

Biohydrogen Production from Complex Organic Substrates Using Extremophile Microorganisms (Dark Fermentation)

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The LMI Biotec-H2 project explores bio-hydrogen from agro-food by-products using marine hyper-thermophilic microorganisms. This study addresses substrate complexity by analyzing four distinct fruit and vegetables wastes (FVW) categories. Comprehensive physico-chemical and biochemical analyses of these substrates were conducted, including pH, COD, carbohydrates, and polyphenol quantification. Pretreatments technics like crushing and enzymatic hydrolysis were used to enhance substrate fermentability. A model waste substrate (MW), with known composition, was defined by coupling physicochemical and biochemical characterization with biogas production by *Thermotoga maritima* MSB8. Optimal conditions achieved a maximum yield of 3.7 mol H₂/mol C₆, 24.2 mM total H₂ production and 0.35 mM.h⁻¹ productivity with enzymatic pretreated model waste. The research aims to develop a predictive model integrating substrate profiles, pretreatments and biokinetics.

1. Introduction

Among the various categories of waste, vegetable and fruit waste (VFW) stands out due to its high organic content. According to the Food and Agriculture Organization (FAO) of the United Nations, VFW accounts for nearly 60% of all food waste. Fruit and vegetable processing operations also produce substantial by-products, representing about 25-30% of the total raw materials (Sagar et al., 2018). However, these residues are mainly disposed of through landfilling or incineration, causing pollution and ecosystem disruption. Therefore, managing vegetable waste is a critical issue that requires appropriate solutions (Abubackar et al., 2019).

Various strategies have been implemented to address environmental sustainability challenges, including advanced waste-to-energy technologies. These technologies valorise organic waste into energy, reducing fossil fuel use and emissions (Muthu et al., 2023). Among these approaches, composting and anaerobic digestion (AD) convert solid or liquid organic waste into biogas.

Other biological methods of energy generation, such as biohydrogen production, though less efficient, are environmentally friendly and utilize a variety of plant-based waste and biomass (Hassan et al., 2024). Special attention has been given to biohydrogen generation through photo- and dark fermentation (Avcioglu et al., 2011). In dark fermentation, hydrogen is produced during acidogenesis and acetogenesis, processes involving anaerobic bacteria (Yassin et al., 2013). The substrates used typically come from the agri-food industry, the sugar industry, or food waste. These substrates mainly consist of lignocellulose, a complex polymer that is difficult to degrade in its natural form. In VFW, lignocellulose comprises cellulose and hemicellulose tightly bound to lignin, forming a recalcitrant matrix. Simple sugars (pentoses and hexoses) within this structure are not easily accessible due to the lignin coating's high resistance to environmental factors such as temperature. Therefore, appropriate pretreatment is required to release these sugars and optimize biogas production (Cieciura-Włoch et al., 2020). Pretreatment methods for lignocellulosic materials fall into three main categories: physical,

chemical, biological, or a combination of these approaches. These pretreatments play a crucial role in increasing substrate availability (Pascualone et al., 2019).

The objective of this study was to characterize the physicochemical and biochemical properties of FVW with different composition and subjected to different pretreatments (mechanical, physical, enzymatic). FVW was classified into four categories based on their origin: class 1 (seed), class 2 (flower), class 3 (tuber), and class 4 (leaf). These categories underwent different pretreatments to release the maximum amount of sugars. A physical, physicochemical, and biochemical characterization was conducted to provide a comprehensive mapping of the waste classes. Then, the hydrogenogenic potential was evaluated using the reference strain *Thermotoga maritima* (TM) to determine which waste class produces the highest amount of biohydrogen. Finally, biohydrogen production was optimized using a standardized model waste (MW) substrate, derived from the optimized combination of four FVW categories, according to TM nutritional requirements.

2. Material and methods

Physical-chemical and biochemical properties analysis of the four FVW substrates categories, with and without pretreatments, were conducted and confronted with bioperformance data from anaerobic cultures of TM in a flask.

2.1 Substrate categories and pretreatment strategies

This study investigated biohydrogen production using two experimental variables: organic substrate classification and pretreatment methods. Four substrates were analyzed: (i) seeded fruits/vegetables (CW1), (ii) flowering vegetables (CW2), (iii) roots/bulbs (CW3) and (iv) leaves (CW4). All these organic substrates were provided from a municipal market of Tunis (Tunisia) in May 2022 and their composition is presented in Table 1.

Table 1: Fruit and Vegetable Waste (FVW) categories composition

| FVW categories | Composition (%) |
|----------------|--|
| CW1 | Eggplant (12), Cucumber (13), Zucchini (7), Melon (22), tomato (11), date (14), orange (11), grapefruit (4), Peach (6) |
| CW2 | Cauliflower (73), broccoli (27) |
| CW3 | Turnip (30), beetroot (9), carrot (17), onion (9), potato (36) |
| CW4 | Parsley (15), chard (29), leeks (56) |

Two different mechanical pretreatments were applied for each FVW category: (i) a grinding treatment (MT) using an electric blender to produce a puree fraction (P), and (ii) solid/liquid extraction treatment (SLT) using juice extractor to obtain separated liquid (L) and solid (S) fractions. Liquid and puree phases were the selected substrates for the dark fermentation process. Subsequently, the L and P fractions were subjected to enzymatic pretreatment (ET) using an enzymatic cocktail derived from *Clostridium thermocellum* DSM 7072, incubated at 55°C for 72 hours (Kothari et al., 2018) and/or thermal pretreatment (TT) involving incubation at 80°C for 1 hour. Following these treatments, the L and P fractions were stored at -20°C for subsequent analysis.

2.2 Substrate Characterization

A physicochemical and biochemical characterization was performed on pre- and post-treated various substrate categories to: (i) quantify key components such as carbohydrates, proteins, polyphenols, and other organic compounds, and (ii) identify specific components influencing microbial growth and dark fermentation performance. Parameters analyzed according to (Raposo et al., 2008) included water content, total and soluble dry matter (tDM, sDM), volatile solids (VS), total Kjeldahl nitrogen (TNK), total and soluble chemical oxygen demand (tCOD, sCOD), conductivity and pH. Total carbohydrate concentration was determined using a modified anthrone-sulfuric acid method (Somani et al., 1987). Briefly, 1 mL samples were mixed with sulfuric acid and anthrone reagent, heated at 105°C, and absorbance was measured at 625 nm. Reducing sugar concentrations were measured according to Kongjan et al. (2011). Fermentable carbohydrates and volatile fatty acids (VFAs) were quantified by HPLC (Agilent 1200) using an Aminex HPX-87H column, RI detector, and 5 mM sulfuric acid as the mobile phase (0.6 mL.min⁻¹, 30°C). Centrifuged and filtered supernatants (0.45 µm) were injected (20 µL).

A physicochemical characterization was also performed on the seawater (pH, conductivity, salinity, major ions and nutrients).

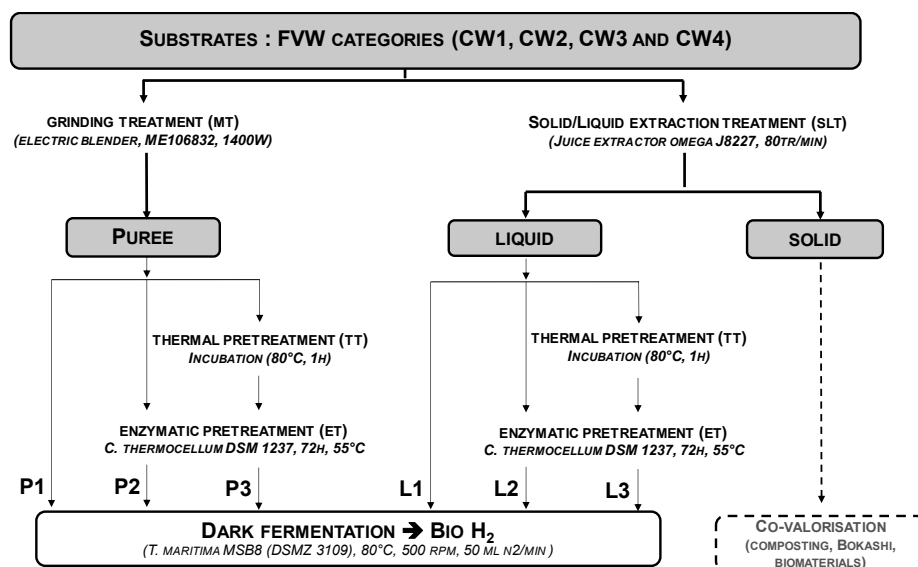


Figure 1: Workflow diagram from Fruit and Vegetable Waste (FVW) categories to Biohydrogen (BioH₂) Production Through Pretreatment Steps.

2.3 Strain and rich medium

The reference strain *Thermotoga maritima* MSB8 (DSMZ 3109), obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, was used in this study. This bacterium is strictly anaerobic, rod-shaped, fermentative, halophilic and extremely thermophilic (optimum growth around 80°C). The basal culture medium (BM) used for its growth was prepared using anaerobic techniques as developed by Hungate and Macy (1973). It contained (per liter): NaCl 20 g, yeast extract 1 g, NH₄Cl 0.5 g, KH₂PO₄ 0.3 g, K₂HPO₄ 0.3 g, MgCl₂ 0.2 g, KCl 0.1 g, CaCl₂ 0.1 g, and Balch trace mineral element solution 10 mL. The medium was adjusted to pH 7.0 with 1 M NaOH and then boiled and cooled down to room temperature under a free N₂. It was then distributed into 100 mL serum bottles (50 mL of medium). After sealing the serum bottles, the gaseous phase was flushed with free N₂ for 30 min. The medium was then autoclaved at 120 °C for 20 min and stored at room temperature. Before inoculation, the culture medium was supplemented with 0.5 mL Na₂S (8%), 1 mL of NaHCO₃ (1%) and 1 mL of glucose (1 mol.L⁻¹) (Auria et al., 2016).

2.4 Flask culture of *Thermotoga maritima*

Anaerobic batch cultures of *Thermotoga maritima* were conducted in 102 mL serum bottles (50 mL working volume). Twenty-four experiments, utilizing 10% (v/v) substrate and natural seawater, were prepared following established substrate and pretreatment variations. The headspace was purged with nitrogen for 25 minutes, and the initial pH was adjusted to 6.9 with 1 M NaOH. To maintain anaerobiosis and pH stability, 1% Na₂S and 2% NaHCO₃ were added. Cultures were inoculated with 10% (v/v) TM preculture and incubated at 80°C for 3 days. All experiments were performed in triplicate.

Headspace hydrogen concentration was determined by gas chromatography (GC, Perichrom) using a thermal conductivity detector (TCD) and a CTR1 column (Alltech), with argon as the carrier gas (20 mL.min⁻¹). The GC was connected to WINILAB III software (Perichrom) (Ben Gaida et al., 2022). Injector, column, and detector temperatures were set at 90, 120, and 100°C, respectively, for biohydrogen and nitrogen measurements. Soluble metabolites produced (acetate, lactate) and residual sugars consumed (sucrose, glucose, fructose) were quantified by HPLC described above.

3. Results and discussion

3.1 Substrate composition and pretreatments impact on bioperformance

Physicochemical and biochemical parameters of the waste classes were characterized to optimize their use as nutrient and energy sources for TM. A control flask with only FVW and seawater was incubated at 80 °C for 24 h to confirm pretreatment efficiency. The absence of biogas production in this control confirmed that the heat treatment effectively suppressed microbial activity and potential contamination risks under the tested conditions.

Among the collected data, special attention was given to the impact of fermentable C6 carbohydrates, phenolic compound concentrations, and pretreatments on biohydrogen (BioH_2) production. Glucose and fructose concentrations (C6) increased after pretreatment across all waste categories (Figure 2), a trend also observed for total carbohydrates (data not shown). The highest C6 concentrations were measured in liquid and puree fractions (L3 and P3) subjected to combined mechanical, enzymatic, and thermal pretreatments. Waste categories 1 (seeds) and 3 (tubers) reached peak fermentable C6 levels of 462.8 mM and 466.6 mM, respectively. These results highlight the synergistic effect of combined pretreatments on sugar release from complex plant matrices and the strong potential of seed and tuber wastes as fermentation substrates.

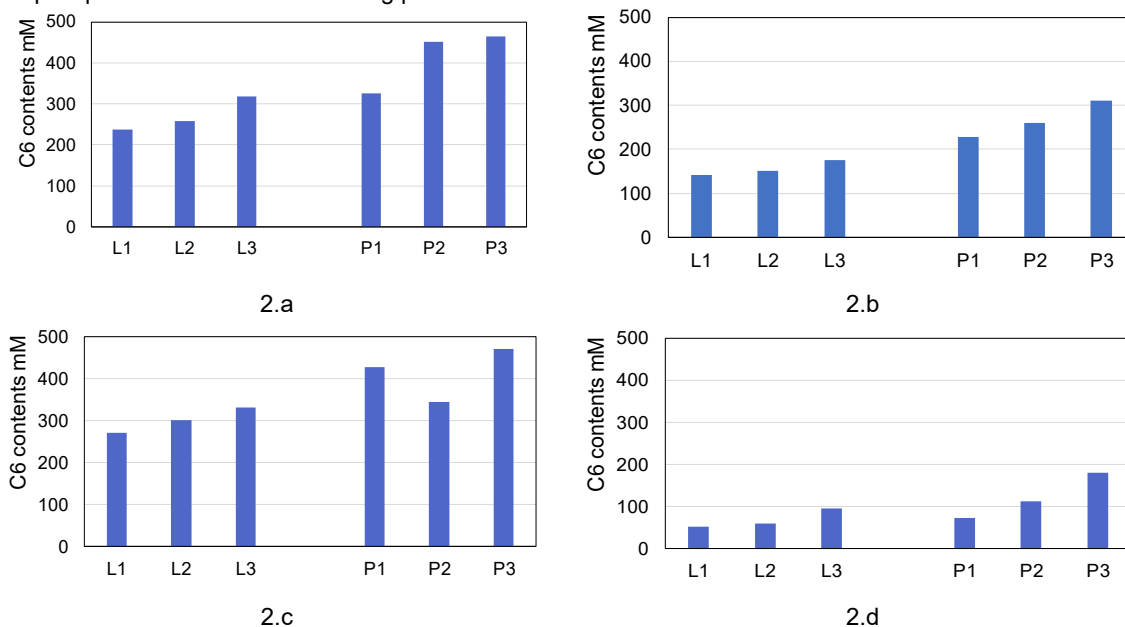


Figure 2: Carbohydrate C6 contents (mM) in the four CW matrices with the various pretreatments technics (2.a: CW1; 2.b: CW2; 2.c: CW3 and 2.d: CW4)

Phenolic compound (PC) concentrations, known for their inhibitory effect on the process, were also analyzed. Puree fractions showed higher PC levels (27–268 $\text{mg}\cdot\text{L}^{-1}$) than liquid fractions (20–132 $\text{mg}\cdot\text{L}^{-1}$). Pretreatments enhanced PC release across all substrates, especially in the leaf class (CW4). The correlation between total H_2 production and PC concentration (Figure 2) revealed a clear inverse relationship: higher PC levels corresponded to lower H_2 yields, with inhibition observed above 100 $\text{mg}\cdot\text{L}^{-1}$. Anaerobic batch cultures of *T. maritima* provided detailed data on total H_2 production, productivity, and yield in relation to C6 carbohydrate concentrations and pretreatments (Table 2).

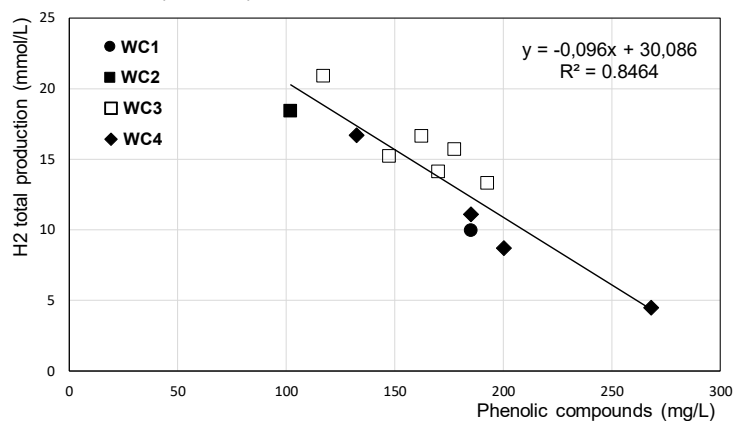


Figure 2: Correlation between Total Hydrogen (H_2) Production and Phenolic Compound Concentration across different Waste Categories

Table 2: kinetic parameters for the four waste classes and the various pretreatments

| Kinetic parameters | Samples | CW 1 | CW 2 | CW 3 | CW 4 | |
|---|--|------------|------------|--------------|------------------|------------|
| Total H ₂ production (mmol.L ⁻¹) | L1 | 7.4 ± 0.19 | 18.4 ± 1.6 | 20.86 | 16.7 ± 0.2 | |
| | L2 | 7.9 ± 0.01 | 15.9 | 16.6 | 19.3 ± 1.2 | |
| | L3 | 6.5 | 15 | 15.7 | 18.9 ± 0.9 | |
| | P1 | 7.26 ± 0.7 | 17.3 | 15.2 | 11.1 ± 0.8 | |
| | P2 | 10.1 ± 1.8 | 15.3 ± 0.6 | 14.1 ± 4.1 | 8.7 ± 1 | |
| | P3 | 9.94 ± 1 | 18.4 ± 1.3 | 13.3 ± 1.4 | 4.48 ± 0.5 | |
| | H ₂ Productivity (mmol.L ⁻¹ .h ⁻¹) | L1 | 0.08 ± 0 | 0.3 ± 0.02 | 0.18 ± 0.2 | 0.2 ± 0.01 |
| | | L2 | 0.08 ± 0.1 | 0.2 ± 0.31 | 0.2 ± 0.03 | 0.2 ± 0.01 |
| | | L3 | 0.06 ± 0.1 | 0.2 ± 0.01 | 0.2 ± 0.01 | 0.3 ± 0.01 |
| P1 | | 0.08 ± 0.1 | 0.2 ± 0.01 | 0.1 ± 1.2 | 0.1 ± 0.01 | |
| P2 | | 0.1 ± 0.1 | 0.1 ± 0.01 | 0.1 ± 0.04 | 0.1 ± 0.01 | |
| P3 | | 0.3 ± 0.1 | 0.3 ± 0.02 | 0.1 ± 0.01 | 0.1 ± 0.1 | |
| Y(molH ₂ /mol C6) | | L1 | 1.6 ± 0.2 | 2.2 ± 0.5 | 3.4 ± 0.7 | 1.1 ± 0.01 |
| | | L2 | 0.9 ± 0.3 | 2.4 ± 0.51 | 1.5 | 1.8 ± 0.6 |
| | | L3 | 2.7 | 2.7 ± 0.3 | 0.72 | 1.4 ± 0.1 |
| | P1 | 0.7 ± 0.1 | 2.2 | 0.35 | 2.3 ± 0.2 | |
| | P2 | 1.3 ± 0.1 | 2.2 ± 0.2 | 0.74 ± 0.3 | 2.1 ± 0.2 | |
| | P3 | 0.9 ± 0.2 | 2.3 ± 0.1 | 1.1 ± 0.06 | 2.2 ± 0.4 | |

This data reveals a complex interplay between substrate type, pretreatment, and biohydrogen (H₂) production across the four waste classes. In CW1, despite consistent H₂ production in puree fractions and interesting C6 carbohydrate contents, polyphenolic inhibition significantly limited overall yields. This highlights the critical role of inhibitory compounds in dictating fermentation efficiency, even in substrates with high sugar content. Conversely, CW2 demonstrated higher H₂ production and yields in the liquid fraction, attributed to an abundance of readily available fermentable sugars and nutrients. CW3 achieved the highest H₂ production, but pretreatments paradoxically decreased production, suggesting that while pretreatments can enhance sugar availability, they may also release inhibitory phenolic compounds, impacting fermentation negatively. This emphasizes the need for optimized pretreatment strategies. Finally, CW4 reproduced the same trend observed in CW1, where polyphenols likely inhibited puree fraction fermentation, despite pretreatments enhancing liquid fraction production. This reinforces the consistent negative impact of polyphenolic compounds on biohydrogen production, particularly in puree fractions.

3.2 Standardized Waste Model

The previous results underscore the necessity of understanding substrate composition and optimizing pretreatments to achieve efficient biohydrogen production from waste materials. This led to establish a model waste (MW) combining waste categories 1, 3 and 4 (CW1, CW3, CW4), previously characterized for composition and biohydrogen potential, excluding CW2 due to limited availability and seasonal variability. The MW was composed (in %) of: onions :35, seasonal fruits (peach, pear, plum): 20, tomatoes: 10, carrots:10, potatoes: 10, apple: 10 and parsley: 5.

The model waste was characterized for physicochemical (pH, conductivity, dry matter, volatile solids, ash, etc.) and biochemical (total/reducing sugars, phenolic compounds, etc.) parameters (data not shown). Fermentable sugar concentrations in the model waste ranged from 473 mM to 501 mM in liquid (L1) and puree (P1) fractions, respectively. Combined mechanical, enzymatic and thermal pretreatments effectively released simple sugars across all waste components. The MW also contained phenolic compounds, with concentrations between 58 mg.L⁻¹ (liquid) and 124 mg.L⁻¹ (puree) remaining within a range that minimizes potential inhibitory effects. TM cultures yielded significant H₂ production, specifically with puree fractions showing the highest H₂ production of 24.3 ± 3.3 mmol.L⁻¹ and a maximum yield of 3.7 mol H₂/mol hexose, similar to bioreactor results (3.8 mol H₂/mol hexose, Reimer et al., 2022). The presence of onions, rich in sugars, phosphorus, potassium, and sulfur (Romano and Zhang, 2008), in the MW improves H₂ production (Ben Gaida et al., 2022). Iron, a key component of TM's hydrogenase (Schut et al., 2014) and an essential component for H₂ production is present in parsley in reduced quantity, its limitation may divert fermentation toward lactate production (Zhang and Shen, 2006). Vitamins from seasonal fruits and vegetables, as replacements for yeast extract vitamins (Childers et al., 1992), also promote bacterial growth.

Beyond demonstrating the efficacy of various pretreatments in enhancing substrate accessibility and reducing inhibitory compounds for increased biohydrogen production, this approach embodies a zero-waste strategy. The residual solid fraction of the FVW, following initial processing for liquid/puree extraction, is not discarded. Instead, it is strategically channeled into solid-state fermentation (SSF) for the production of valuable

biocatalysts (enzymes), or it can be valorized through composting or biomaterial production processes. By transforming a problematic waste into both bioenergy (hydrogen) and valuable bioproducts (enzymes or biomaterials), this research illustrates how integrated biorefineries can contribute to a more resilient and environmentally responsible bioeconomy.

4. Conclusions

This study focused on biohydrogen (bioH₂) production from the strain *Thermotoga maritima* MSB8 (DSMZ 3109), a halophilic and hyperthermophilic bacterium using anaerobic conditions in batch mode. Four categories of fruit and vegetable wastes (CW1, CW2, CW3, CW4) were mixed with seawater.

The study examined the impact of variations in wastes composition and pretreatment on the degradation of lignocellulosic matrices and the release of fermentable sugars. The variability in H₂ production performance is attributed to the diversity of waste composition, inhibitory phenolic compounds, and the impact of pretreatments. Finally, a standardized model was proposed achieving 24.3 ± 3.3 mmol.L⁻¹ of H₂ 3.7 mol H₂/mol hexose taking into account the essential nutrients for TM growth and reducing the presence of inhibitory contents. Liquid fractions generally outperformed puree fractions in H₂ production. Furthermore, while pretreatments can improve sugar availability, their impact on releasing inhibitory compounds must be carefully considered.

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