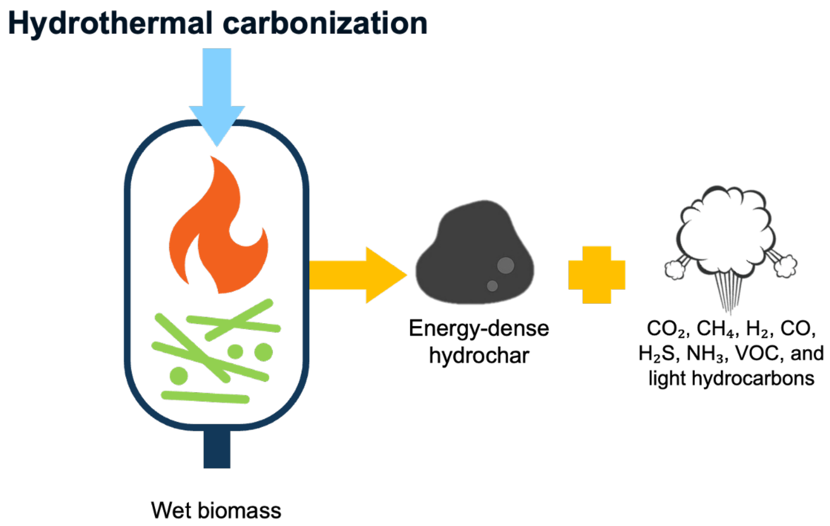

Figure 1.2. Schematic representation of hydrothermal carbonization of biomass.

1.2.2 Hydrothermal Liquefaction

Hydrothermal liquefaction (HTL) stands out as a particularly attractive option for treating wet organic waste, as it avoids the energy-intensive drying step required by many other technologies (Fig. 1.3). By processing biomass in hot, pressurized water (280–380 °C), HTL produces a bio-crude oil that can later be upgraded into fuels or specialty chemicals (Rao et al., 2018). Despite its promise, HTL exhibit severe challenges. In this context, issues such as complex system control, equipment wear, wastewater generation, and energy demand currently limit its scalability (Anastasakis and Ross, 2015; Nelson et al., 2013). However, HTL could play a key role in future sustainable waste management systems with further technological refinements.

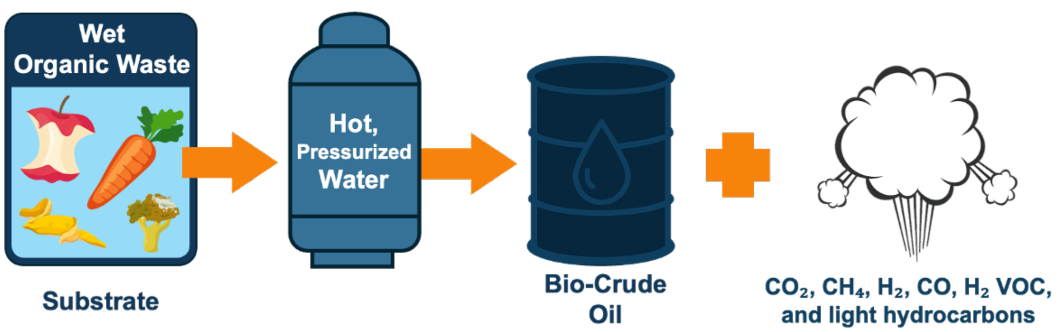

Figure 1.3. Schematic representation of hydrothermal liquefaction of organic waste.

1.2.3 Pyrolysis and Gasification

Both processes are thermochemical methodologies that can convert organic waste into useful products like biochar, syngas, and bio-oil. Pyrolysis focuses on thermal decomposition in the absence of oxygen, while gasification operates at higher temperatures and in limited oxygen conditions to generate syngas (Hervy et al., 2018).

These methods (Fig. 1.4) not only reduce waste volume but also create pathways for energy recovery. However, their viability depends heavily on economic and operational factors, including energy efficiency, system complexity, and the cost of implementation (Zeng et al., 2015; Liu et al., 2021). Both pyrolysis and gasification require pre-treatment of organic residues, including drying to reduce moisture content, size reduction, and sometimes homogenization, to ensure efficient thermal conversion and consistent product quality. These technologies are generally considered to be at a TRL between 6 and 8, with several pilot and demonstration-scale projects in operation worldwide. However, their industrial deployment still faces challenges related to feedstock variability, tar management, and integration with downstream valorization pathways.

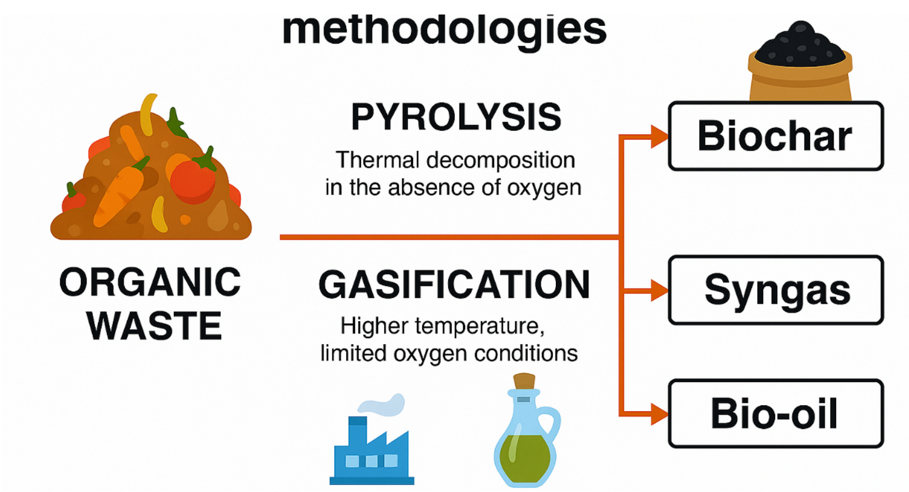

Figure 1.4. Comparative diagram of pyrolysis and gasification as thermochemical methodologies.

1.2.4 Incineration

Incineration (Fig. 1.5) is a well-established thermal treatment for organic waste that significantly reduces volume and enables energy recovery through high-temperature combustion (Liu et al., 2020; Nikku et al., 2019). Although effective in destroying pathogens and minimizing residual organics, incineration can produce harmful gas pollutants like dioxins and Poly-Chlorinated Biphenyls (PCBs), requiring advanced emission control systems (Caneghem et al., 2010; Nzihou et al., 2012).

The resulting ash residues also demand further treatment to prevent heavy metal leaching (Rocca et al., 2012). While incineration offers lower GHG emissions, its high costs and environmental concerns remain key challenges for implementation at full scale (Münster and Lund, 2010; Chen et al., 2016).

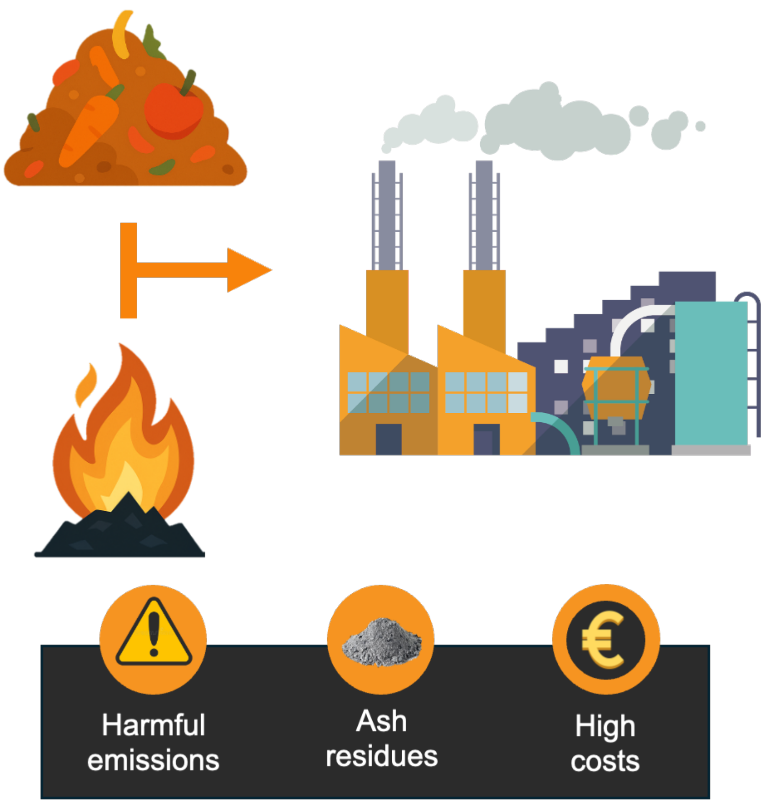

Figure 1.5. Incineration as a high-temperature thermal treatment for organic waste.

1.2.5 Landfilling

Landfilling is still widely used for organic waste disposal, where anaerobic microbial degradation produces landfill gas; mainly composed of 50–60% CH_4 and 40–50% CO_2, along with trace organic compounds (Duan et al., 2021; Pantini et al., 2015; Scheutz et al., 2008). CH_4 generation in landfills depends heavily on waste composition, landfill age, and microbial activity (Frank et al., 2017; Fei et al., 2016; Ishii and Furuichi, 2013).

Although modeling CH_4 production remains difficult due to the heterogeneous nature of landfill materials, recent advances have enhanced prediction accuracy and gas recovery methods (Emkes et al., 2015). Environmental variables such as moisture, temperature and air intrusion also influence CH_4 yield and can increase impacts like leachate formation and GHG emissions (Fig. 1.6). Understanding these dynamics is essential to improve CH_4 recovery and reduce the environmental footprint of landfilling (Duan et al., 2021; Pantini et al., 2015).

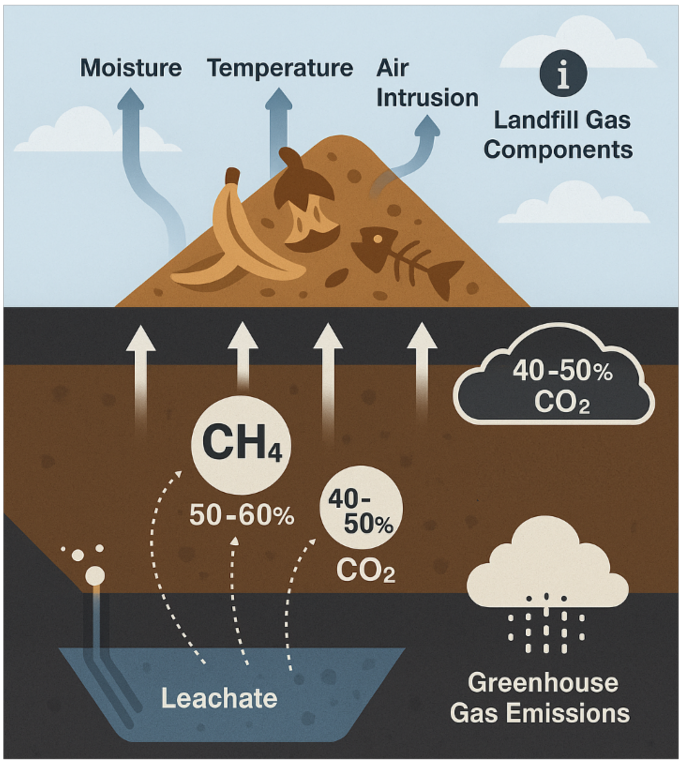

Figure 1.6. Schematic of landfill process for organic waste management and methane formation.

1.3 Biological Treatments for Organic Waste Valorization

Biological treatments offer a promising and environmentally friendly approach to transforming organic waste into valuable resources. These processes are based on the natural capabilities of the microorganisms to break down complex organic matter and generate products such as biofertilizers, biogas, organic acids (OA), and H_2. Techniques like composting, AD, and dark fermentation (DF) not only help reducing the environmental impact of organic waste but also contribute to the creation of a circular economy by giving new life to discarded materials. These biological strategies are especially well-suited for valorizing diverse and variable organic waste streams, such as FW including FVW, based on their flexibility and adaptability.

1.3.1. Enzymatic Treatment

Enzymes can mediate a biological and environmental friendly route to enhancing waste valorization (Fig. 1.7). By breaking down complex organic molecules into simpler, more digestible forms, enzymes such as lipases can boost biogas production from FVW during anaerobic digestion and improve the overall efficiency of fermentation processes (Meng et al., 2017). Yet, their broader application faces obstacles such as high costs, sensitivity to operating conditions, and variable effectiveness across different types of waste (Costa et al., 2012; Wagland and Tyrrel, 2010).

Even so, ongoing innovations in enzyme engineering and process optimization continue to expand their potential (Meng et al., 2017). These treatments can maximize the efficiency of subsequent fermentation processes, making them a valuable addition to waste-to-energy technologies. The challenges related to cost, environmental sensitivity, feedstock variability, inhibitory compounds, efficiency limitations, and handling difficulties must be addressed prior full-scale implementation (Costa et al., 2012; Wagland and Tyrrel 2010).

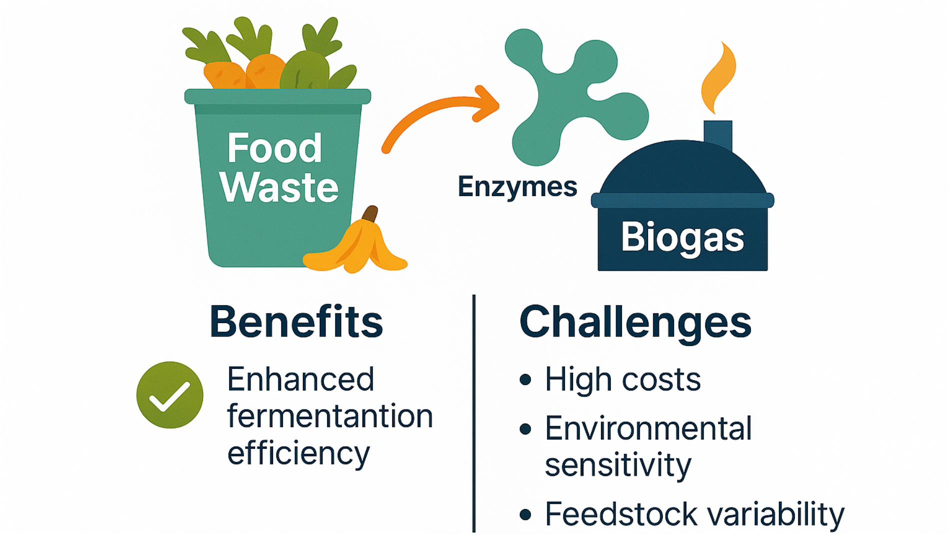

Figure 1.7. Diagram of enzymatic treatment of FVW and principal challenges.

1.3.2. Composting

Although often categorized under biological treatments, composting processes can also incorporate physicochemical aspects, particularly in how materials are pre-processed and the management of operational conditions (e.g., temperature, moisture, and aeration). Composting involves the aerobic decomposition of organic waste materials (such as food scraps, yard trimmings, and agricultural residues) into a stable, nutrient-rich soil amendment known as compost (Vargas-Estrada et al., 2025). This transformation is mediated by microorganisms (Fig. 1.8), including bacteria and fungi, and invertebrates like earthworms, which collectively degrade organic matter (Shrestha et al., 2011).

Composting contributes to sustainable waste management by significantly reducing the volume of organic waste devoted for landfills, thus alleviating pressure on municipal systems and lowering disposal costs (Batool and Chuadhry, 2009). The resulting compost enhances soil structure, water retention, and nutrient content, promoting higher crop productivity and reducing the need for chemical fertilizers (Barrena et al., 2014; Shrestha et al., 2011).

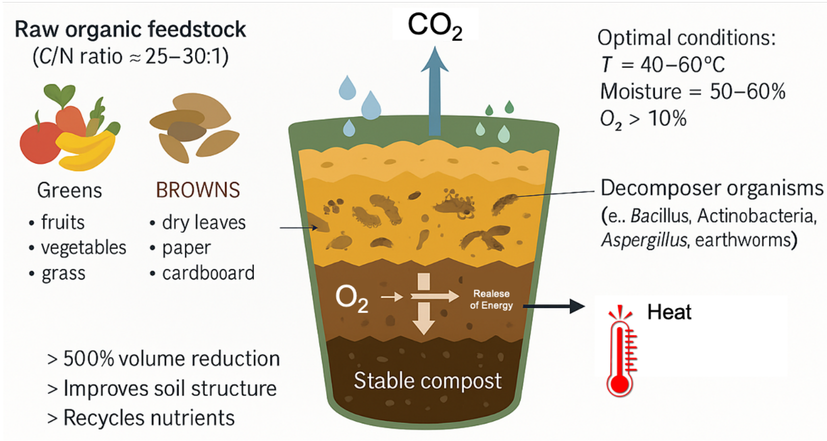

Figure 1.8. Visual representation of composting as anaerobic biological process for organic waste management.

From an environmental point of view, composting emits fewer GHG than landfilling or incineration, particularly CH_4, thus contributing to climate mitigation strategies (Edjabou et al., 2016; Walker et al., 2009). Composting also aligns with circular economy principles by recycling nutrients back into agricultural systems (Jiang et al., 2015). However, composting is time-consuming, often requiring weeks to months, and demands significant space; posing implementation challenges in urban environments (Begum et al., 2007; López-González et al., 2015). Odor and dust emissions may arise from poorly managed piles, affecting surrounding communities (Galgani et al., 2014), while the inclusion of unsuitable feedstocks, such as meat or plastics, can lead to contamination risks and reduced compost quality (Kim and Oh, 2011). Moreover, compost quality can be inconsistent due to feedstock variability and operational conditions, influencing its agronomic effectiveness (Cerda et al., 2018; Jiang et al., 2015).

Despite these limitations, when properly implemented, composting offers substantial environmental, agronomic, and economic benefits, making it a cornerstone of sustainable organic waste management. Composting is a mature technology (TRL 9), widely implemented at commercial scale, though it presents limitations such as long processing times, odor generation, and limited control over end-product quality.

1.3.3. Anaerobic Digestion

1.3.3.1. Anaerobic Digestion Pathways

CH_4 is recognized not only as a potent GHG but also as a valuable renewable energy source and the main component of biogas. It is primarily generated through AD, a natural biological process in which complex organic matter is degraded in the absence of oxygen by a syntrophic microbial consortium composed of bacteria and methanogenic archaea. This multi-stage process is essential for converting organic substrates into CH_4, with methanogenic archaea playing a key role during the final step (Steinmetz et al., 2016; Poirier et al., 2020; Lansing et al., 2016). Expanding on the foundational role of CH_4 as a renewable energy vector in anaerobic systems, AD remains the most common and efficient biological route for converting organic materials (such as FVW) into CH_4-rich biogas.

This multi-stage biochemical process involves four interrelated phases (Fig. 1.9): hydrolysis, acidogenesis, acetogenesis, and methanogenesis, each driven by specific microbial communities responsible for the stepwise degradation of complex organic matter (Ruffino et al., 2015; Nguyen et al., 2019). During the initial hydrolysis phase, hydrolytic bacteria secrete extracellular enzymes that break down particulate macromolecules like carbohydrates, proteins, and lipids into simpler compounds such as sugars, amino acids, and volatile fatty acids (VFAs). The efficiency of this stage is crucial, as it determines the availability of soluble substrates for downstream microbial metabolism (Nguyen et al., 2019). In the subsequent acidogenesis phase, these soluble products are fermented by acidogenic bacteria into VFAs (Dahiya et al., 2015; Kandylis et al., 2016; Khan et al., 2016), H_2, and CO_2, which serve as key intermediates in CH_4 formation (Vargas-Estrada et al., 2025; Meng et al., 2017; Lin et al., 2016). Acetogenesis then further converts these VFA intermediates into acetic acid (HAc), H_2, and CO_2 via acetogenic bacteria, effectively preparing substrates for the final step of methanogenesis (Ruffino et al., 2015; Nguyen et al., 2019).

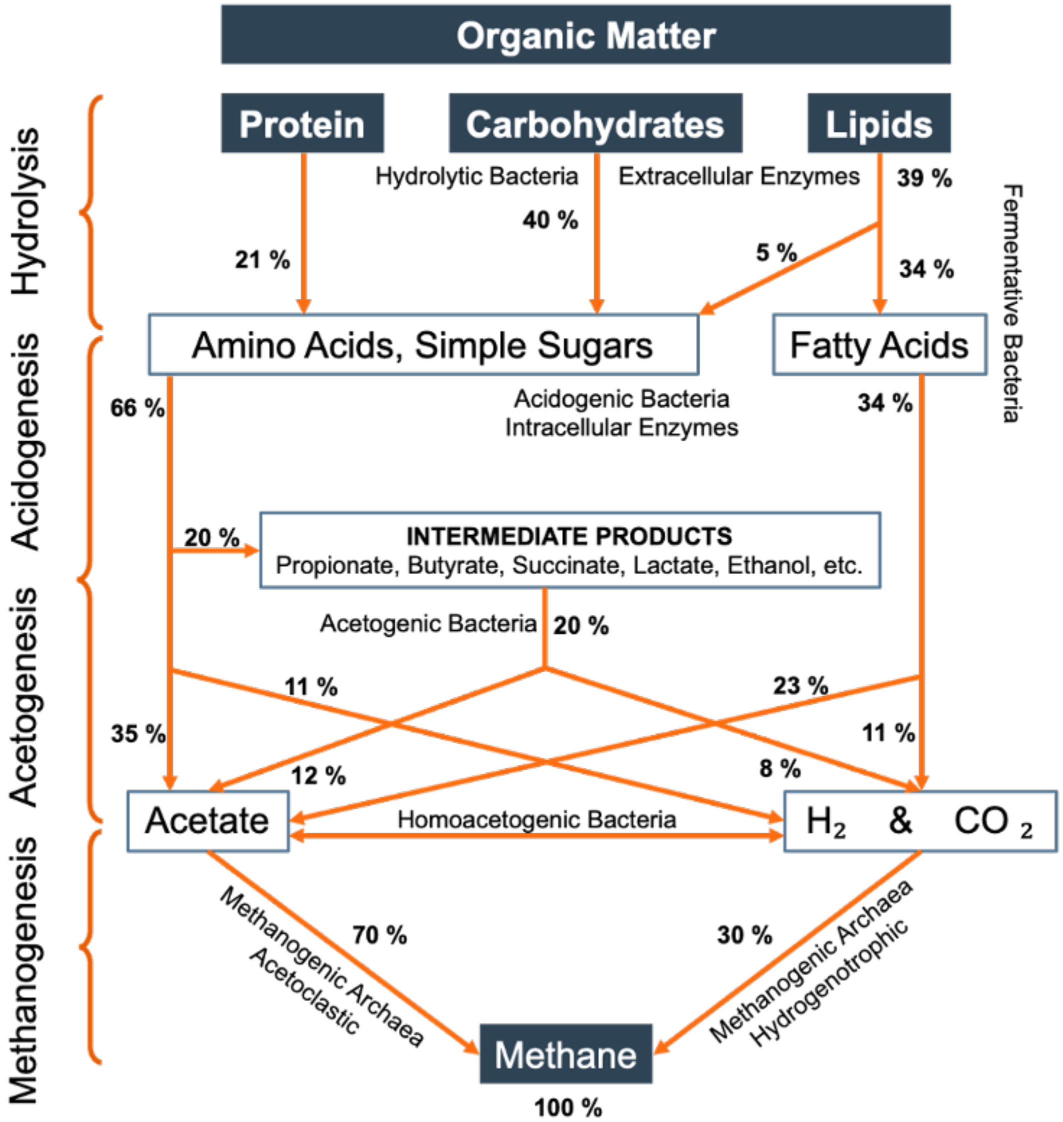

Figure 1.9. Overview of AD steps from organic matter to CH_4. Adapted from Torres-Lozada & Pérez, 2010.

This final phase is performed by *methanogenic archaea*, a group of microorganisms within the domain archaea, which convert HAc, H_2, and CO_2 into CH_4. Depending on the metabolic pathway, some methanogens (acetoclastic) produce CH_4 directly from HAc, while others (hydrogenotrophic) reduce CO_2 using H_2 as electron donor (Nguyen et al., 2019; Arias et al., 2018). Therefore, methanogenic archaea are central to the success of this process, especially under high-loading rates like those associated with FVW treatment, which rapidly ferments and may acidify the digester cultivation broth. These archaea are well-adapted to strictly anaerobic conditions and play a stabilizing role in the digestion system by effectively transforming intermediary products into CH_4 (Nguyen et al., 2019; Oliveira et al., 2015; Ahamed et al., 2015). Therefore, the microbial pathways underlying AD are essential for turning organic waste into valuable energy, while simultaneously mitigating environmental impacts and supporting circular economy initiatives. In addition, Figure 1.9 illustrates the conversion pathways of organic matter during AD. The percentages indicate the relative distribution of each metabolic route: 40% of carbohydrates, 39% of lipids, and 21% of proteins are initially transformed into simple sugars, amino acids, or fatty acids. Subsequently, about 66% of these compounds are directed towards intermediate products (propionate, butyrate, succinate, lactate, or ethanol), while 35% are directly converted into acetate. At the acetogenesis and methanogenesis stages, acetate contributes 70% to the final CH_4 production, whereas the remaining 30% derives from H_2 and CO_2, highlighting the predominance of the acetoclastic over the hydrogenotrophic pathway.

Beyond its contribution to waste stabilization, AD provides a sustainable pathway for organic waste valorization, transforming residues into biogas that can be used for electricity generation, heating, and as a transportation fuel (Poirier et al., 2020; Youngsukkasem et al., 2015; Crocamo et al., 2015). The use of CH_4 as an energy vector contributes to a reduction in fossil fuel dependency and supports the transition toward low-carbon energy systems, particularly when its production is integrated into circular economy concepts (Youngsukkasem et al., 2015). However, due to its high global warming potential (up to 28 times more impactful than CO_2 over a 100-year period) its controlled production, capture, and use are crucial to minimize negative environmental consequences (Crocamo et al., 2015; Strong et al., 2016). Thus, the biological generation of CH_4 represents both a challenge and an opportunity, making it a central topic in sustainable waste management and renewable energy research.

1.3.3.2. Fruit-Vegetable Waste as a Feedstock for Methane Production

Building on CH_4 central role as a renewable energy carrier, FVW has emerged as a cost-effective feedstock for biogas production through AD. Its high biodegradability, moisture content (70–95%), and sugar-rich composition promote microbial activity and an efficient breakdown of organic matter into CH_4 (Quiroga et al., 2014; Meng et al., 2015; Capson-Tojo et al., 2017; Haider et al., 2015; Zhang et al., 2016). Table 1.1 summarizes reported CH_4 yields and biodegradability values for FVW under AD. The abundance of simple carbon sources supports methanogenic archaea, enhancing gas yields and minimizing residual waste (Meng et al., 2017; Owamah and Izinyon, 2015). Economically, FVW is low-cost and widely available, making it a viable substrate for large-scale biogas systems (Xie et al., 2016). Its use also reduces CH_4 and CO_2 emissions from landfills, supporting circular economy models and sustainable waste management (Haider et al., 2015).

Table 1.1. Summary of CH_4 productivity and biodegradability from FVW under AD.

Substrate	Methane productivity / Biodegradability	Reference
Organic waste (Including FVW)	High biodegradability	Khalid et al. (2011)
FVW + fish waste + cattle slurry	0.38 L CH_4/g VS	Eiroa et al. (2012)
FVW + cattle manure	230–450 L CH_4/kg VS	Quiroga et al. (2014)
FVW + sewage sludge	0.97–2.40 L CH_4/L-d; 56–57%	Mazareli et al. (2016)
Food waste (including FVW)	421 ± 15 mL CH_4/g VS; 73% biodegradability	Moñino et al. (2016)
Food waste	0.96 NL/L-d and 398 NmL/g VS_{FED}	Chapter 6 of this thesis

However, despite its benefits, the AD of FVW presents operational challenges. One of the most common is process acidification caused by excessive accumulation of VFAs, which can inhibit methanogens and reduce the overall system performance (Meng et al., 2017; Zhong et al., 2015). Furthermore, the low carbon-to-nitrogen (C/N) ratio of FVW can lead to ammonia inhibition, necessitating adjustments through co-digestion with nitrogen-rich substrates or external nutrient addition to ensure microbial balance and optimal CH_4 yields (Peces et al., 2016; Razaviarani and Buchanan, 2015). Various strategies have been developed to overcome these limitations. Co-digestion with complementary feedstocks in terms of elemental composition has proven effective in improving nutrient balance and microbial stability (Strong et al., 2016; Ahamed et al., 2015). Pretreatment techniques (such as thermal, enzymatic, ultrasound, or chemical treatments) can enhance substrate accessibility and hydrolysis efficiency, often leading to improved CH_4 yields (Patinvoh et al., 2017; Yahmed et al., 2021). Additionally, a careful control of operational conditions such as temperature (mesophilic 25–40°C or thermophilic 50–65°C) and pH (7-8) helps creating an optimal environment for microbial communities, particularly methanogens (Lin et al., 2016; Zahedi et al., 2018).

1.3.3.3. Operational Parameters

Building on the relevance of microbial pathways and substrate characteristics in CH_4 generation, the AD of FVW is highly sensitive to a series of operational, compositional, and inhibitory factors that influence process performance. pH plays a central role in maintaining microbial balance. Methanogens generally thrive at pH 6.5–7.5, while acidogenic bacteria prefer slightly more acidic conditions. Deviations from optimal pH can lead to VFA accumulation and methanogenic inhibition, reducing CH_4 yields and destabilizing the process (Tampio et al., 2016).

Temperature impacts both microbial metabolism and process kinetics. Mesophilic conditions (25–40°C) offer stable microbial performance, while thermophilic digestion (50–65°C) can increase reaction rates and CH_4 output but demands tighter control to avoid microbial inhibition and ammonia toxicity (Cavinato et al., 2012; Li et al., 2016). HRT determines the time available for microbial communities to metabolize the substrates. Too low HRTs can cause biomass washout, particularly of slow-growing methanogens, while excessively long HRTs may reduce system efficiency because of an extended endogenous metabolism (Young et al., 2013; Zahedi et al., 2017). An optimal HRT ensures adequate digestion and CH_4 production without overloading the system. Finally, the OLR, which reflects the daily mass input of organic matter per reactor volume, also influences microbial activity and CH_4 yield. Moderate OLR supports biogas generation, but organic overloading may result in acid accumulation and microbial inhibition, especially under suboptimal HRT or pH conditions (Orzi et al., 2010; Ruile et al., 2015). Together, these parameters must be carefully optimized and balanced to sustain a high CH_4 productivity and ensure long-term operational stability of AD systems treating FVW.

1.3.3.4. Strategies to Enhance Methane Yields

Maintaining an appropriate pH range is essential, as methanogenic archaea are particularly vulnerable to acidification. The rapid fermentation of FVW (rich in simple carbohydrates) can lead to the accumulation of VFAs, triggering a drop in buffering capacity and ultimately in pH that compromises methanogenic activity (Shin et al., 2015). Thus, stabilizing pH through buffer capacity or co-digestion is key to balancing acid production and consumption. Temperature is another determining factor: thermophilic conditions (around 55 °C) can accelerate reaction rates and enhance hydrolysis, but they require strict control, as microbial consortia can become unstable under thermal stress (Ruile et al., 2015). Similarly, while increasing the OLR may improve volumetric CH_4 production, this must be matched with an adequate HRT to avoid acid accumulation and incomplete substrate degradation (Tampio et al., 2016; Mazareli et al., 2016).

In AD of organic waste, typical HRT ranges from 15–30 days and OLR from 2.4–6.0 g VS/L-day, ensuring stable CH_4 production and avoiding VFA inhibition (Mazareli et al., 2016; Cardona et al., 2019; Tampio et al., 2016). The nature of the substrate also plays a pivotal role. Although mono-digestion of FVW can yield high CH_4 outputs due to its high biodegradability and sugar content, co-digestion with substrates like animal manure or sludge often leads to greater process stability. This synergistic approach enhances nutrient balance and dilutes potential inhibitors, fostering favorable conditions for microbial consortia and CH_4 generation (Kiran et al., 2014). In contrast, relying solely on FVW may expose the system to nutrient deficiencies or imbalances that impair performance. In this context, inhibitory compounds remain one of the major bottlenecks in AD. Accumulation of VFAs and elevated ammonia concentrations (often resulting from nitrogen-rich or proteinaceous waste) can severely inhibit methanogens by disrupting their metabolic functions (Liu et al., 2015; Lerm et al., 2012). Additionally, competition for substrates between sulfate-reducing bacteria and methanogens may further constrain CH_4 production pathways (Lerm et al., 2012). Therefore, anticipating and managing these risks through adequate process monitoring and control is crucial for maintaining functional microbial ecosystems.

The availability of micronutrients and trace elements is essential for enzymatic activity and microbial resilience. In many cases, especially when using FVW as a primary substrate of AD, essential elements such as cobalt, nickel, or selenium may be present in suboptimal concentrations. Supplementing these micronutrients has been shown to stabilize methanogenic populations and enhance methane yields, particularly in co-digestion setups where nutrient variability is common (Moestedt et al., 2016). Addressing nutrient limitations through tailored supplementation is thus critical to support a robust microbial performance and unlock the full potential of FVW as a feedstock for sustainable CH_4 production.

Two-phase AD (Fig. 1.10) enhances biogas production and process stability by separating hydrolytic-acidogenic processes from acetogenic-methanogenic ones. In the first stage, hydrolytic and fermentative bacteria (e.g., *Clostridium, Bacteroides*) convert complex particulate organics into VFAs, H_2, and CO_2 (Xiao et al., 2015). In the second stage, acetogenic bacteria (e.g., *Syntrophomonas*) and methanogens (e.g., *Methanosarcina*) convert these intermediates into CH_4 (Zhang et al., 2019; Cavinato et al., 2011). This phase separation allows for tailored conditions.

Thus, acidogenesis benefits from slightly acidic pH (5.5–6.5), while methanogenesis thrives near neutrality (6.5–8.0), which enhances microbial efficiency (Grimberg et al., 2015). As a result, two-phase systems demonstrate greater process stability, reduce inhibition risks (Nasr et al., 2012; Wang et al., 2024), and often produce higher biogas yields (Da Silva Júnior et al., 2025; García-Depraect et al., 2023; García-Depraect et al., 2022; Amodeo et al., 2021; Alonso et al., 2016; Yeung et al., 2017). Additionally, they offer operational flexibility for treating complex or variable waste streams (Wang et al., 2012; Fagbohungbe et al., 2017; Ohdoi et al., 2024; Akimoto et al., 2025; Chatterjee and Mazumder 2024).

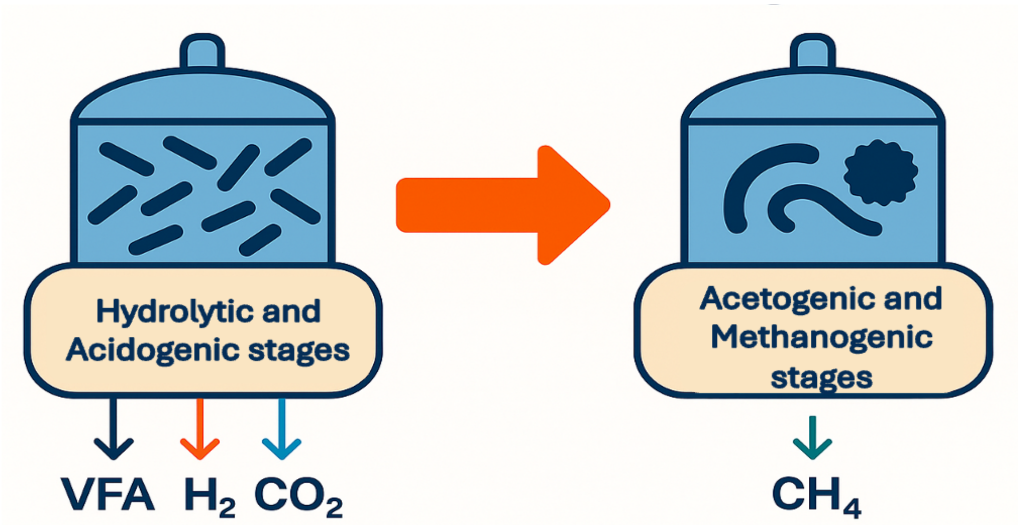

Figure 1.10. Schematic representation of the two-phase AD process, separating hydrolytic-acidogenic and acetogenic-methanogenic stages for enhanced biogas production.

1.3.3.5. The Role of Methane in Integrated Biorefinery Systems

Building on the growing relevance of anaerobic digestion for CH_4 generation, integrated models for organic waste valorization increasingly place CH_4 production at the core of multistage treatment systems aimed at improving efficiency and sustainability (García-Depraect et al., 2023; García-Depraect et al., 2022). In these multistage configurations, CH_4 not only serves as a renewable energy carrier but also plays a stabilizing role in the overall treatment of complex waste streams, enabling the recovery of nutrients and the integration of downstream biorefinery processes (Poirier et al., 2020; Khalid et al., 2011). Notably, two-phase systems that couple DF for H_2 production with subsequent CH_4 generation through AD have demonstrated enhanced bioenergy recovery, particularly under cascade setups that maximize substrate conversion (Hou et al., 2025; Jariyaboon et al., 2015; Quéméneur et al., 2011).

Furthermore, aligning CH_4 production with biorefinery platforms and fertilizer recovery systems supports circular economy principles by transforming digestate into a valuable soil amendment, thereby strengthening the environmental and economic outcomes of the process (Kuisma et al., 2013; Lee et al., 2024). Importantly, CH_4 energy potential makes it especially relevant in rural or high-waste-producing regions, where it can contribute to decentralized energy generation while addressing broader challenges such as energy access and climate mitigation (Marín et al., 2021; Omar et al., 2019).

The experimental findings presented in Chapter 6 of this thesis provide robust validation of the conceptual basis discussed in Section 1.3.3, particularly concerning the advantages of lactate-driven two-stage AD for enhancing CH_4 production. The comparison between a single-stage AD system and a lactate-based two-stage AD system using food waste as substrate revealed that the two-stage configuration achieved significantly higher CH_4 yields (398.1 ± 35.2 NmL CH_4/g VS_{FED}) and productivity (959.3 ± 75.3 NmL CH_4/L-d), exceeding those of the conventional one-stage AD system by over 30%.

These results confirm the thermodynamic advantage of lactic acid (HLac) as a fermentative intermediate, which was predominantly produced during the acidogenic phase (up to 6.5 ± 0.9 g/L) and almost completely oxidized during the methanogenic stage. The efficient conversion of HLac to HAc and CH_4 aligns with the predicted energetic benefits and demonstrates the value of physically separating the acidogenic and methanogenic stages to optimize microbial performance.

1.3.4. Dark Fermentation

H_2 is increasingly regarded as a clean and versatile energy carrier, essential in transitioning towards low-carbon energy systems. The main merits of H_2 are its high gravimetric energy content (≈ 142 kJ/g), rapid energy conversion kinetics, and its harmless combustion yielding only water vapor, making it an environmentally benign fuel (Haroun et al., 2016; Lopes et al., 2015).

In the context of climate change mitigation and energy diversification, H_2 stands out as a strategic solution, especially when produced from renewable or waste-derived sources (Sekoai et al., 2020). The integration of H_2 into the global energy matrix holds significant promise for decarbonizing hard-to-abate sectors such as transportation, industry, and distributed power generation.

1.3.4.1. Fundamentals, Potential and Metabolic Pathways

Among the various methods available for H_2 production, DF (Fig. 1.11) has recently gained traction as a biological, low-energy alternative, capable of converting the chemical energy contained in organic waste into H_2 under anaerobic and light-independent conditions (Abreu et al., 2016). In contrast with thermochemical processes, DF operates under mild conditions and relies on the metabolic activity of anaerobic microbial consortia, primarily hydrolytic and acidogenic bacteria (Xiao et al., 2010).

These microorganisms ferment carbohydrates, proteins, and lipids into OA, alcohols, CO_2, and H_2. The simplicity of this process, along with the use of naturally occurring bacteria, makes it a viable option for decentralized H_2 production (Haroun et al., 2016). In addition, the organic effluent generated during DF can be further valorized to produce biogas, bioplastics, among others (García-Depraect et al., 2025).

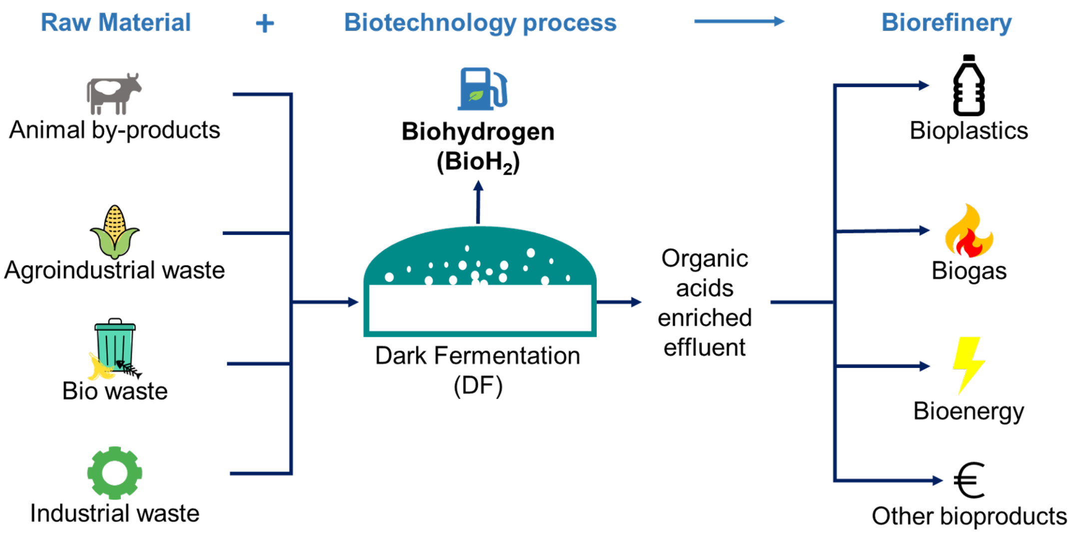

Figure 1.11. Visual representation of dark fermentation process.

At a biochemical level, DF proceeds through a series of enzymatic pathways that begin with the hydrolysis of complex organic compounds into monomeric sugars, which then enters the glycolytic pathway. Here, the resulting pyruvate acts as a central intermediate and is directed through various acidogenic routes. This metabolic conversion results in the production of VFAs, mainly HAc, butyric (HBu), propionic (HPr), and formic acids (HFor), and molecular H_2. In this context, the HAc pathway is considered the most hydrogen-efficient, as it facilitates a balanced release of electrons that are captured by hydrogenase enzymes to generate H_2 (Guo et al., 2010; Mugnai et al., 2021). However, when electrons are instead diverted toward the synthesis of other VFAs like HPr, HAc (via homoacetogenesis), or toward HLac via Lactic Acid Bacteria (LAB), the net H_2 yield is significantly reduced.

FVW represents a promising substrate for DF due to its high-water content, high fermentable sugars content (e.g., glucose, fructose, sucrose) (Ebrahimian et al., 2022). These components not only support a rapid microbial proliferation but also trigger enzymatic activities critical to H_2 production (Ebrahimian et al., 2022). Compared to lignocellulosic biomass, which requires energy-intensive pretreatment, FVW is relatively easy to hydrolyze, thereby accelerating the onset of glycolysis and acidogenesis. Nonetheless, this rapid fermentation rate also makes FVW susceptible to metabolic imbalances (especially the accumulation of HLac) which can disrupt the proton gradient, reduce pH, and impair hydrogenase function (Gioannis et al., 2013).

The accumulation of HLac during fermentation is closely linked to the proliferation of LAB, such as *Lactobacillus*, *Weissella*, and *Enterococcus*, which metabolize sugars rapidly and favor HLac production over H_2. Under unregulated conditions (such as excess sugar concentration, low buffer capacity, or suboptimal pH) LAB can dominate the microbial consortium, leading to a metabolic redirection that suppresses hydrogenogenesis. As HLac builds up, the medium becomes increasingly acidic, thereby inhibiting key metabolic enzymes and collapsing the proton motive force required for energy transduction in H_2-producing bacteria (HPB) (Mugnai et al., 2021). This disruption limits ATP generation, nutrient transport, and electron transfer processes, ultimately resulting in a dramatic decrease in H_2 yields. Effective control of HLac accumulation is essential for the success of DF. Strategies to achieve this include the use of buffer systems to stabilize pH, controlled feeding of FVW to prevent sugar overload, and microbial management practices such as selective inoculation with hydrogenogenic bacteria or the introduction of LAB inhibitors. Moreover, co-cultivation techniques and the design of microbial consortia that promote metabolic balance, can help redirecting fermentation pathways toward VFAs like HAc and HBu, acids more compatible with H_2 production (Guo et al., 2010; Gioannis et al., 2013).

From a biotechnological standpoint, integrating DF with other anaerobic processes, such as methanogenesis, can also mitigate HLac-related inhibition. In two-stage or cascade systems, HLac and other residual metabolites from DF are further processed into CH_4 by methanogenic archaea, thereby improving the overall energy recovery and waste stabilization. This sequential configuration not only enhances the energy output (H_2 + CH_4) but also promotes resource circularity by utilizing fermentation effluents instead of discarding them (Ebrahimian et al., 2022). Maintaining the proton gradient across microbial membranes is also central to the success of DF. This electrochemical potential is responsible for ATP synthesis and influences the activity of hydrogenases, particularly the FeFe-hydrogenases that catalyze the final step of H_2 evolution. When HLac over accumulates, the elevated concentration of protons outside the cell disrupts this gradient, undermining the energy economy of the microbial system. Consequently, the loss of membrane integrity and decreased enzymatic activity become significant barriers to efficient H_2 production (Mugnai et al., 2021).

FVW's high content of fermentable sugars also means that fermentation systems must be equipped to handle fast acidogenesis without collapsing under acid stress. Compared to more recalcitrant substrates, such as lignocellulosic residues, FVW undergoes fermentation much more quickly, often leading to volatile shifts in microbial activity. Therefore, while the energetic and operational potential of FVW is high, these systems demand fine-tuned control mechanisms, including pH regulation, adaptive microbial inocula, and possibly metabolic engineering approaches to limit the pathways leading to HLac accumulation.

1.3.4.2. Fruit-Vegetable Waste as a Feedstock for Hydrogen Production

Table 1.2 summarizes the reported H_2 yields obtained from FVW under various DF conditions, highlighting its effectiveness as a feedstock. Additionally, DF offers advantages in terms of lower energy input and minimal equipment requirements compared to water electrolysis, and relatively short HRT compared AD, further supporting its feasibility for on-site or small-scale applications (Xiao et al., 2010).

Table 1.2. Summary of H_2 yields from organic waste under DF.

Substrate	H_2 yield / productivity	Reference
FVW	14.0 ± 1.0 NL-H_2/L-d	Chapter 7 of this thesis
FW	9.6 ± 0.9 L H_2/L-d	Regueira-Marcos et al. (2024)
FW	70.1 ± 7.7 NmL-H_2/g-VS_{FED}	Martínez-Fraile et al. (2024)
FVW	11.8 NL H_2/L-d and 95.6 NmL H_2/g VS_{FED}	Chapter 5 of this thesis
FW	4.2 ± 0.6 NL H_2/L-d	Regueira-Marcos et al. (2023)
FW	13 NL H_2/L-d	Regueira-Marcos et al. (2023)
FVW	50 mL H_2/g VS and 976.4 mL H_2/L-h	Chapter 4 of this thesis
FVW + corn stover hydrolysates	289 mL H_2/g COD	Rodríguez-Valderrama et al. (2020)
Garden and food waste	0.40–0.60 L H_2/g VS	Abreu et al. (2019)
FVW	4.5 L H_2/g VS	Saidi et al. (2018)
Cheese whey + FVW	1.8 L H_2/g VS	Gómez-Romero et al. (2014)
Agricultural waste mix	2.6 L H_2/L reactor	Kumar et al. (2012)
FVW + sucrose adaptation	0.55–0.75 L H_2/g VS	Li et al. (2012)
Glucose (FVW model)	0.67 L H_2/g COD	Xiao et al. (2010)

Organic residues such as FVW, agro-industrial byproducts, and food scraps have been widely explored as feedstocks for DF due to their high biodegradability and sugar content (Arizzi et al., 2016; De Menezes et al., 2024). These substrates are metabolically favorable for H_2-producing bacteria and can serve a dual purpose: producing renewable energy and reducing the environmental burden of waste accumulation. In fact, the valorization of such organic wastes via DF supports circular economy principles by recovering energy, carbon and nutrients from materials that would otherwise be landfilled or incinerated (Abreu et al., 2016, Adamu et al., 2023).

1.3.4.3. Operational Parameters

Key operational parameters such as HRT, OLR, pH, and temperature (Fig. 1.12) play a critical role in DF processes. Among these, HRT, which reflects the average residence time of the substrate within the reactor, is particularly influential in optimizing H_2 Production Rate (HPR). While shorter HRTs are generally associated with enhanced waste to H_2 conversion efficiency, excessively low retention times may result in biomass washout and system instability.

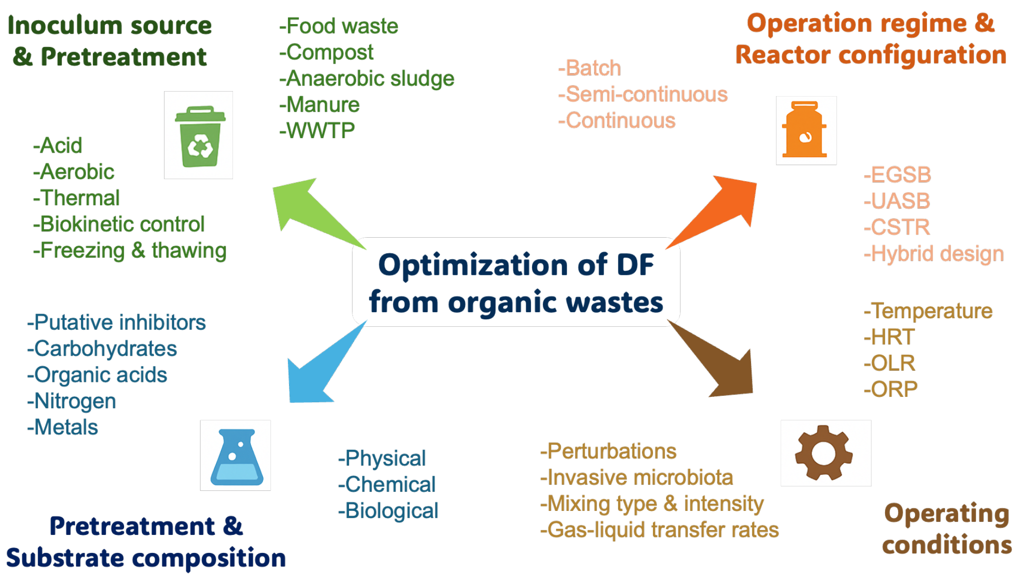

Figure 1.12. Comprehensive overview of the variables requiring optimization to enhance the efficiency of H_2 production in DF. WWTP: wastewater treatment plant; CSTR: continuous stirred-tank reactor; EGSB: expanded granular sludge bed reactor; UASB: up-flow anaerobic sludge blanket reactor; AFBR: anaerobic fluidized bed reactor; HRT: hydraulic retention time; SRT: solid retention time; OLR: organic loading rate; and ORP: oxidation–reduction potential adapted from García-Depraect et al., 2025.

The OLR determines the amount of organic matter supplied to the reactor, where moderate levels enhance microbial activity and H_2 yields, while excessive loading can lead to acid accumulation and system inhibition (Ghimire et al., 2016). Maintaining an optimal OLR range is thus essential for balancing substrate availability and reactor stability (Groof et al., 2021). Similarly, pH regulation is crucial for microbial performance, as H_2-producing bacteria, particularly *Clostridium*, thrive in near-neutral to slightly alkaline conditions. Deviations from this range affect both microbial growth and product distribution, reducing H_2 generation and increasing inhibitory metabolites (Mudhoo et al., 2018; Yahmed et al., 2021). Temperature also influences metabolic rates and microbial community structure. While mesophilic conditions (30–40°C) support diverse microbial consortia, thermophilic settings (>45°C) may accelerate H_2 production. However, fluctuations in temperature may compromise process efficiency, highlighting the importance of thermal stability (Sivagurunathan et al., 2016; Okonkwo et al., 2019).

Despite its importance, limited research has systematically investigated these parameters to maximize H_2 yields from organic waste. The conceptual framework introduced in Section 1.3.4 is strongly validated by the experimental results detailed in Chapter 5 of this thesis. In this study, the continuous DF of FVW was optimized by modulating the HRT, achieving unprecedently high HRT and H_2 yields at a 9 h HRT.

1.3.4.4. Challenges: Lactate Accumulation and Process Inhibition

From a technological perspective, advancements in reactor design, such as upflow anaerobic sludge blanket (UASB) or continuous stirred-tank reactors (CSTR) tailored for DF, or dynamic membrane dark fermenters (Tang et al., 2017) have improved process stability and microbial retention. Additionally, real-time monitoring and control systems are being developed to track critical variables like pH, oxidation-reduction potential, and metabolite concentration, allowing for rapid adjustments that prevent metabolic imbalances and ensure steady H_2 production (Abreu et al., 2019). Substrate selection also plays a crucial role in optimizing H_2 yields. Carbohydrate-rich wastes tend to favor hydrogenogenic pathways, and excessive sugar concentrations can induce HLac fermentation (Xiao et al., 2010). Therefore, pretreatment strategies (such as thermal hydrolysis, enzymatic digestion, or acid/alkali treatments) are often applied to enhance substrate bioavailability while minimizing pathway diversion (Arizzi et al., 2016).

Furthermore, microbial strain engineering and adaptive evolution are being explored to develop robust microbial consortia with higher resistance to inhibitors and improved H_2 production capacities (Sekoai et al., 2020).

Building on the potential of H_2 as a clean energy vector, DF has emerged as one of the most promising biological routes for converting organic wastes into renewable H_2 (García-Depraect et al., 2022). This process involves the anaerobic metabolism of carbohydrates, primarily derived from biomass such as FVW, and is carried out by specialized microbial communities that operate under oxygen-free conditions. The core advantage of DF lies in its ability to produce H_2 at moderate temperatures (37-55 ºC) without the need for light or complex pretreatment stages, making it highly applicable for decentralized waste-to-energy systems and circular economy models (Gioannis et al., 2013). The experimental findings, discussed in Chapter 5 of this thesis, not only confirm the inhibitory potential of HLac under suboptimal conditions but also highlight its metabolic versatility when managed appropriately. In particular, the observed shifts in metabolite profiles across varying HRTs demonstrated that the presence of HLac was consistent among the predominant soluble by-products. Under optimal operational conditions, its conversion was positively correlated with enhanced H_2 production, reinforcing the theoretical premise introduced lately.

1.3.4.5. Lactate-Driven Dark Fermentation: A New Perspective

In recent years, lactate-driven DF (LDDF) has emerged as a transformative approach in the field of H_2 production (García-Depraect et al., 2022). Traditionally, HLac was regarded as a by-product with inhibitory effects on H_2-producing microorganisms due to its role in acidifying the fermentation medium. However, new perspectives have highlighted its potential as a fermentable intermediate capable of generating additional H_2 when metabolized by specialized microbial consortia under controlled conditions (Jürgensen et al., 2015; Mudhoo et al., 2018; García-Depraect et al., 2022; Pengadeth et al., 2024). This approach entails a reconfiguration of metabolic pathways, where HLac formed during acidogenesis is not allowed to accumulate, but rather undergoes further oxidation into butyrate and CO_2; liberating additional molecular H_2 in the process (García-Depraect et al., 2022). Rather than surpassing the yields of glucose-based DF, this strategy contributes to mitigating the inhibitory effects associated with lactic acid bacteria. The conversion of HLac, a relatively energy-dense molecule, represents a strategy to enhance substrate conversion efficiency and overall energy recovery (Nasr et al., 2015).

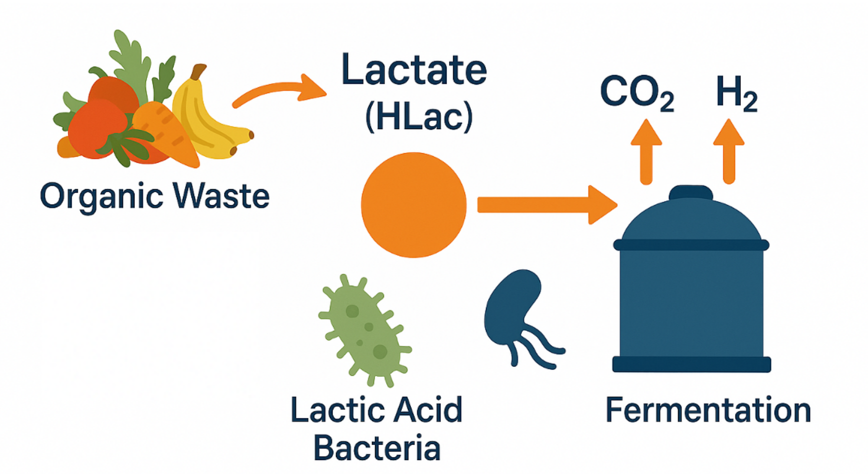

Figure 1.13. Diagram of the lactate-driven dark fermentation (LDDF) process.

Biochemically, HLac results from pyruvate reduction by HLac dehydrogenase, an essential process for NAD^+ regeneration in LAB, which does not directly contribute to H_2 production. To overcome this bottleneck, HLac-oxidizing bacteria, often from the *Clostridium* genus or other strict anaerobes, are introduced or enriched to metabolize HLac into H_2 and HBu (García-Depraect et al., 2022). These organisms utilize oxidative enzymes and hydrogenases in syntrophic relationships with LAB, converting a metabolite previously seen as inhibitory into an asset. The thermodynamics of HLac oxidation are favorable when the H_2 partial pressure is kept low, achievable through continuous gas removal or reactor optimization. This enables redirection of electron flow through Fe-Fe hydrogenases, facilitating efficient H_2 production (Mudhoo et al., 2018).

Lactate-based fermentation plays a central role in DF and subsequent AD, acting as a key metabolic intermediate between primary acidogenic reactions and methanogenesis. Mechanistically, HLac is oxidized to pyruvate by lactate dehydrogenase, with the concomitant transfer of reducing equivalents (NADH/NAD^+) (Garvie, 1980). Pyruvate can then be further converted into acetyl-CoA, releasing CO_2 and producing reduced cofactors that support H_2 generation (Detman et al., 2019). Acetyl-CoA is ultimately metabolized into acetate, a major substrate for both hydrogenogenic and acetoclastic pathways. This metabolic shift explains the observed co-production of acetate, butyrate, and H_2 under lactate-driven conditions (Wu et al., 2020; Kucek et al., 2016). In the methanogenic phase, acetate serves as the dominant precursor for CH_4 formation by *Methanothrix* (Smith & Ingram-Smith, 2007), while H_2 and CO_2 are consumed by hydrogenotrophic archaea such as *Methanobacterium* (Thauer et al., 2008). Therefore, HLac functions not only as an intermediate derived from carbohydrate fermentation but also as a bioenergetic substrate that enhances redox balancing, stabilizes microbial interactions, and improves CH_4 yields in phase-separated systems (Wang et al., 2009).

The strategy thereby turns metabolic competition into synergy by engineering co-cultures where LAB produce HLac and HPB consume it, maximizing energy recovery (Pérez-Rangel et al., 2021). Thus, LDDF is an attractive platform for sustainable H_2 production. Firstly, it supports relatively high H_2 yields per mole of substrate, with theoretical values consistent with the maximum of 4 mol H_2 per mol of glucose reported for conventional DF (Zagrodnik and Łaniecki, 2015). Secondly, it enables better pH management. Whereas uncontrolled HLac accumulation leads to acidification and microbial inhibition, converting HLac reduces proton buildup, thus protecting hydrogenase activity and maintaining membrane potential (Zagrodnik and Łaniecki, 2015).

1.3.4.5.1. Reactor Engineering & Control Tools in Lactate-Driven Dark Fermentation

Process stability and efficiency can be further enhanced through bioaugmentation, introducing lactate-oxidizing strains into the fermentation system. These tailored consortia rebalance the microbial population, outcompeting or cooperating with native LAB to prevent excessive acidification and maintain H_2 yields (Park et al., 2021; Pérez-Rangel et al., 2021; García-Depraect and León-Becerril 2023).

Likewise, co-digestion with substrates rich in buffering capacity or complementary nutrients helps moderate acid accumulation and improve community diversity (Silva et al., 2023). Advanced process engineering also plays a central role in the success of LDDF. Reactor configurations and the application of gas stripping enhance mass transfer and remove inhibitory gases. Real-time monitoring of key parameters such as pH, HLac levels, and H_2 yields allows for dynamic adjustments that stabilize and optimize the process (Mudhoo et al., 2018).

From a biochemical standpoint, hydrogenogenesis from HLac relies on the oxidative reversal of the HLac dehydrogenase pathway, producing pyruvate and releasing electrons. These are subsequently used by hydrogenases to reduce protons into H_2. The integration of this metabolic route into existing fermentation systems provides a pathway to improve the energetic output while reducing system inhibition (Nasr et al., 2015). In terms of microbial ecology, maintaining the balance between hydrogenogenic and HLac-producing populations is essential. LAB such as *Lactobacillus*, *Weissella*, and *Enterococcus* are competitive sugar fermenters, often dominating when sugar levels are high. In contrast, HPB are more sensitive to pH and redox changes, requiring deliberate management of environmental conditions and inoculum structure (Jürgensen et al., 2015; Mudhoo et al., 2018).

The proton motive force, crucial for ATP synthesis and metabolic homeostasis, is also directly affected by HLac accumulation. High extracellular proton concentrations collapse the gradient, disrupting hydrogenase activity and redirecting metabolic flux toward non-productive pathways. By actively oxidizing HLac, microbial communities can restore proton gradients and redirect energy toward H_2 generation (Zagrodnik and Łaniecki, 2015). Recent research has emphasized the potential of integrating LDDF into broader biorefinery frameworks, especially when coupled with systems like AD, photofermentation, microalgae growth or microbial electrolysis (Silva et al., 2023). Such hybrid processes enable the valorization of not just H_2 but also co-products like HAc or CH_4, making the approach economically attractive and environmentally sustainable.

Furthermore, the genetic and enzymatic underpinnings of LDDF are being explored through omic-level studies, targeting overexpression of key enzymes, metabolic pathway redirection, and strain optimization (Abreu et al., 2016). These insights are crucial for tailoring consortia that efficiently channel electrons toward H_2 rather than competing end-products. In summary, LDDF represents a compelling advancement in H_2 production. By repositioning HLac from inhibitor to intermediate, it unlocks a new layer of substrate conversion efficiency and system resilience. With the combined use of microbial engineering, bioaugmentation, co-digestion, and precise pH and process control, LDDF systems can play a vital role in transitioning toward renewable, waste-based H_2 energy production (Abreu et al., 2016; Nasr et al., 2015; Mudhoo et al., 2018).

The strategy proposed in Section 1.3.4.5 is strongly supported by the experimental findings presented in Chapter 4 of this Thesis. Notably, experiments conducted under controlled conditions demonstrated that HLac is not merely an inhibitory by-product. While HLac accumulation was indeed correlated with reduced hydrogen yields and microbial inhibition (validating its role as a limiting metabolite in DF) the results also revealed that HLac can serve as a fermentable intermediate capable of being further converted into molecular H_2. This conversion was particularly evident in fermentation setups involving co-cultures enriched with lactate-oxidizing bacteria.

The metabolic dynamics observed throughout the fermentation process showed a clear transformation of HLac into HAc and H_2, accompanied by a significant increase in H_2 production. These findings underscore a syntrophic relationship between HLac-producing bacteria (e.g., *Lactobacillus*) and hydrogenogenic species (e.g., *Clostridium*), in line with thermodynamic predictions that HLac oxidation under low H_2 partial pressure releases electrons that fuel hydrogenase activity.

1.3.4.6. Scaling High-Performance Dark Fermentation

The next frontier in dark fermentative H_2 production lies in the incorporation of advanced biotechnologies and predictive process management tools (Fig. 1.14). These innovations are geared not only towards enhancing H_2 yield but also improving the energy and cost-efficiency of the overall system. As the global energy landscape shifts towards more sustainable solutions, the continued development of robust and responsive DF platforms remains critical. In this context, the transition of DF technology from laboratory-scale research to industrial application hinges on the development and implementation of robust, reproducible, and scalable strategies.

Among the most promising complementary approaches are: bioaugmentation with HLac-consuming bacterial cultures, rigorous pH control, precise nutrient and trace metal supplementation, and advanced process automation, particularly through artificial intelligence (AI) and digital twins. These strategies not only increase H_2 yields but, crucially, enhance the long-term reproducibility of bioprocesses and create a solid foundation for industrial deployment.

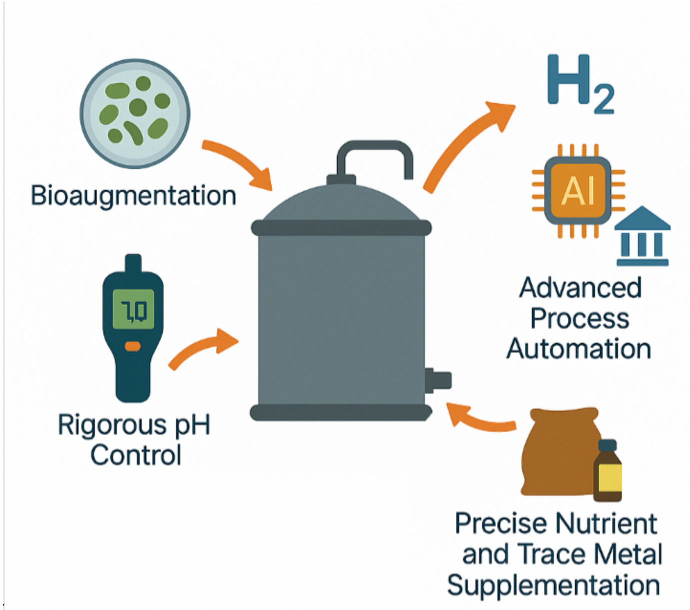

Figure 1.14. Strategies to enhance dark fermentative hydrogen production.

1.3.4.6.1. Boosting Dark Fermentation Via Bioaugmentation and pH Control

A recurring bottleneck in DF, as previously mentioned, is the unpredictable accumulation of HLac, a metabolic by-product that disrupts H_2 production by diverting electrons away from hydrogenase enzymes. Bioaugmentation with HLac-oxidizing cultures, such as *Clostridium*, has been proven to redirect metabolic fluxes toward acetate and H_2 production (Marone et al., 2017). This method enhances redox balance and creates a syntrophic microbial network where HLac-consuming and HPB support one another, resulting in a more stable and reproducible microbial ecosystem (Quéméneur et al., 2010). Specially in reactors treating carbohydrate-rich wastes such as FVW, the integration of tailored bioaugmented cultures mitigates pH drops and enzymatic inhibition, supporting steady H_2 yields over extended operational periods.

Additionally, in Chapter 4 the bioaugmentation of HLac-consuming bacterial cultures significantly redirected metabolic fluxes toward HAc and H_2 formation. This syntrophic restructuring of the microbial consortium directly reflects the mechanisms outlined in Section 1.3.4.5 regarding LDDF and validates the hypothesis that HLac can be valorized as a beneficial intermediate rather than simply mitigated as an inhibitor. However, bioaugmentation alone is insufficient to ensure system reproducibility. A precise pH control (typically maintained between 5.5 and 6.5) is critical to preserving hydrogenase activity and supporting stable electron transport across microbial membranes (Guo et al., 2010). pH fluctuations can destabilize microbial communities, lower energy yields, and increase the accumulation of inhibitory intermediates.

Automated buffering systems, guided by online pH sensors, allow for real-time corrections that maintain optimal biochemical conditions. These are now frequently integrated into digital twin architectures, which simulate reactor behavior and guide pH regulation strategies based on predictive analytics (Tomczak et al., 2018). In addition, the results obtained in Chapter 4 of this thesis confirmed the crucial role of pH regulation. Optimal H_2 production consistently occurred within the expected pH window of 5.5–6.5, whereas deviations from this range led to acidification and suppressed hydrogenogenic activity. These findings reinforce the importance of implementing effective buffering systems and real-time monitoring tools to maintain favorable fermentation conditions.

Complementarily, the data presented in Chapter 5 further supported this conclusion by showing that maintaining the pH near neutrality was essential for stimulating microbial activity and sustaining metabolic equilibrium. Taken together, these insights underscore that pH regulation is not only fundamental for process stability but also pivotal in managing metabolite profiles, particularly in relation to HLac. While HLac has traditionally been regarded as an inhibitory by-product in DF systems, both chapters demonstrate that, under well-controlled conditions and with appropriate microbial management, it can be transformed into a fermentable intermediate that significantly contributes to H_2 generation.

The automation of pH and nutrient regulation plays a pivotal role in maintaining reproducibility, particularly under conditions of variable substrate input. FVW often vary in composition and buffering capacity. Thus, supplementation of macronutrients (e.g., nitrogen and phosphorus) and trace metals (e.g., iron, cobalt, and nickel) is crucial for maintaining enzymatic function and microbial growth (Hallenbeck and Liu, 2016). Trace metals serve as cofactors for Fe-Fe- and Ni-Fe-hydrogenases (key enzymes in H_2 metabolism). Optimizing their concentrations using real-time monitoring systems and periodic spectrometric analyses (e.g., ICP-MS) ensures consistent enzyme activity and minimizes the risk of nutrient-related inhibition or toxicity (Zhao et al., 2019; Adebo et al., 2020).

The results presented in Chapter 7 provide strong empirical support for the conceptual framework outlined in Section 1.3.4.5 regarding LDDF, pH regulation, and bioaugmentation. HLac emerged as one of the dominant metabolites throughout all experimental stages. While its initial accumulation correlated with decreased H_2 productivity, its subsequent conversion (particularly under conditions involving HLac-oxidizing bacterial enrichment) led to notable increases in H_2 production. This pattern reflects a syntrophic interaction between LAB and HPB. Furthermore, maintaining the pH close to neutrality proved essential for preserving metabolic balance and microbial performance, reinforcing the importance of real-time monitoring and automated control systems. Bioaugmentation, although transient in effect, facilitated a favorable metabolic shift toward HAc and H_2 production, further validating the potential of HLac as a fermentable intermediate rather than merely an inhibitor. The empirical findings so far obtained also reinforce the principles discussed in Section 1.3.4.6 concerning tailored nutrient supply. Indeed, controlled supplementation of key nutrients enhanced both microbial stability and H_2 output.

Overall, these results obtained in this thesis emphasize that process reproducibility (a cornerstone for industrial scalability) depends heavily on the integration of microbial management, pH control, and nutrient optimization. The consistent trends observed across different experimental phases confirm that when these strategies are properly applied, DF systems can achieve stable and predictable H_2 production.

1.3.4.6.2. Bioprocess Automation and Microbial Tools

To optimize these complex systems, omics technologies have also emerged as indispensable tools. Metagenomics enable the identification of dominant H_2-producing and HLac-consuming species, providing insight into the structure of microbial consortia under different operational conditions (Jung et al., 2020; Quéméneur et al., 2010). Complementary transcriptomic and proteomic studies further reveal how gene and protein expression respond to pH changes, nutrient availability, or metal supplementation. For instance, hydrogenase gene expression has been shown to decrease sharply under low pH or micronutrient-limited conditions. This knowledge informs targeted nutrient interventions (Hallenbeck and Liu, 2016). These omics-driven insights feed into AI-assisted control systems, enabling precise, data-informed optimization of reactor parameters (Li et al., 2025).

Synthetic biology provides a powerful extension to the omics-guided strategies. Genetic engineering of *Clostridium* and other hydrogenogenic strains has yielded variants with increased tolerance to low pH, resistance to HLac accumulation, and enhanced hydrogenase expression (Quéméneur et al., 2010). These strains, when introduced through bioaugmentation, integrate into the reactor ecosystem and reinforce performance even under suboptimal conditions. The potential to design strains with programmable responses to environmental cues opens new pathways toward fully controlled, self-regulating hydrogenogenic systems. Importantly, synthetic biology allows the tailoring of microbial function in concert with digital process control, facilitating real-time adaptation and prolonged reactor stability. The scalability of DF is inextricably tied to process reproducibility. Reactor systems that incorporate bioaugmentation, pH regulation, and nutrient supplementation show markedly reduced variability in H_2 yields, making them ideal for industrial expansion (Guo et al., 2010). Pilot-scale studies have demonstrated that such systems experience shorter startup times, reduced lag phases, and extended operational lifespans compared to traditional setups. Standardized reactor designs that embed modular control components (pH regulation, nutrient dosing, and AI decision layers) further promote scalability by allowing consistent performance replication across different facilities.

Digital twins and AI-based automation are at the forefront of this scalability push. Digital twins act as real-time, virtual models of the reactor, continuously updated with sensor data and used to simulate responses to various control strategies (Tomczak et al., 2018). Machine learning algorithms analyze these datasets to detect anomalies, predict performance shifts, and recommend preemptive corrective actions (Pengadeth et al., 2024). For example, if HLac accumulation is detected through real-time monitoring, the AI system can suggest additional bioaugmentation, alter nutrient feed, or adjust the pH buffer flow, all before inhibitory effects impair reactor performance. Such predictive capabilities are vital for minimizing downtime, reducing human intervention, and ensuring long-term reliability of H_2 output (Jung et al., 2020; Pinu et al., 2019. Hybrid energy systems, such as those coupling DF with microbial electrolysis cells (MECs), also benefit from these reproducible upstream processes. In these systems, fermentation effluents rich in VFAs or HLac are further converted to H_2 electrochemically. The stability of the upstream DF stage is critical: only with consistent effluent composition can MECs operate efficiently and predictably (Marone et al., 2017). Thus, reproducibility achieved via complementary strategies directly contributes to the viability of integrated energy recovery platforms, reinforcing DF’s role in the circular bioeconomy. In parallel, microbial electrochemical technologies (METs) leverage microbial metabolism to produce electricity and biochemical products from waste, and when coupled with anaerobic digestion, they enhance the breakdown of volatile fatty acids and methane yield (Rocha et al., 2018; Alonso et al., 2020; Poirier et al., 2020; Chenebault et al., 2022). In this context, residue valorization becomes an added benefit of system reproducibility. The digestate left after fermentation, enriched with nutrients and low in inhibitory compounds, can serve as a high-quality biofertilizer; another valuable product stream that supports the economic case for DF (Marone et al., 2017). Ensuring the stability of H_2 yields and by-product quality allows facilities to pursue integrated biorefinery models that align with circular economy principles.

In conclusion, enhancing reproducibility and scalability in dark fermentative H_2 production relies on a cohesive framework built upon microbial engineering, precision control, and advanced data analytics. Bioaugmentation with HLac consumers, rigorous pH and nutrient control, and trace metal supplementation together can stabilize microbial activity. When enriched with omics-based insights, synthetic biology innovations, and AI-driven digital twins, these strategies unlock a new generation of smart, self-regulating hydrogenogenic systems. These developments not only improve process performance but also pave the way towards industrial-scale, economically viable H_2 production from organic residues.

1.4 Emerging Technologies for Organic Waste Valorization

The field of organic waste valorization is advancing rapidly, driven by the urgent need to enhance environmental sustainability and resource recovery. A diverse range of emerging technologies is reshaping how organic waste is managed, transforming it from a burden into a valuable resource (Fig. 1.15). Among these innovations, the production of bioplastics and biomaterials from food and agricultural waste reduces dependence on fossil-based plastics and supports a circular production model (Ojha et al., 2020; Moretto et al., 2019; Thomassen et al., 2018).

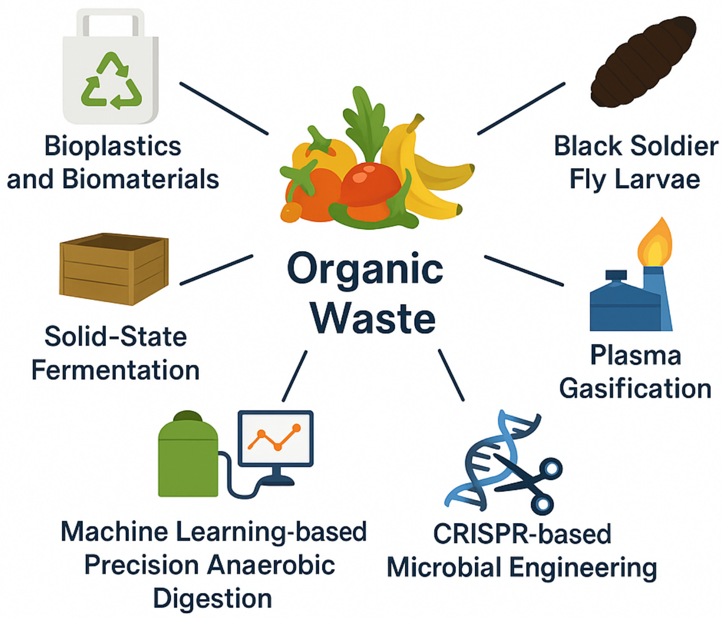

Figure 1.15. Innovative pathways for organic waste valorization.

The use of black soldier fly larvae (BSFL) has also emerged as a promising bioconversion method, converting organic residues into protein-rich biomass and organic fertilizers, while simultaneously reducing pathogenic loads (Looveren et al., 2023; Ojha et al., 2020). Another notable process, solid-state fermentation (SSF), utilizes minimal water and high-solid substrates like agricultural waste to generate bioactive compounds under energy-efficient conditions (Ebrahimian et al., 2022; Thomassen et al., 2018).

Advanced thermal technologies such as plasma gasification offer a route to convert complex or non-biodegradable organic waste into syngas, with high energy efficiency and potential for carbon capture, positioning it as a sustainable alternative to incineration (Rocha et al., 2018; Tabu et al., 2022; Morena et al., 2023). Meanwhile, machine learning-based precision AD brings data science into waste management by improving process control and biogas productivity through predictive analytics (Said et al., 2023; Vanierschot et al., 2023; Iglesias-Iglesias et al., 2019). On the frontier of biotechnology, CRISPR-based microbial engineering is revolutionizing bioprocesses by enhancing microbial degradation and metabolite synthesis for products like biofuels and bioplastics

(Awasthi et al., 2022; Alonso et al., 2020; Chenebault et al., 2022; Moretto et al., 2019). Together, these emerging technologies present a compelling vision for the future of organic waste management one where innovation transforms environmental challenges into circular economy opportunities, fostering cleaner, more resilient, and resource-efficient societies.

In this context, the present thesis focuses on the study of complementary strategies aimed at improving the efficiency, reproducibility, and resilience of H_2 production from organic waste. Particular attention is given to the role of bioaugmentation, precise control of pH and nutrients. These tools are essential for scaling fermentative processes in an efficient, reliable manner, aligned with the principles of the circular bioeconomy.

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Chapter 2

Aims and scope of the thesis

2.1. Justification of the Thesis

The growing generation of FVW represents a pressing environmental, economic, and social challenge worldwide. In the European Union alone, approximately 21 kg of unavoidable FVW are generated per capita annually, arising throughout the entire agri-food chain. Although this biomass is rich in biodegradable compounds, its underutilization contributes to resource inefficiencies and greenhouse gas emissions. In line with the Circular Bioeconomy Strategy and the 2030 Sustainable Development Goals, the valorization of FVW into bio-based products and renewable energy has emerged as a key strategy for sustainable waste management.

Among the various technologies available, DF stands out as a low-energy biological route for converting carbohydrate-rich residues into H_2 and short-chain organic acids. H_2 is considered a promising clean energy vector due to its high energy content and zero-emission profile upon combustion. However, the large-scale implementation of DF is hindered by operational and biological bottlenecks, including instability under continuous operation, accumulation of inhibitory metabolites such as HLac, and low process reproducibility, particularly when using complex substrates like FVW. These challenges are amplified by the rapid acidification potential of FVW, resulting from its high biodegradability and sugar content.

Recent advances in LDDF propose HLac acts as a direct H_2 precursor, potentially improving the overall energy recovery and systems robustness. However, the influence of key operational parameters; including HRT, OLR, solid concentration, pH, and temperature, on H_2 production in LDDF systems remains insufficiently explored. Understanding these variables is essential for developing stable, high-rate DF processes that are both scalable and resilient. At the same time, conventional single-stage AD for CH_4 production from FVW has shown limitations in microbial efficiency and substrate conversion. Two-phase AD systems, particularly those incorporating HLac-type fermentation in the acidogenic stage, offer enhanced thermodynamics and CH_4 yields. Nevertheless, this configuration has yet to be systematically compared to traditional AD systems under equivalent operational conditions.

This research will contribute to the development of robust and scalable fermentative bioenergy platforms by addressing process reproducibility, biological variability, and energy conversion efficiency. Ultimately, the integration of DF and AD into circular biorefinery models can unlock the full potential of FVW as a renewable feedstock, promoting cleaner energy production, reducing reliance on fossil fuels, and advancing eco-innovative solutions aligned with circular economy principles.

2.2. Main Objectives

GENERAL OBJECTIVE:

This thesis work aimed at optimizing the valorization of FVW through innovative configurations of DF and AD processes, with the goal of maximizing H_2 and biogas production. The study specifically elucidated the key role of the main operational parameters in LDDF and addressed key challenges related to process efficiency and operational reproducibility under continuous operation.

SPECIFIC OBJECTIVES:

1. To investigate the influence of critical operational parameters, including pH, total solids concentration, and initial biomass concentration, on the metabolic pathways involved in H_2 production through LDDF.
2. To evaluate the effect of HRT on H_2 production from FVW via continuous DF, in order to optimize substrate conversion and maximize the HPR, while analysing the role of HLac metabolism in enhancing process efficiency.
3. To perform a comprehensive energy and mass balance analysis of the DF process using FVW, in order to establish a baseline for the future design, assessment, and optimization of next-generation biorefineries.
4. To perform a comparative assessment of energy yields and process stability in single-stage and two-stage anaerobic digestion systems, with particular focus on the role of HLac-type fermentation in the acidogenic phase.
5. To evaluate the efficiency and reproducibility of LDDF using FVW as a substrate through continuous parallel reactor operation, assessing H_2 productivity, yield, metabolite profiles, and microbial communities, while exploring bioaugmentation and tailored nutrient supplementation as enhancement strategies.

2.3. Development of the Thesis

In the present thesis, the production of H_2 and CH_4 from FVW was investigated through DF and AD, focusing on operational optimization and process reproducibility. The work (Fig. 2.1) explores the potential of LDDF and two-stage AD systems as advanced strategies for improving energy recovery and aligning with circular bioeconomy principles.

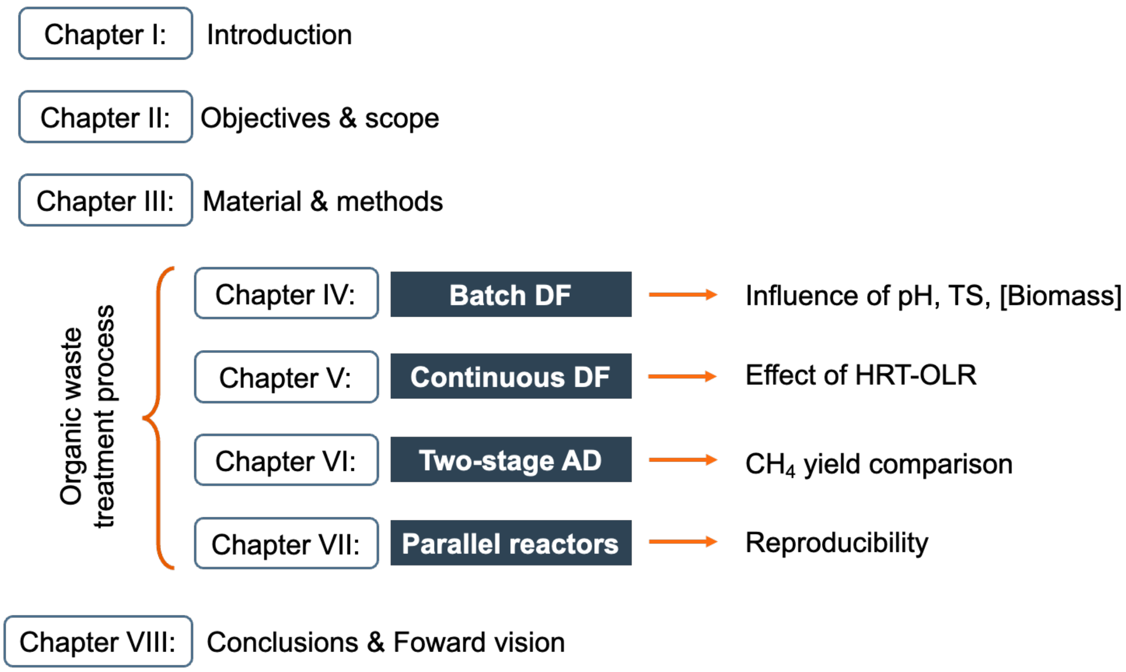

Figure 2.1. Thesis structure highlighting the experimental chapters on FVW treatment through DF and AD processes.

Chapter 1 of this thesis provided the general introduction, while **Chapter 2** presented the objectives and scope of the study, establishing the scientific and practical motivations behind the work. **Chapter 3** then described the general materials and methods used throughout the thesis, serving as the methodological foundation for the experimental work presented in the following chapters.

Chapter 4 evaluates the effect of key operational parameters (such as pH, TS content, and initial biomass concentration) on H_2 production from FVW in DF systems. Special attention was given to the role of HLac as both an inhibitory metabolite and a fermentable intermediate under controlled conditions. **Chapter 5** investigates the influence of HRT and the progressively OLR increase, on the performance of continuous DF in stirred tank reactors. A stepwise modulation of HRT was implemented to determine its effect on HPR, metabolite profiles, and microbial dynamics. The findings contribute to defining an optimal operational window for maximizing FVW-to-H_2 conversion efficiency. **Chapter 6** introduces a comparative study of single-stage versus HLac-based two-stage AD configurations for FW treatment. The performance of both systems was assessed in terms of CH_4 yield, substrate conversion, and system stability. Results highlighted the thermodynamic benefits of HLac-type fermentation in enhancing CH_4 production. **Chapter 7** focuses on the reproducibility and stability of the DF process by operating three parallel continuous reactors under identical conditions. The study analysed H_2 yields, OA profiles, and microbial community structures across replicates. Strategies such as nutrient supplementation and transient bioaugmentation were evaluated to reduce biological variability.

Finally, **Chapter 8** summarizes the major conclusions and provides recommendations for future research aimed at scaling up LDDF and two-phase AD technologies. The integration of these processes into biorefinery platforms is proposed as a sustainable solution for valorising FVW, enhancing bioenergy production, and advancing circular economy objectives.

Chapter 3

Materials and methods

3.1. Materials

3.1.1. Inoculum

A mesophilic anaerobic digestate derived from a 100 L pilot-scale reactor treating restaurant food waste was used as the primary inoculum source across Chapters IV, V, VI, and VII. This digestate underwent heat-shock pretreatment (90 °C for 20 min) to irreversibly inactivate methanogenic populations (García-Depraect et al., 2022). For the H_2 production experiments (Chapters IV, V, and VII), the enrichment of hydrolytic and acidogenic bacteria was achieved through successive culture transfers using an aliquot of the preserved inoculum. The inoculum (Fig. 3.1) was reactivated at 37 ± 1 °C for 19 hours in a 2.1-L fermenter operated in batch mode without pH control, resulting in an active hydrogenogenic culture with a concentration of 180 mg VSS/L (Martínez-Mendoza et al., 2022). A mineral medium was used, containing (g/L): lactose 10.0, NH_4Cl 2.4, K_2HPO_4 2.4, $MgCl_2{\cdot}6H_2O$ 2.5, KH_2PO_4 0.6, $CaCl_2{\cdot}2H_2O$ 0.15, and $FeCl_2{\cdot}4H_2O$ 0.035 (García-Depraect et al., 2019a). The resulting microbial consortium, mainly composed of lactic acid bacteria and lactate-utilizing, hydrogen-producing bacteria, was capable of carrying out LDDF (García-Depraect et al., 2022).

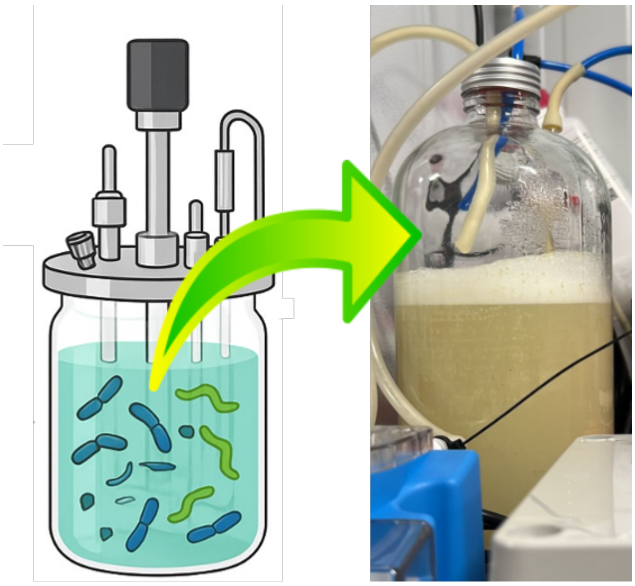

Figure 3.1. Representation of the batch fermentation setup and microbial culture conditions for LDDF development.

In Chapter VI, a dual-inoculum strategy was adopted. The acidogenic phase used the same pretreated culture described above, while the methanogenic phase employed fresh anaerobic sludge obtained from the municipal WWTP of Valladolid, Spain. The methanogenic inoculum was preincubated for 7 days at 37 °C and presented typical characteristics: pH 7.5, total solids 29.7 g/L, and volatile solids 14.9 g/L. In Chapter VII, in addition to the hydrogenogenic culture described, a bioaugmentation strategy was implemented. This involved sourcing inoculum from a stable, continuously operated 1.4-L reactor treating powdered cheese whey (Sueromancha S. L., Spain) with an H_2 productivity of 11.5 ± 1.1 NL H_2/L-d and a volatile suspended solids concentration of 2.6 g/L. The reactor featured automatic pH regulation (EvopH-P5), gas and liquid sampling ports, and was maintained at 37 ± 1 °C by a thermostatic bath.

3.1.2. Substrate

The substrates used throughout this thesis consisted mainly of simulated and representative FVW or food waste, blended and preserved to ensure uniformity and prevent degradation. The preparation procedures were standardized across all experiments, with slight variations tailored to the objectives of each chapter.

For Chapters IV, V, and VII, simulated FVW was prepared based on the formulation described by Martínez-Mendoza et al. (2022), which was adapted from Abubackar et al. (2019). The formulation included (% w/w): banana (14.5), eggplant (12.8), carrot (9.7), tomato (8.4), cucumber (7.5), onion (7.1), radish (6.5), potato (6.2), capsicum (5.7), apple (5.3), cabbage (4.7), grape (3.1), orange (3.1), lemon (2.7), and pumpkin (2.4). All ingredients were purchased fresh from a local marketplace, blended (Fig. 3.2) without added water using a semi-industrial blender (Sammic, XM-32, Azkoitia, Spain), and stored in 1 L plastic bags at –20 °C to prevent degradation.

Figure 3.2. Representation of FVW preparation.

In Chapter VI, a food waste substrate simulating restaurant waste was prepared following the recipe described in Regueira-Marcos et al. (2020), consisting of (% w/w): potato (78), chicken breast (14), white cabbage (4), and pork lard (4). This mixture was homogenized using a blender and stored at −20 °C until use. The physicochemical properties of each substrate used are described in Table 3.1. Prior to use, the substrate was diluted with tap water to the desired final TS content (Martínez-Mendoza et al., 2022).

Table 3.1. Summary of physicochemical properties of the substrates used in this thesis.

Parameter	FVW	Food waste
pH	4.6 ± 0.1	6.3
Total chemical oxygen demand, g/L	111.4 ± 0.1	295
Total solids, g/L	100.7 ± 7.8	211
Volatile solids, g/L	94.7 ± 7.4	189
Total carbohydrates, g/L	80.9 ± 2.8	102
Lipid content, %	1.2 ± 0.0	20.03
Total Kjeldahl nitrogen, g/L	2.8 ± 0.5	4.1
Protein, %	17.3 ± 2.5	25.4
Phosphorus, g/L	3.7 ± 0.0	0.3
Ash content, %	6.0 ± 0.1	4.7

3.2. Experimental Set-Up

All experimental procedures described in this thesis were conducted in custom-built laboratory-scale systems designed to evaluate the fermentative and AD performance of FVW under varying operational conditions. The experiments included both batch and continuous operations, as well as single-stage and two-stage configurations, depending on the objectives of each chapter.

3.2.1. Reactor Designs

Custom-built fermenters were employed, with working volumes ranging from 0.7 to 5.0 L. Reactors were fabricated from transparent polyvinyl chloride and were equipped with the following standard components: gas-tight sealing systems to ensure anaerobic conditions; liquid and gas sampling ports for periodic monitoring; custom-made biogas flow meters, based on the liquid displacement principle; magnetic stirrer, typically operated at ~300 rpm to ensure homogeneity; and temperature-controlled rooms, maintaining mesophilic conditions (37 °C).

3.2.2. pH Control and Monitoring

When required, fermenters were equipped with automated pH control systems (EvopH-P5, BSV Electronic, Spain) and pH electrodes (HO35-BSV01, BSV Electronic, Spain) to maintain stable operational pH values. pH setpoints varied depending on experimental goals (e.g., 5.5–7.0 for dark fermentation, or unregulated in acidogenic/methanogenic phases), and were adjusted using NaOH (3–6 M) or HCl (3 N) solutions.

3.2.3. Operating Modes and Conditions

A brief overview (Fig. 3.3) of the experimental setups for Chapters IV to VII is presented below. Detailed descriptions of the experimental designs, including operational setpoints and specific configurations, are provided in their respective sections.

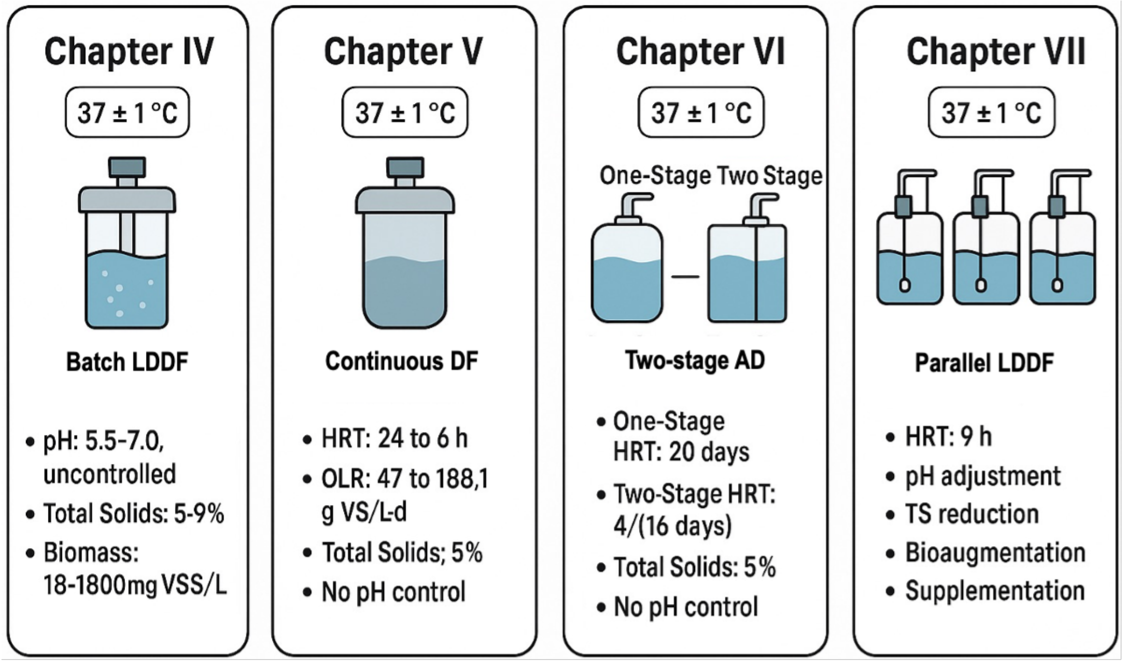

Figure 3.3. Summary of operational conditions and modes of fermentation applied in Chapters IV–VII, including batch, continuous, and anaerobic digestion configurations.

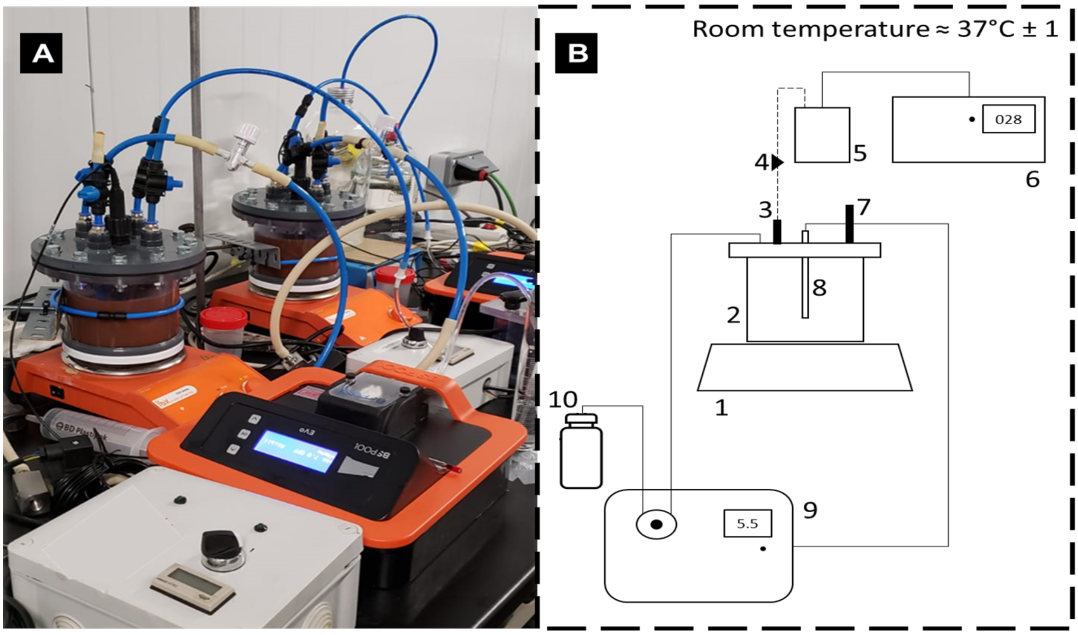

Figure 3.4. Photograph (A) and scheme (B) of the batch dark fermentation set-up. Magnetic stirrer (1), fermenter (2), gas outlet (3), gas sampling port (4), water column (5), gas counter (6), liquid sampling port (7), pH probe (8), pH controller (9), NaOH solution (10).

In Chapter IV (Fig. 3.4), batch fermentations were conducted at 37 ± 1 °C in 1.25-L reactors (0.7 L working volume) to evaluate the effects of pH (5.5–7.0 and uncontrolled), total solids (5–9%), and biomass concentration (18–1800 mg VSS/L) on hydrogen production from simulated FVW. In Chapter V (Fig. 3.5), continuous fermentation was performed in a 1.25-L PVC reactor over 47 days, with HRT reduced from 24 to 6 h and OLR increased from 47 to 188 g VS/L-d.

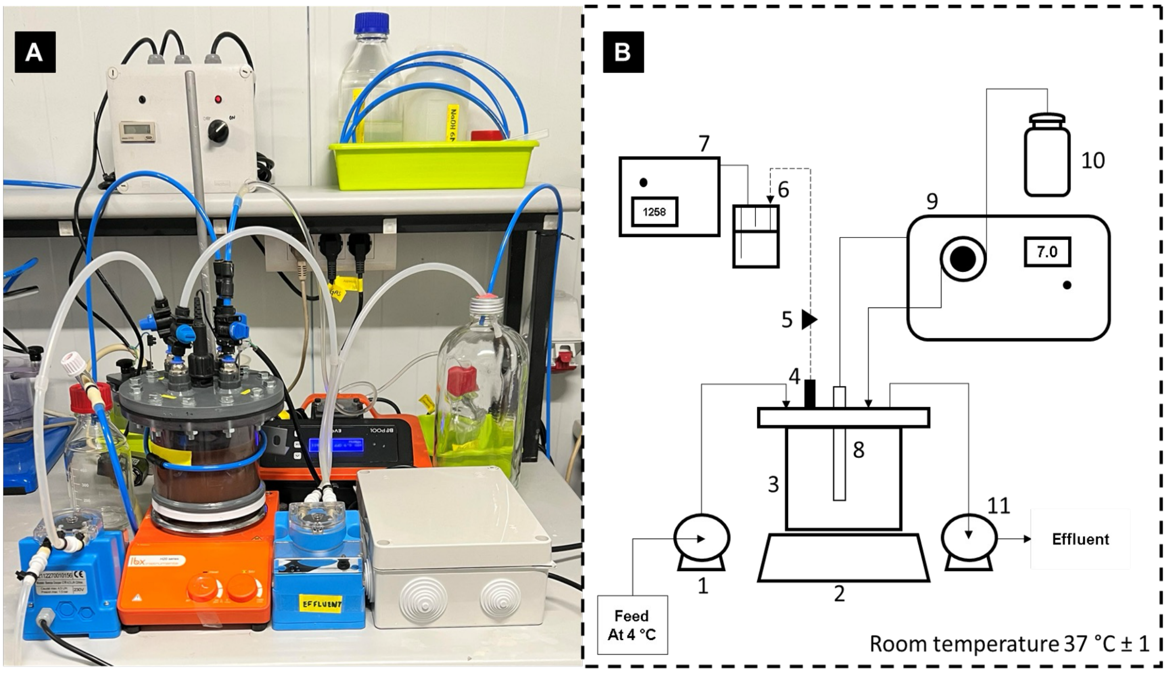

Figure 3.5. Photograph (A) and scheme (B) of the continuous dark fermentation set-up used to investigate the effect of hydraulic retention time on the FVW-to-hydrogen biotransformation. Peristaltic pump (1 and 11), magnetic stirrer (2), dark fermenter (3), gas outlet (4), gas sampling port (5), water column (6), gas counter (7), pH probe (8), pH controller (9), 6 N NaOH solution (10).

In Chapter VI (Fig. 3.6), one- and two-stage AD systems were operated continuously for 40 days at 37 ± 1 °C. The one-stage was operated at an HRT of 20 days, while the two-stage exhibited 4 days for acidogenesis and 16 days for methanogenesis. TS was kept at 5%, OLR at 2.3 g VS/L-d, and no pH control was applied.

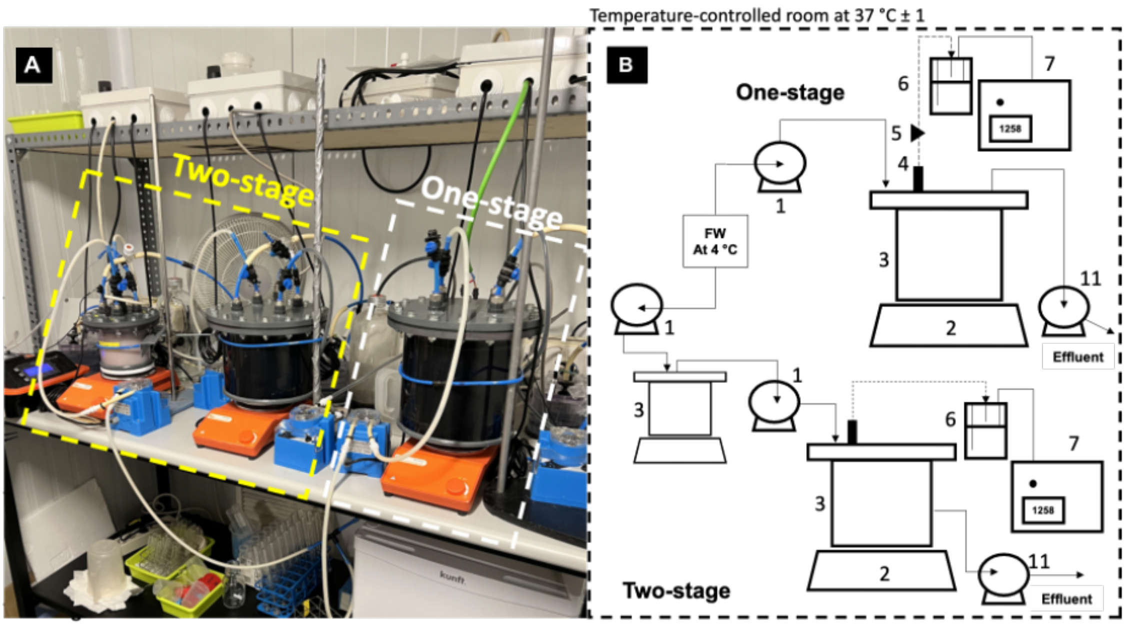

Figure 3.6. Photograph (A) and scheme (B) of the anaerobic digestion set-up used to systematically compare the one vs two-stage systems of food waste treatment. Peristaltic pump (1 and 11), magnetic stirrer (2), digester (3), gas outlet (4), gas sampling port (5), water column (6), gas counter (7).

In Chapter VII (Fig. 3.7), three parallel 0.8-L CSTRs were operated for 90 days. The HRT was shortened from 18 to 9 h during de first two operational periods. Strategies to enhance hydrogen production were explored, which included pH adjustment (7 to 6.5), TS reduction (5% to 3%), bioaugmentation (20% broth replaced), and supplementation with micronutrients and cheese whey (25 g COD/L).

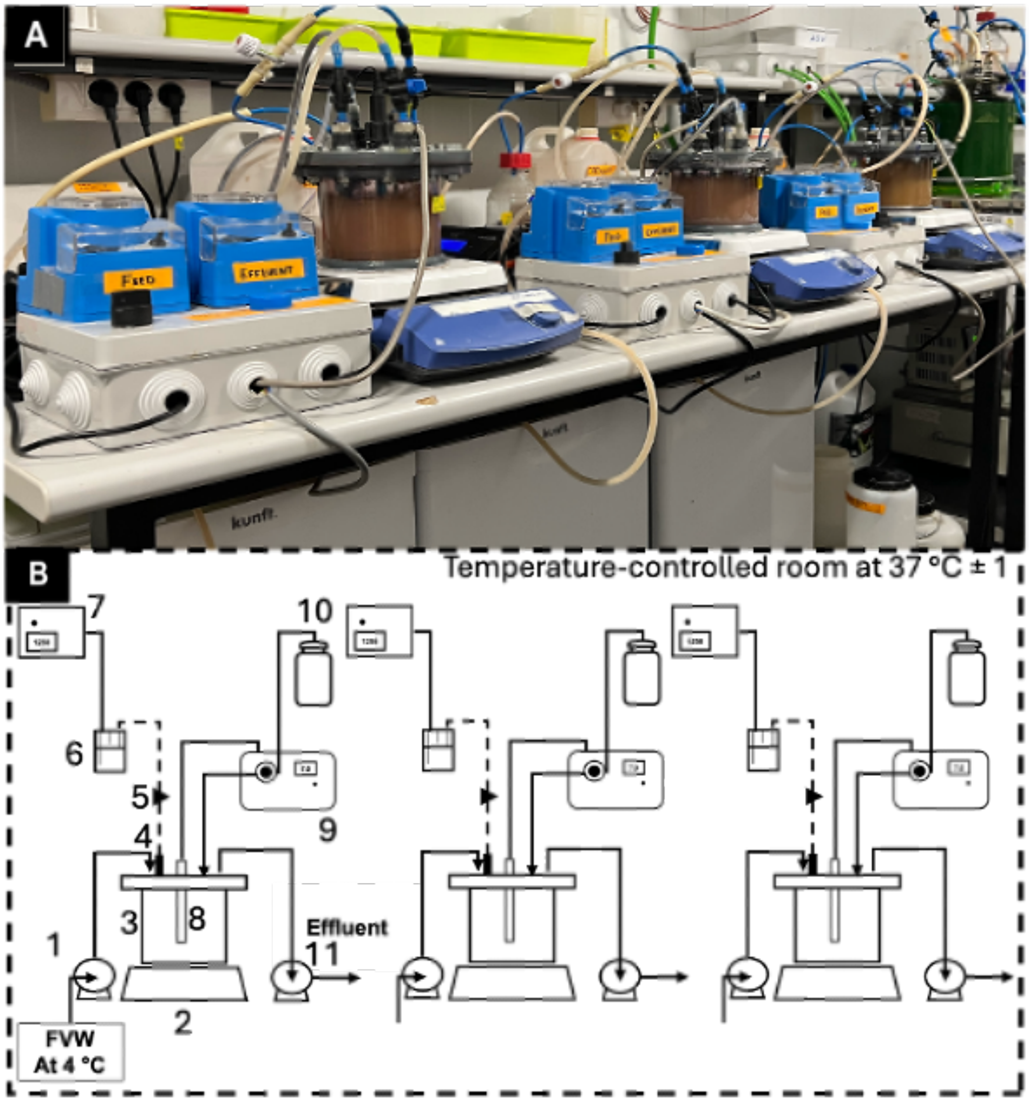

Figure 3.7. Photograph (A) and scheme (B) of the continuous dark fermentation set-up used to investigate the reproducibility of the effect of the hydraulic retention time on the FVW to hydrogen biotransformation. Peristaltic pump (1 and 11), magnetic stirrer (2), dark fermenter (3), gas outlet (4), gas sampling port (5), water column (6), gas counter (7), pH probe (8), pH controller (9), 6 N NaOH solution (10).

3.2.4. Process Monitoring and Performance Indicators

Liquid and gas samples were collected periodically and analysed for biogas composition, volumetric production rates for hydrogen, and methane productivity, H_2 and CH_4 yields, organic acids profile, volatile solids removal, and total carbohydrate consumption.

3.3. Analytical Techniques

All physicochemical and biological analyses performed throughout this thesis followed internationally recognized protocols and were consistent across all experimental chapters unless otherwise specified. The physicochemical characterization according to Standard methods described by APHA (2005) were used for the determination of: pH, total solids, volatile solids, and chemical oxygen demand, total Kjeldahl nitrogen for estimating protein content (N-to-protein factor: 6.25), total carbohydrates via the phenol–sulfuric acid method, lipid content following the PNTNAG-006 SERIDA protocol.

The concentrations of H_2, CO_2, and CH_4 in the gas phase were determined using a Varian CP-3800 gas chromatograph (Varian, USA), equipped with a thermal conductivity detector and two connected capillary columns: a CP-Molsieve 5A (15 m × 0.53 mm × 15 µm) and a CP-PoraBOND Q (25 m × 0.53 mm × 10 µm). The system was calibrated using certified gas standards with known compositions (e.g., 70.0% H_2/30.0% CO_2 and 70.53% CH_4/24.0% CO_2/2.99% N_2/2.0% H_2S/0.49% O_2). High-purity helium was employed as the carrier gas at a flow rate of 13 mL/min.

Methane production was quantified using both manometric and chromatographic approaches, following the methodology described by García-Depraect et al. (2022). The analysis of organic acids (such as formate, acetate, isobutyrate, butyrate, propionate, lactate, isovalerate, valerate, isocaproate, hexanoate, and heptanoate) was carried out using high-performance liquid chromatography (HPLC). A Waters Alliance e2695 system (Massachusetts, USA) equipped with a Waters 2998 PDA UV–vis detector (operating at 210 nm), an infrared detector for ethanol quantification, and a HyperREZ XP Carbohydrate H^+ 8 µm column (Thermo Scientific, UK) was employed. The column was maintained at 75 °C, and the eluent consisted of 25 mM H_2SO_4 delivered at a flow rate of 0.7 mL/min. Quantification was performed using standards of sodium L-lactate (Sigma-Aldrich, 71718, USA) and a mixed organic acid solution (Sigma-Aldrich

CRM46975, USA). In parallel, the composition of the gaseous phase was determined by gas chromatography using a Varian CP-3800 GC-TCD (Palo Alto, USA), following the procedure described by García-Depraect et al. (2022). Gas volumes were standardized to conditions of 0 °C and 1 atm.

The structure of the microbial community was assessed by amplifying the V3–V4 region of the 16S rRNA gene using the primer pair 341F-805R, following the Illumina 16S Metagenomic Sequencing Library protocol (Illumina15044223 B) as described by Klindworth et al. (2013). The resulting sequencing data were processed using the QIIME2 bioinformatics pipeline (Bolyen et al., 2019). Amplicon sequencing variants (ASVs) were initially annotated against the NCBI 16S rRNA database (version 2021) at a 97% identity threshold; for ASVs with lower identity (<97%), the SILVA database version 138 was used for taxonomic classification.

To ensure data consistency for diversity analysis, rarefaction was applied using the Phyloseq package in R, following the approach proposed by Weiss et al. (2017). Alpha diversity was evaluated using the Shannon-Wiener and Simpson (1-D) indices, calculated with PAST software (version 4.09). Furthermore, to characterize archaeal diversity in the methanogenic reactors, the hypervariable V4 region of the 16S rRNA gene was sequenced according to the protocol outlined by Pausan et al. (2019).

3.4. Data Analysis

In chapter IV, hydrogen production kinetics were analyzed using the modified Gompertz model (Eq. 1) previously described by Ramos et al. (2012), where, H (t) represents the total amount of hydrogen (in NmL) produced at time t (h), $Hmax$ represents the maximal amount (in NmL) of hydrogen produced, $Rmax$ is the maximum hydrogen production rate (in mL/h), and λ stands for the lag time (in h). Each experimental condition was tested in duplicate, and the plotted data corresponds to the average and standard deviation recorded. The acidification degree was calculated according to Eq. 2, where COD eq. is the net sum of COD equivalent (in g/L) of all the organic acids measured at the end of fermentation and $TCOD_{FVW}$ is the total COD concentration (in g/L) of the FVW fed. Finally, a COD mass balance analysis was performed according to Eq. 3. COD equivalent for biomass was estimated as 5% of the total COD of the influent (García-Depraect et al., 2019b).

$$H(t) = H_{max} * exp\left[-exp\left(\frac{2.71828 * R_{max}(\lambda - t)}{H_{max}} + 1\right)\right] \quad (1)$$

$$Acidification\ degree\ (\%) = \frac{\text{COD eq.}}{TCOD_{FVW}} x\ 100 \quad (2)$$

$$Total\ initial\ COD\ = COD_{organic\ acids} + COD_{residual\ sugars} +\ COD_{H2} + COD_{biomass} + COD_{Not\ determined} \quad (3)$$

In chapter V, the hydrogen production stability index (HPSI) was calculated as reported by García-Depraect et al. (2020) using Eq. (4). The HPSI calculation considers variations in HPR during each operational stage (not including results from the first 3 HRTs in each operational stage). A stability index equals to 1 means a constant HPR, while a deviation value in HPR as large as the average HPR represents a stability index equals to 0. Thus, the higher the HPSI index, the lower the dispersion of hydrogen production.

$$HPSI = 1 - \frac{Standard\ deviation\ HPR}{Average\ HPR} \quad (4)$$

The energy analysis was estimated in terms of energy production rate (EPR) (kJ/L-d) and energy yield (EY) (kJ/g VS), calculated using Eq. (5), (6), respectively, where HPR is expressed in NL H_2/L-d, HV_{H_2} is the hydrogen heating value (286 kJ/mol), and HY stands for the hydrogen yield (NL H_2/g VS fed) (Kumar et al., 2016).

$$EPR = \frac{Average\ HPR}{22.4} \times HV_{H_2} \quad (5)$$

$$EY = \frac{Average\ HY}{22.4} \times HV_{H_2} \quad (6)$$

Analysis of variance (ANOVA) followed by Tukey's post hoc test ($p < 0.05$) was performed to assess significant differences across experimental conditions. The Shapiro-Wilk test was applied to confirm the normality of data distributions prior to variance analysis. All statistical tests were conducted using Statgraphics Centurion version 19.2.01. Additional data modeling and chapter-specific indices, such as the HPSI and homoacetogenic contributions, are detailed in the respective experimental chapters (IV–VII).

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Chapter 4

Influence of key operational parameters on biohydrogen production from fruit and vegetable waste via lactate-driven dark fermentation

This chapter was adapted after its publication in Bioresource Technology:

Martínez-Mendoza, L.J., Lebrero, R., Muñoz, R., García-Depraect, O., 2022. Influence of key operational parameters on biohydrogen production from fruit and vegetable waste via lactate-driven dark fermentation, Bioresource Technology. 364 128070. https://doi.org/10.1016/j.biortech.2022.128070

Influence of key operational parameters on biohydrogen production from fruit and vegetable waste via lactate-driven dark fermentation

Leonardo J. Martínez-Mendoza [a,b], Raquel Lebrero [a,b], Raúl Muñoz [a,b,] Octavio García-Depraect [a,b,*]

[a] Institute of Sustainable Processes, University of Valladolid, Dr. Mergelina s/n., Valladolid, 47011, Spain

[b] Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina s/n., Valladolid 47011, Spain

[*]**Corresponding author:** octavio.garcia@uva.es

Highlights:

•First study exploring the effect of 3 key process parameters on the LD-DF of FVW.

•Boosted H_2 production at pH 7, 5% total solids and 1.8 g VSS/L cell concentration.

•H2 production agreed with the lactate uptake and acetate and butyrate production.

•Moderate H_2 yield (50 mL/g VS) but an outstanding rate (976.4 mL/L-h) was achieved.

•Lactate-based DF is a promising route to transform FVW into H_2 at high rates.

Keywords:

Dark fermentation

Fruit and vegetable waste

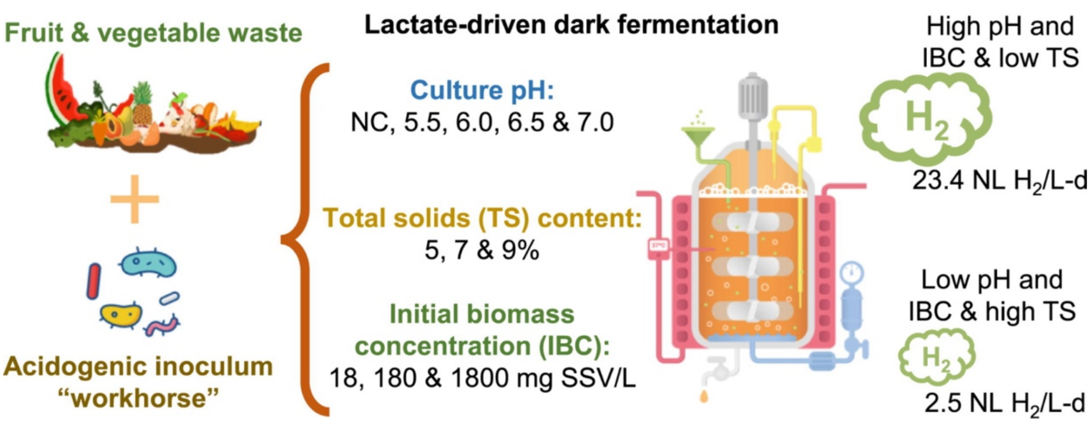
Hydrogen production

pH effect

Total solids

Abstract

This study aims at investigating the influence of operational parameters on biohydrogen production from fruit-vegetable waste (FVW) via lactate-driven dark fermentation. Mesophilic batch fermentations were conducted at different pH (5.5, 6.0, 6.5, 7.0, and non-controlled), total solids (TS) contents (5, 7, and 9%) and initial cell biomass concentrations (18, 180, and 1800 mg VSS/L). Higher hydrogen yields and rates were attained with more neutral pH values and low TS concentrations, whereas higher biomass densities enabled higher production rates and avoided wide variations in hydrogen production. A marked lactate accumulation (still at neutral pH) in the fermentation broth was closely associated with hydrogen inhibition. In contrast, enhanced hydrogen productions matched with much lower lactate accumulations (even it was negligible in some fermentations) along with the acetate and butyrate co-production but not with carbohydrates removal. At pH 7, 5% TS, and 1800 mg VSS/L, 49.5 NmL-H_2/g VS_{FED} and 976.4 NmL-H_2/L-h were attained.



Chapter 5

Unlocking the high-rate continuous performance of fermentative hydrogen bioproduction from fruit and vegetable residues by modulating hydraulic retention time

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Unlocking the high-rate continuous performance of fermentative hydrogen bioproduction from fruit and vegetable residues by modulating hydraulic retention time

Leonardo J. Martínez-Mendoza, Octavio García-Depraect, Raúl Muñoz*

Institute of Sustainable Processes, University of Valladolid, Dr. Mergelina s/n., 47011 Valladolid,Spain.

***Corresponding author:** mutora@iq.uva.es

Highlights

•HRT determined H_2 productivity and yield and the profile of soluble end-products.

•H_2 productivity of 11.8 NL/L-d and H_2 yield of 95.6 NmL/g VS_{FED} achieved at 9 h HRT.

•The major organic acids were lactate (key to metabolism), acetate and butyrate.

•Max energy recovery from fruit-vegetable waste (FVW): 1.2 kJ/g VS_{FED} and 150 kJ/L-d.

•Source-separated FVW is a good feedstock to produce H_2 via dark fermentation.

Keywords

Biohydrogen

Biorefinery

Dark fermentation

Fruit-vegetable waste

Process optimization

Abstract

Harnessing fruit-vegetable waste (FVW) as a resource to produce hydrogen via dark fermentation (DF) embraces the circular economy concept. However, there is still a need to upgrade continuous FVW-DF bioprocessing to enhance hydrogen production rates (HPR). This study aims to investigate the influence of the hydraulic retention time (HRT) on the DF of FVW by mixed culture. A stirred tank reactor under continuous mesophilic conditions was operated for 47 days with HRT stepwise reductions from 24 to 6 h, leading to organic loading rates between 47 and 188 g volatile solids (VS)/L-d. The optimum HRT of 9 h resulted in an unprecedented HPR from FVW of 11.8 NL/L-d, with a hydrogen yield of 95.6 NmL/g VS_{FED}. Based on an overarching inspection of hydrogen production in conjunction with organic acids and carbohydrates analyses, it was hypothesized that the high FVW-to-biohydrogen conversion rate achieved was powered by lactate metabolism.

Chapter 6

Enhanced methane production from food waste: A systematic comparison between conventional single-stage and lactate-based two-stage anaerobic digestion processes

This chapter was adapted after its publication in Biomass and Bioenergy:

Martínez-Mendoza, L. J., Muñoz, R., García-Depraect, O., 2024. Enhanced methane production from food waste: A systematic comparison between conventional single-stage and lactate-based two-stage anaerobic digestion processes, Biomass and Bioenergy, 188, 107312. https://doi.org/10.1016/j.biombioe.2024.107312

Enhanced methane production from food waste: A systematic comparison between conventional single-stage and lactate-based two-stage anaerobic digestion processes

Leonardo J. Martínez-Mendoza [a b], Raúl Múñoz [a b], Octavio García-Depraect [* a b]

[a] Institute of Sustainable Processes, University of Valladolid, Dr. Mergelina S/n., 47011, Valladolid, Spain

[b] Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina S/n., 47011, Valladolid, Spain

***Corresponding author:** octavio.garcia@uva.es

Highlights:

- Systematic comparison of one-stage and two-stage lactate-based AD of food waste.
- The highest CH_4 productivity and yield achieved: 0.96 NL/L-d and 398 NmL/g VS_{FED}.
- Biogas production was significantly enhanced by efficient lactate utilization.
- *Lactobacillus* and *Methanobacterium* dominated the two-stage AD process.

Keywords:

Acidogenesis

Anaerobic digestion

Biogas

Food waste

Lactic-type fermentation

Two-stage process

Abstract

The increasing generation of food waste (FW) poses significant environmental and management challenges, requiring efficient and sustainable treatment methods. This study presents the first systematic comparison between a conventional single-stage anaerobic digestion (AD) process and a lactate-based two-stage AD process using food waste (FW) as the substrate. Both AD configurations were operated in parallel under identical operating conditions, i.e., 37 °C, 20 days hydraulic retention time, 2.3 g volatile solids (VS)/L-d organic loading rate, and pH 8. The two-stage AD system exhibited a methane productivity of 959 NmL CH_4/L-d and a methane yield of 398 NmL CH_4/g VS_{FED}, which were 32.0 ± 5.6 % and 35.9 ± 0.6 % higher than those of the single-stage AD process, respectively. The two-stage AD system also showed significant lactate accumulation in the acidogenic stage, which was almost completely oxidized in the methanogenic stage. Furthermore, molecular analysis of the acidogenic stage revealed diverse bacterial communities, with a prevalence of lactate-producing bacteria such as *Lactobacillus*. In the methanogenic stage, various bacteria and archaea, including *Methanobacterium* and *Methanothrix*, were identified as major contributors to methane production. The enhanced methane production performance of the two-stage AD system was attributed to the physical separation of the acidogenic stage from methanogenesis and the occurrence of lactate-type fermentation in the acidogenic stage.

Chapter 7

Continuous fermentative bio-hydrogen production from fruit-vegetable waste: A parallel approach to assess process reproducibility

This chapter was adapted after its publication in Fermentation:

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Continuous fermentative biohydrogen production from fruit-vegetable waste: A parallel approach to assess process reproducibility

Leonardo J. Martínez-Mendoza [1,2], Raúl Múñoz [1,2], Octavio García-Depraect [1,2,*]

[1] Institute of Sustainable Processes, University of Valladolid, Dr. Mergelina s/n., 47011, Valladolid, Spain

[2] Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina s/n., 47011, Valladolid, Spain

***Corresponding author:** octavio.garcia@uva.es

Keywords:

Dark fermentation
Biohydrogen
Reproducibility
Microbial community
Bioaugmentation
Bioenergy
Fruit and vegetable waste

Abstract

Dark fermentation (DF) has gained increasing interest over the past two decades as a sustainable route for biohydrogen production; however, understanding how reproducible the process can be, both from macro- and microbiological perspectives, remains limited. This study assessed the reproducibility of a parallel continuous DF system using fruit- vegetable waste as a substrate under strictly controlled operational conditions. Three stirred-tank reactors were operated in parallel for 90 days, monitoring key process performance indicators. In addition to baseline operation, different process enhancement strategies were tested, including bioaugmentation, supplementation with nutrients and/or additional fermentable carbohydrates, and modification of key operational parameters such as pH and hydraulic retention time, all widely used in the field to improve DF performance. Microbial community structure was also analyzed to evaluate its reproducibility and potential relationship with process performance and metabolic patterns. Under these conditions, key performance indicators and core microbial features were reproducible to a large extent, yet full consistency across reactors was not achieved. During operation, unforeseen operational issues such as feed line clogging, pH control failures, and mixing interruptions were encountered. Despite these disturbances, the system maintained an average hydrogen productivity of 3.2 NL H_2/L-d, with peak values exceeding 6 NL H_2/L- d under optimal conditions. The dominant microbial core included *Bacteroides*, *Lactobacillus*, *Veillonella*, *Enterococcus*, *Eubacterium*, and *Clostridium*, though their relative abundances varied notably over time and between reactors. An inverse correlation was observed between lactate concentration in the fermentation broth and the amount of hydrogen produced, suggesting it can serve as a precursor for hydrogen. Overall, the findings presented here demonstrate that DF processes can be resilient and broadly reproducible. However, they also emphasize the sensitivity of these processes to operational disturbances and microbial shifts. This underscores the necessity for refined control strategies and further systematic research to translate these insights into stable, high-performance real-world systems.

Chapter 8

From waste to value:

Lessons and next steps

CONCLUSIONS

This doctoral thesis has advanced the valorisation of FVW through LDDF and lactate-based two-stage AD, addressing both technical feasibility and environmental relevance. The research outcomes are the result of a systematic exploration of operational parameters, process configurations, and reproducibility assessments, which together build a comprehensive framework for the integration of fermentative and methanogenic platforms.

The influence of key operational conditions was first examined, revealing that pH, total solids concentration, and inoculum density govern the metabolic dynamics of H_2-producing microbial consortia. Under neutral pH (7.0), low TS (5%), and elevated inoculum concentrations (1800 mg VSS/L), LDDF achieved its maximum performance, with volumetric H_2 productivity reaching 976 NmL H_2/L-h and yields of 49.5 NmL H_2/g VS. Importantly, H_2 generation was not directly associated with carbohydrate degradation, but rather with HLac consumption coupled to the co-production of HAc and HBu, which reflects a metabolic redirection toward HLac-dependent pathways. Building on this, the evaluation of HRT under continuous conditions confirmed its pivotal role in stabilizing microbial activity and maximizing gas production. A gradual reduction of HRT demonstrated that 9 h represented the optimal value, delivering the highest H_2 production rate (11.8 NL H_2/L-d) and yield (95.6 NmL/g VS_{FED}). These findings underscore the need for finely tuned HRT control when operating LDDF in continuous mode. Complementarily, the establishment of a detailed energy and mass balance demonstrated that the management of 1000 kg of FVW per day would require a reactor volume of ~0.8 m^3, producing up to 9.4 m^3 H_2 daily. This provides a practical baseline for the future design and techno-economic evaluation of biorefineries integrating DF.

A comparative analysis of process configurations further elucidated the benefits of HLac-based two-stage AD. In contrast to the single-stage system, the phase-separated configuration improved CH_4 productivity and yield by 32% and 36%, respectively, while maintaining comparable process stability and CH_4 purity. The superior efficiency of the two-stage setup derived from enhanced substrate conversion and microbial specialization, with HLac emerging as a key bioenergetic intermediate. The acidogenic phase fostered the proliferation of HLac-producing bacteria, whereas the methanogenic phase supported stable populations of *Methanobacterium* and *Methanothrix*, central to efficient CH_4 generation.

Finally, reproducibility and enhancement strategies were assessed through the parallel operation of three continuous bioreactors. The results confirmed that LDDF delivers consistent H_2 content (65 ± 5%), production rates, and metabolite profiles under stable conditions. Temporary improvements were observed following bioaugmentation and micronutrient supplementation, with productivity peaking at 7.4 NL H_2/L-d. However, these enhancements were not sustained, reflecting the inherent complexity of microbial interactions and the need for adaptive operational strategies to secure long-term stability.

In summary, this thesis validates LDDF and HLac-based two-stage AD as robust and sustainable biotechnological platforms for converting organic waste into clean energy carriers, namely bioH_2 and biogas. The sensitivity of LDDF to operational parameters, the central role of HRT, the reproducibility demonstrated under parallel operation, and the superior performance of the two-stage configuration all provide critical insights into process optimization and scalability. Beyond the specific experimental findings, the work contributes to the broader development of reproducible, efficient, and scalable fermentative bioenergy systems that align with circular economy principles and global climate mitigation goals.

FORWARD VISION

While the current findings entailed important advancements in the field of DF and AD, several research directions remain open to further improve the robustness, environmental performance, and industrial applicability of this biotechnological platform:

• Life Cycle Assessment (LCA): Conduct a comprehensive LCA to evaluate the environmental impact, energy efficiency, and carbon footprint of the integrated LDDF and AD processes under different configurations and scales. This is essential for benchmarking against conventional waste management and energy recovery systems.

• Process Scale-Up: Transition from laboratory-scale systems to pilot- and full-scale reactors to assess process performance under real operational conditions. Scaling up will enable a better understanding of microbial dynamics, system stability, and energy/product recovery efficiency at higher loads.

• Process Automation: Implement fully automated process control systems to maintain stable conditions in long-term operations. This includes adaptive pH control, feedstock monitoring, crucial for minimizing process variability and enhancing operational reliability.

• Advanced Microbial Characterization: Employ metagenomics and transcriptomics to better understand the functional roles of microbial communities, particularly under variable operating conditions and in response to enhancement strategies. This will inform the design of more targeted bioaugmentation or selective enrichment protocols.

• Integration with other bioproduct recovery technologies: Explore the coupling of LDDF and AD systems with downstream processes for the recovery of value-added biochemicals (e.g., VFA, bioplastics precursors) or CO_2 capture technologies, contributing to a more holistic biorefinery approach.

Through these future efforts, the technology platform presented in this thesis can evolve into a highly adaptable, economically viable, and ecologically sound solution for organic waste valorisation, reinforcing its role within the global transition toward sustainable energy systems and circular resource management.

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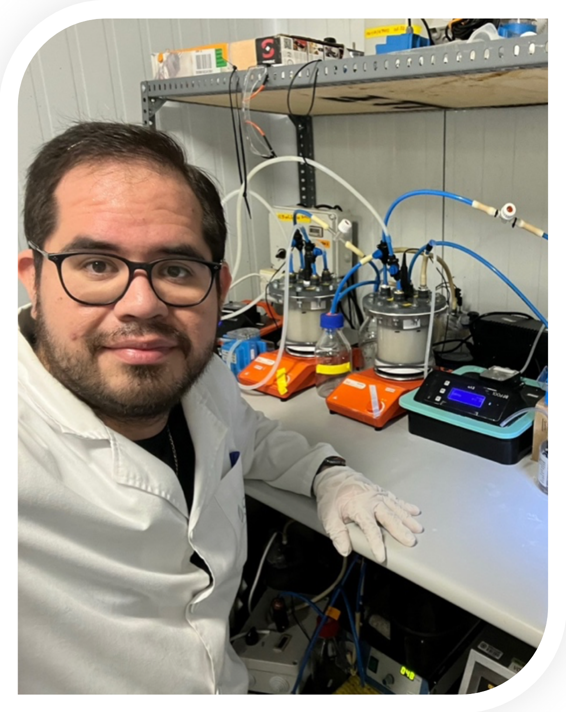
Chapter 9

About the author

BIOGRAPHY

Leonardo José Martínez Mendoza was born in Tepic, Nayarit, México. He earned his Bachelor's degree in Chemical Engineering from the Instituto Tecnológico de Tepic in 2017.



During his undergraduate studies, his growing interest in scientific research led him to participate in the *XXV Verano de la Investigación Científica* (2015), completing a two-month research stay at the University of Guanajuato in México.

Leonardo holds a Master's degree in Biotechnological Innovation from the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. (CIATEJ). His master's thesis focused on evaluating the presence of micro-contaminants in the surface waters of Lake Chapala, along with preliminary treatability tests using an advanced oxidation process based on ozone (2021).

In 2022, Leonardo joined the VOC and Odour Treatment Group and the Institute of Sustainable Processes, both at the University of Valladolid, to develop his thesis project *Integral valorization of food waste through the recovery of biohydrogen and biomethane*.

PUBLICATIONS

1. **Martínez-Mendoza, L.J.**, Lebrero, R., Muñoz, R., García-Depraect, O., 2022. Influence of key operational parameters on biohydrogen production from fruit and vegetable waste via lactate-driven dark fermentation, Bioresource Technology. 364 128070. https://doi.org/10.1016/j.biortech.2022.128070
2. **Martínez-Mendoza, L. J.**, García-Depraect, O., Muñoz, R.,2023. Unlocking the high-rate continuous performance of fermentative hydrogen bioproduction from fruit and vegetable residues by modulating hydraulic retention time, Bioresource Technology, 373, 128716. https://doi.org/10.1016/j.biortech.2023.128716
3. **Martínez-Mendoza, L. J.**, Múñoz, R., García-Depraect, O., 2024. Enhanced methane production from food waste: A systematic comparison between conventional single-stage and lactate-based two-stage anaerobic digestion processes, Biomass and Bioenergy, 188, 107312, https://doi.org/10.1016/j.biombioe.2024.107312
4. **Martínez-Mendoza, L. J.**, García-Depraect, O. Muñoz, R., 2025. Continuous fermentative biohydrogen production from fruit-vegetable waste: A parallel approach to assess process reproducibility. Manuscript submitted in Fermentation.
5. García-Depraect, O., Castro-Muñoz, R., Muñoz, R., Rene, E. R., León-Becerril, E., Valdez-Vazquez, I., Kumar, G., Reyes-Alvarado, L. C., **Martínez-Mendoza, L. J.**, Carrillo-Reyes, J., Buitrón, G., 2021. A review on the factors influencing biohydrogen production from lactate: The key to unlocking enhanced dark fermentative processes. Bioresource Technology, 324, 124595, https://doi.org/10.1016/j.biortech.2020.124595

6. García-Depraect, O., Lebrero, R., Rodríguez-Vega, S., Bordel, S., Santos-Beneit, F., **Martínez-Mendoza, L. J.,** Aragão Börner, R., Börner, T., Muñoz, R., 2022. Biodegradation of bioplastics under aerobic and anaerobic aqueous conditions: Kinetics, carbon fate and particle size effect. Bioresource Technology, 344 B, 126265, https://doi.org/10.1016/j.biortech.2021.126265
7. García-Depraect, O., **Martínez-Mendoza, L. J.**, Diaz, I., Muñoz, R., 2022. Two-stage anaerobic digestion of food waste: Enhanced bioenergy production rate by steering lactate-type fermentation during hydrolysis-acidogenesis. Bioresource Technology, 358, 127358. https://doi.org/10.1016/j.biortech.2022.127358
8. García-Depraect, O., Mirzazada, I., **Martínez-Mendoza, L. J.**, Regueira-Marcos, L., Muñoz, R. 2023. Biotic and abiotic insights into the storage of food waste and its effect on biohydrogen and methane production potential. Journal of water process engineering, 53, 103840, https://doi.org/10.1016/j.jwpe.2023.103840
9. Leroy-Freitas, D., Muñoz, R., **Martínez-Mendoza, L.J.**, Martínez-Fraile, C., García-Depraect, O., 2024. Enhancing Biohydrogen Production: The Role of Iron-Based Nanoparticles in Continuous Lactate-Driven Dark Fermentation of Powdered Cheese Whey. Fermentation, 10, 6, 296. https://doi.org/10.3390/fermentation10060296
10. García-Depraect, O., **Martínez-Mendoza, L. J.**, Aragão Börner, R., Zimmer, J., Muñoz, R., 2024. Biomethanization of rigid packaging made entirely of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate): Mono- and co-digestion tests and microbial insights. Bioresource Technology, 408, 131180, https://doi.org/10.1016/j.biortech.2024.131180

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1. **<u>Martínez-Mendoza, L. J.</u>**, García-Depraect, O., Muñoz, R. "Valorización de residuos alimenticios a través de la producción fermentativa de biohidrógeno". Best poster and oral presentation, XIV Reunión de Jóvenes Investigadores Iberoamericanos, Tordesillas, Spain. March 2022.
2. García-Depraect, O., **<u>Martínez-Mendoza, L. J.</u>**, Díaz, I., Muñoz, R. “Two-stage anaerobic digestion of food waste: improving bioenergy recovery rate by steering acidogenesis”, Co-Author. 9th International Conference on Engineering for Waste and Biomass Valorisation, Copenhagen, Denmark. June 2022.
3. **<u>Martínez-Mendoza, L. J.</u>**, García-Depraect, O., Lebrero, R., Muñoz, R. ”Influence of culture parameters on hydrogen production from fruit and vegetable waste by lactate-driven dark fermentation”. Oral presentation in the 6th International Congress on Water, Waste and Energy Managemen (WWEM-22), Niccolo Cusano University, Rome, Italy. July 2022.
4. García-Depraect, Leroy Freitas, **<u>Martínez-Mendoza, L. J.</u>**, Muñoz, R. “Impact of iron-based nanoparticles on the continuous lactate-driven dark fermentation of cheese whey”. Co-Author 4th International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability, Lake Garda, Italy. May 2023.
5. **<u>Martínez-Mendoza, L. J.</u>**, Muñoz, R., Lebrero, R., García-Depraect, O. “Biohydrogen production from fruit and vegetable waste via dark fermentation: From batch to continuous lab-scale operation. Virtual oral presentation on 3rd International Conference on Pollution Prevention and Clean Technologies (ICPPCT 2023) Institute of Technology and Business in České Budějovice, Czech Republic. September 2023.

6. **<u>Martínez-Mendoza, L. J.</u>**, Muñoz, R., García-Depraect, O. “Improved biogas production from food waste: Systematic comparison of conventional single-stade vs lactate-based two-stage anaerobic digestion. Oral presentation in XIV Latin American Workshop and Symposium on Anaerobic Digestion, Instituto de Ingeniería UNAM, Querétaro, Mexico. October 2023.
7. **<u>Martínez-Mendoza, L. J.</u>**, García-Depraect, O., Muñoz, R. “Comparación sistemática de la digestión anaerobia de una etapa frente a dos etapas basada en lactato para la producción de biogás". Best poster and oral presentation, XIV Reunión de Jóvenes Investigadores Iberoamericanos, Tordesillas, Spain. May 2024.
8. **<u>Martínez-Mendoza, L. J.</u>**, García-Depraect, O., Muñoz, R. “Valoración integral de residuos alimenticios mediante la recuperación de hidrógeno y biogas”. Poster presentation in III Encuentro de Jóvenes Investigadores 2024, University of Valladolid, Spain. June 2024

COURSES & SEMINARS

1. Iniciación a la escritura y publicación de artículos científicos, Universidad de Valladolid, 2020
2. Formación en comunicación y soft skills, Universidad de Valladolid, 2020
3. 8ª jornada de doctorandos del programa de doctorado en ingeniería química y ambiental, Universidad de Valladolid, 2020
4. Introducción a las tecnologías de secuenciación y bases de datos genómicas, Universidad de Valladolid, 2020
5. Recursos de información para doctorandos, Universidad de Valladolid, 2021
6. Análisis de ciclo de vida: Fundamentos y casos prácticos, Universidad de Valladolid, 2021
7. Taller de técnicas analíticas, Universidad de Valladolid, 2021
8. Workshop Anaerobic digestion quo vadis, Universidad de Valladolid y Universidad de Santiago de Compostela, 2021
9. 9ª jornada de doctorandos del programa de doctorado en ingeniería química y ambiental, Universidad de Valladolid, 2021
10. Valorización de resultados de investigación y creación de EBTS, Universidad de Valladolid, 2022
11. Acceso Abierto y estrategias de publicación, Universidad de Valladolid, 2022
12. Tratamiento de datos personales en la investigación, Universidad de Valladolid, 2022
13. Análisis de datos con SPSS, Universidad de Valladolid, 2022
14. Excel para investigadores, Universidad de Valladolid, 2022
15. Introducción al diseño de experimentos, Universidad de Valladolid, 2022

16. Principios básicos de cromatografía líquida de alta resolución (HPLC) y manejo del LC-2050C de Shimadzu, Universidad de Valladolid, 2022
17. 1er encuentro de egresados del Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A. C., México, 2022
18. Semana de la Ciencia de Castilla y León, Universidad de Valladolid, 2022
19. Ciencia en Familia, Universidad de Valladolid, 2022
20. 10ª jornada de doctorandos del programa de doctorado en ingeniería química y ambiental, Universidad de Valladolid, 2022
21. Figuras de calidad para artículos científicos, Universidad de Valladolid, 2023
22. Biological carbon capture technologies BIP Course, Universidad de Valladolid, 2023
23. El arte de ser profesional, Universidad de Valladolid, 2023
24. Procesos térmicos de valorización de residuos, Universidad de Valladolid, 2023
25. Elaboración de una propuesta de Proyecto, Universidad de Vallaolid, 2023
26. Coffee with science, Universidad de Valladolid, 2023
27. Jornadas de digestión anaerobia para el aprovechamiento de residuos, Universidad de Valladolid, 2023
28. Characterization of microbial communities in anaerobic systems, challenges and opportunities, Universidad Nacional Autónoma de México campus Juriquilla, 2023
29. 11ª jornada de doctorandos del programa de doctorado en ingeniería química y ambiental, Universidad de Valladolid, 2023
30. Iniciación a la escritura de propuestas de proyecto de investigación, Universidad de Valladolid, 2024
31. Emprende con tu investigación, Universidad de Valladolid, 2024
32. Excel para investigadores. Estadística y modelado, Universidad de Valladolid, 2024

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