THERMODYNAMIC EVALUATION OF DARK FERMENTATIVE HYDROGEN PRODUCTION: EFFECTS OF SLUDGE TYPE AND PRETREATMENT

Oscar F. Herrera Adarme[1]*, Paula Fontoura Procopio[1,3], Michelle F. Araújo[1,4], Maria P. Cardeal Volpi[2], Marcelo Falsarella Carazzolle[3] Gonçalo Pereira[3] Gustavo Mockaitis[1]

[1]Interdisciplinary Research Group on Biotechnology Applied to the Agriculture and the Environment, School of Agricultural Engineering, University of Campinas (FEAGRI/UNICAMP), Av. Candido Rondon, 501 - Cidade Universitária, Campinas - SP, 13083-875, Brazil.
[2]Department of Biosystems Engineering, LEB, Luiz de Queiroz College of Agriculture - ESALQ, University of São Paulo - USP, Av. Pádua Dias, 11 - Agronomy, Piracicaba - SP, 13418-900, Brazil
[3] Laboratory of Genomics and BioEnergy (LGE), Institute of Biology, Department of Genetics, Evolution, and Bioagents, University of Campinas (UNICAMP), 13083-864 Campinas, SP, Brazil
[4]School of Chemical Engineering, University of Campinas, (FEQ/UNICAMP) Av. Albert 19 Einstein, 500 - Cidade Universitária, Campinas - SP, 13083-852
** Corresponding author's email address: oscarf@unicamp.br*

Highlights

- Thermal pretreatment enhanced ΔG to –6.0 kJ/e⁻ eq, boosting H_2 production.
- Poultry sludge yielded 25% of electrons as H_2, highest among tested systems.
- Activated sludge with acid PT showed lowest H_2 yield and least exergonic ΔG.

Introduction

Dark fermentation is a biological process that converts organic substrates (e.g. carbohydrates) into biohydrogen (H_2) and metabolites (primarily volatile fatty acids (VFA), alcohols, and biomass) under anaerobic conditions. It offers a sustainable route to hydrogen production, but the efficiency (hydrogen yield) is strongly influenced by the metabolic pathways that microbial systems employ. Different fermentation pathways produce different end-products and H_2 yields; for example, fermenting glucose to acetate yields a maximum of 4 mol H_2 per mol glucose, whereas conversion to butyrate yields only 2 mol H_2 ((WRESTA, WIDYARANI, *et al.*, 2021)). More reduced fermentation products like butyrate, ethanol, or lactate indicate that electrons from the substrate are being diverted away from H_2, lowering the hydrogen yield. Thermodynamic analysis can elucidate these differences: pathways that oxidize the substrate more completely (to H_2 and CO_2 with acetate) release more free energy (more exergonic), whereas pathways generating reduced metabolites (e.g. butyrate or propionate) are less exergonic. This work examines four dark fermentation systems to evaluate, from a thermodynamic perspective, which inoculum source and pretreatment favor H_2 production. Thus, two sludge inocula, an activated sludge and a poultry manure (obtained from upflow anaerobic sludge blanket system) were each subjected to two common pretreatment methods (acid shock vs. thermal heat shock) prior to fermentation. Acid and heat pretreatments are widely used to enrich hydrogen-producing bacteria by suppressing H_2-consuming archaea and non-spore-forming competitors (ALEXANDROPOULOU, ANTONOPOULOU, *et al.*, 2022). The goal is to determine which sludge–pretreatment combination yields the most favorable Gibbs free energy change (ΔG) per electron equivalent and the highest electron flow toward H_2 (as opposed to other metabolites). We also discuss how these thermodynamic insights relate to metabolic shifts in the microbial community and the implications for reactor design and energy recovery.

Material and Methods

Substrates and inocula

Glucose was used as a reference substrate and its concentration was adjusted in the range from 4-15 g/L for maintain the substrate to inoculum ratio in 2 g VS/g VS. Aerobic (activated) sludge (AS) was obtained in the recirculation system of an aerated tank from an urban wastewater treatment plant. Anaerobic sludge was sourced *from an up-flow anaerobic sludge blanket bioreactor (UASB) working at mesophilic conditions to treat poultry slaughterhouse effluents (PS).* Biomass formation was estimated using the empirical formula, obtained by elemental analysis. Thus, was used $C_5H_7O_2N$ and $C_{6.64}H_{15.07}O_{10.53}N$ for UASB poultry and activated sludge respectively. Using these two inocula allows assessment of how an anaerobic versus aerobic sludge origin influences fermentation pathways after appropriate pretreatment.

Sludge Pretreatments

The inoculums used were maintained at 4ºC until use and slowly acclimatized to 55ºC a week before being subjected to pretreatments. To perform acidic pretreatment, a hydrochloric acid (HCl) solution (12 mol/L) was added to the inocula until it reached pH 3 and maintained as this for 24 hours. For thermal pretreatment, inocula was subjected to 100°C for one hour. Inocula was then cooled at room temperature and kept at 55 °C for subsequent hydrogen production assays.

Biochemical Hydrogen Production assay (BHP)

The BHP assays were conducted in serum flasks (315 mL), hermetically sealed with rubber stoppers and aluminium caps, and maintained at 55°C under dark conditions with continuous agitation at 150 rpm. All experiments were conducted in triplicate, and the pH of each flask was adjusted to 5-5.5 with a NaOH solution. The inoculum to substrate ratio was set in 2:1 in terms of VS to ensure maximum substrate consumption. A negative control, i.e. a bottle incubated without substrate, was also included in all the experiments. The biogas pressure was measured, and hydrogen production were evaluated daily through gas chromatography. After the experiment finished, the content of each flask was centrifuged at 3600 rpm for 10 minutes to separate digestate from inocula, for subsequent characterization of volatile fatty acids using HPLC.

Thermodynamic analysis of dark fermentation

The distribution of electrons in the dark fermentation system was assessed using the method of electron equivalents (e^- eq) as proposed by (RITTMANN, MCCARTY, 2020). The stoichiometry of the overall metabolic reaction was estimated using $R_{Overall}=f_eR_A + f_sR_C - R_D$,from the semi-reactions of each compound based on their redox transformations (RITTMANN, MCCARTY, 2020).The methodology treats each bioconversion as the sum of three types of reactions: Electron-donating half-reaction (substrate oxidation, R_D), Electron-accepting half-reactions (product formation, R_A) and Biomass synthesis half-reaction (R_C). On the other hand, f_e is the fraction of electron donor moved to the electron acceptor, and f_S is the portion of donor electrons transferred to cell synthesis, in which $f_e + f_S = 1$. Finally, thermodynamic feasibility was analyzed using the TEEM approach (Theoretical Energy Equivalent Model (MCCARTY, 2007)), integrating McCarty's redox framework with Gibbs free energy theory.

Results and Discussion

Electron Equivalents Distribution and Metabolic Products

Beyond the aggregate free energy, it is informative to examine where the electrons from glucose ended up in each system – i.e. the distribution of electron-equivalents among H_2, various fermentation products, and biomass. Figure 1 presents radar charts of the **electron-equivalent allocation** to key sinks: H_2 gas, VFAs, ethanol, biomass, and "other" metabolites (with "other" mainly capturing any residual electrons in solvents like lactate or propionate, or unaccounted minor products). The percentages show what fraction of glucose's electrons are contained in each product pool at the end of fermentation (summing to ~100% for each system). Several important differences in electron routing are evident. First, the **share of electrons ending in H_2** (the H_2 axis) was highest for the poultry sludge with thermal pretreatment (B), where about 25% of the substrate electrons were released as hydrogen gas. The PS-Acid case (B) had slightly lower H_2 allocation (~20%), followed by the activated sludge Thermal (A) ~15%, and the lowest was activated sludge Acid (A) with only ~8% of electrons in H_2. These percentages mirror the hydrogen yields: more electrons to H_2 means more H_2 produced per mole of glucose. Indeed, the ranking matches the observed H_2 yields (qualitatively noted during the experiments): PS-Thermal > PS-Acid > AS-Thermal > AS-Acid in hydrogen mol/mol glucose. In system A (AS-Acid), the vast majority of electrons (50%) fell into the "Other" category, indicating a large portion went into non-H_2, non-VFA products (MOSTAFA, IM, *et al.*, 2022). This suggests that in the acid-pretreated activated sludge, pathways like the **lactate–propionate route** were significant – those end-products (lactate, propionate) would appear in "Other." Such pathways do not produce H_2 (e.g. lactate fermentation yields no H_2), consistent with the low H_2 fraction. The dominance of "Other" electrons explains why this case had the least favorable ΔG: forming lactate or propionate leaves much of glucose's reducing power unused for H_2, resulting in less energy release. In contrast, system B (PS–Thermal) has only ~5% in "Other," implying minimal flux to the lactate/propionate sink. Most electrons in B were split between **VFAs (~50%)** and **biomass (~15%)** besides the 25% in H_2. The high VFA electron fraction in B is chiefly acetate and butyrate production. Notably, PS–Thermal shows a slightly higher biomass electron fraction (15%) compared to others (around 5–10%). This could mean that the greater energy availability (more exergonic ΔG) enabled more cell synthesis – thermodynamically, a more favorable catabolism allows microbes to allocate more electrons to growth (anabolism). Systems A and B both channeled roughly 50–60% of electrons to VFAs, but there is a subtle difference: Activated–Thermal had a bit more "Other" (10%) and slightly less H_2, whereas Poultry–Acid had higher H_2 and minimal "Other." This suggests that even without thermal treatment, the poultry sludge naturally directed electrons more toward acetate/butyrate pathways than the activated sludge did with a heat shock. The **acetate-to-butyrate ratio** is a useful indicator of metabolic route: higher acetate (relative to butyrate) means more H_2 produced (LEE, SALERNO, *et al.*, 2008). Although we have combined VFAs, one can infer that PS–Thermal likely had the highest acetate:butyrate ratio (given its high H_2), followed by PS–Acid. Activated–Thermal probably produced a mix with moderate acetate (some H_2) and some butyrate, whereas Activated–Acid likely produced more reduced acids (propionate) instead of butyrate or acetate, which aligns with a low acetate/butyrate and presence of propionate (again pointing to the lactate pathway). Overall, the electron distribution analysis reinforces that **poultry sludge with heat pretreatment** most strongly favors the desired H_2 + acetate route (maximizing H_2 and leaving little in undesired products), while **activated sludge with acid pretreatment** had electrons "stuck" in reduced organic products, reflecting an unfavorable metabolic shift for H_2 production.

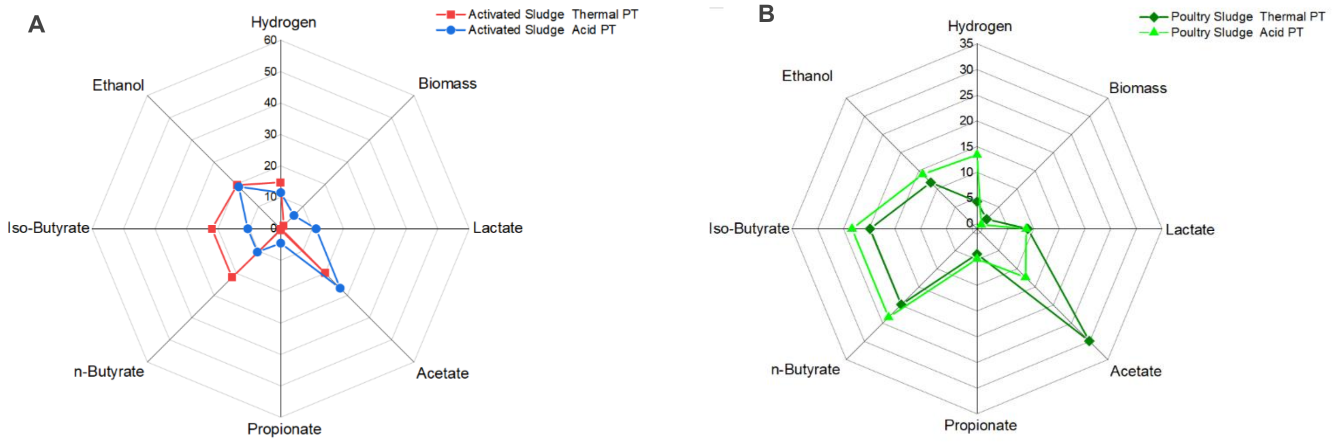

Figure 1 Electron-equivalent distribution (in %) among major product categories for each system (radar plots). Each chart corresponds to one fermentation: (A) Activated sludge – Acid and Thermal pretreatment, (B) Poultry sludge – Acid and Thermal pretreatment.

Figure 2 compares the computed ΔG (per e^- eq) for the four experimental scenarios. Several clear trends emerge. Thermal pretreatment resulted in more exergonic fermentations than acid pretreatment for a given sludge type (more negative ΔG per e^-). For the activated sludge inoculum, thermal pretreatment increased the energy release from around −4.0 to −5.0 kJ/e^- eq. An even larger improvement was seen with the poultry sludge: ΔG went from about −5.5 kJ/e^- eq with acid pretreatment to roughly −6.0 kJ/e^- eq with thermal pretreatment. Meanwhile, comparing the two sludge sources, the poultry UASB sludge consistently achieved more negative ΔG than the activated sludge under the same pretreatment. The poultry sludge with thermal pretreatment yielded the most exergonic reaction of all (approximately −6 kJ per electron), indicating that this combination most effectively directed the glucose electrons toward oxidized products (like H_2/CO_2) rather than leaving them in reduced metabolites. In contrast, the activated sludge with acid pretreatment was the least favorable thermodynamically (around −4 kJ/e^-). Notably, all ΔG values are negative, confirming that all four fermentations were exergonic and thus thermodynamically feasible. However, the magnitude of ΔG matters for microbial energy gain: a more negative ΔG means more energy is available to the microbes per electron transferred, which often correlates with higher possible cell yield or higher driving force for the reaction to proceed (AMIN, BINA, *et al.*, 2016). The differences here suggest that the microbial community in PS–Thermal was able to carry the fermentation closer to the high-energy-yield pathway (high H_2 production, likely acetate-dominant), whereas the AS–Acid community's metabolism left more energy in the products (suggesting more reduced products like lactate or propionate were formed, yielding less energy). These calculations align with known bioenergetics: fermenting glucose to acetate + H_2 (the high-H_2 pathway) has a standard free energy change around −206 kJ per mol glucose, equivalent to roughly −8.6 kJ per electron (24 electrons per glucose), whereas glucose fermentation to mixed acids or alcohols yields less energy (for instance, a glucose-to-ethanol + acetate pathway gives ~–205 kJ/mol). Our results per e^- eq reflect these proportions, with the best case (PS–Thermal) approaching −6 kJ/e^- (indicating a substantial fraction of the pathway was acetate-centric) and the worst case (AS–Acid) near −4 kJ/e^- (indicating much more reduced product formation). Overall, thermophilic heat-shock pretreatment of an anaerobic sludge created the thermodynamically most favorable condition for H_2 production, while acid-shocked activated sludge was the least favorable.

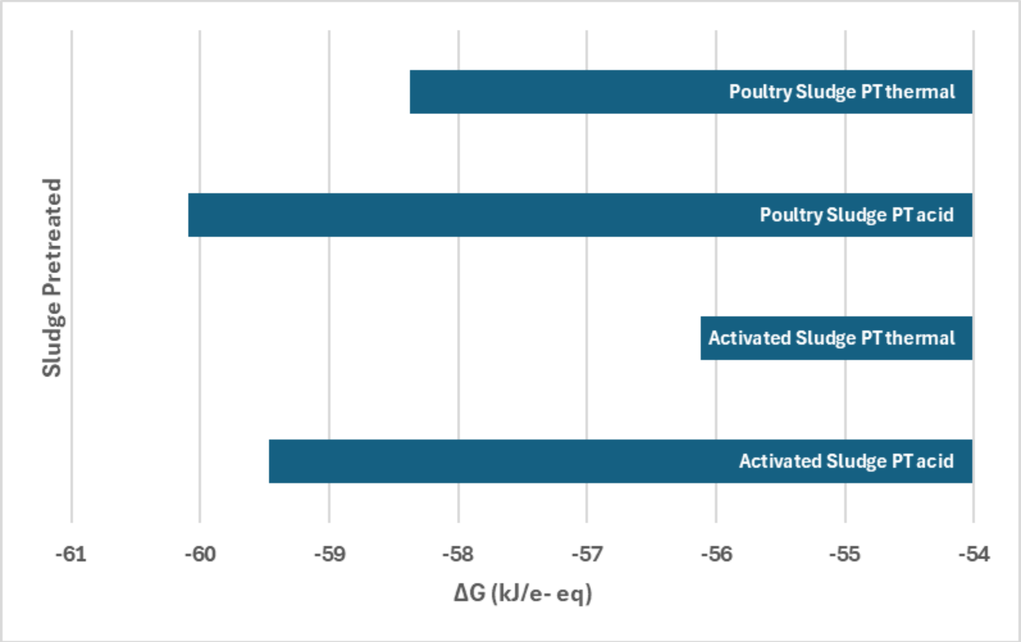

Figure 2 Calculated Gibbs free energy changes (ΔG) for the global reactions.

Conclusion

In an evaluation of four dark fermentation systems (two sludge inocula × two pretreatments), the **poultry manure UASB sludge with thermal pretreatment** emerged as the most thermodynamically and metabolically favorable for hydrogen production. This system achieved the highest hydrogen yield (as evidenced by the largest fraction of electrons flowing to H_2) and the most exergonic overall reaction (most negative ΔG per electron equivalent). Thermodynamic analysis using global stoichiometries and McCarty's electron-equivalent method provided quantitative backing to these observations, connecting the microscopic electron flows to macroscopic energy release.

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