

Enhancing Dark Fermentative Hydrogen Evolution via Tailored Water Hyacinth Iron-Biochar Additives

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Abstract—Dark fermentation is a promising and sustainable renewable energy generating method, however poor hydrogen yield and unstable gas production limit its efficiency, requiring improved measures. Water hyacinth (*Eichhornia crassipes*), a common invasive aquatic weed, was used to generate biohydrogen in this study. Batch fermentation studies using sewage sludge in 400 mL reactors were performed. Heat-shock pretreatment at 90–100°C for 15–20 minutes suppressed methanogenic activity and enriched hydrogen-producing bacteria in the inoculum. The substrate-to-inoculum ratio was 2 and the substrate concentration was 80 g VS/L. To promote acidogenic hydrogen-producing bacteria, the fermentation medium pH was set at 5.5. A control reactor with simply water hyacinth and inoculum produced 70–80 mL of gas per day. In comparison, the reactor with iron-loaded biochar produced 150 mL of gas in 24 hours, a nearly two-fold increase over the control. Due to fast acidogenesis and volatile fatty acid formation, the iron charcoal reactor pH decreased from 5.5 to 4.69 in 12 hours. To continue hydrogen production, the pH was readjusted to 5.5. Improved microbial activity, iron catalysis, and electron transfer processes boost gas generation. The results show that iron-loaded biochar improves water hyacinth biohydrogen generation and is environmentally friendly.

Index Terms—Keywords: Biohydrogen production, Dark fermentation, Water hyacinth, Gas production, Sustainable bioenergy

I. INTRODUCTION

Demand for clean and sustainable energy has sparked an interest in alternative fuels that lessen reliance on petroleum. Hydrogen is a viable renewable energy carrier due to its high energy content and zero-emissions nature, with only water as a byproduct. However, traditional hydrogen production processes are energy-intensive and emit greenhouse emissions, making biological hydrogen generation a more environmentally friendly option. Dark fermentation is an efficient biological method for producing hydrogen since it is quick and can use a variety of biodegradable substrates. However, its large-scale applicability is constrained by poor hydrogen yield, process instability, and inhibitory metabolite accumulation, necessitating improvement measures. Water hyacinth (*Eichhornia crassipes*), an invasive aquatic weed, provides a low-cost and abundant feedstock for biohydrogen generation; however, its lignocellulosic structure inhibits microbial breakdown, lowering hydrogen yield.

II. LITERATURE SURVEY

Biohydrogen is being widely studied as a clean energy carrier because it produces only water during combustion and offers a sustainable alternative to fossil fuel-based energy systems. Due to increasing global energy demand and rising concerns about greenhouse gas emissions, renewable hydrogen production methods have gained strong importance. Among the biological routes, dark fermentation is considered one of the most practical processes because it can operate without light under anaerobic conditions and is suitable for continuous hydrogen generation.

Dark fermentation involves the microbial conversion of carbohydrate-rich organic substrates into hydrogen gas along with carbon dioxide and soluble metabolites such as volatile fatty acids. Hydrogen yield is strongly influenced by microbial metabolic pathways, where acetic acid and butyric acid formation is generally associated with higher hydrogen generation, while propionic acid formation is linked with reduced hydrogen efficiency. Operating parameters such as pH and organic loading rate significantly affect microbial activity and fermentation stability. Dursun (2024) reported that hydrogen production from quinoa residue increased with higher substrate loading and that acidic conditions supported improved performance [1]. Similarly, Tiegam Tagne et al. (2024) demonstrated that pH optimization is substrate-dependent, with pH 6 producing better hydrogen yields from coffee and pineapple residues [4]. Agricultural and agro-industrial wastes are considered promising feedstocks for dark fermentation due to their low cost and high carbohydrate availability. However, lignocellulosic residues often require pretreatment to improve sugar release and enhance microbial utilization. Tiegam Tagne et al. (2024) showed that alkaline hydrogen peroxide pretreatment improved the fermentability of coffee and pineapple skins and increased hydrogen yield by enhancing cellulose and hemicellulose accessibility. In addition, microbial community composition plays an important role in fermentation performance [4]. Dursun (2024) identified hydrogen-producing *Clostridium* species as dominant microorganisms in high-yield reactors, confirming their importance in fermentative hydrogen pathways [1]. Recent studies suggest that nanoma-

terials can enhance dark fermentation by improving electron transfer and stimulating microbial enzymatic activity. Iron-based nanoparticles are especially relevant because iron is a key component of hydrogenase enzymes involved in hydrogen evolution. Green synthesis approaches using plant extracts have gained attention because they avoid toxic chemicals and rely on natural phytochemicals for nanoparticle formation and stabilization. Jain et al. (2021) successfully synthesized iron nanoparticles using jackfruit peel extract [5], while Nair et al. (2025) highlighted *Eichhornia crassipes* (water hyacinth) as a promising biogenic source due to its rich phenolic and flavonoid content [7].

Water hyacinth is a highly invasive aquatic weed that causes serious ecological problems, but its high biomass availability makes it suitable for resource recovery applications. Reports show that water hyacinth extracts can be used to synthesize iron nanoparticles effectively, supporting its potential for sustainable nanomaterial production. In addition, biochar has gained importance as a nanoparticle support material due to its porous structure, surface functional groups, and ability to act as a microbial carrier. Geetha et al. (2024) demonstrated that iron oxide-loaded biochar composites possess high surface area and magnetic properties, making them useful for separation and reuse [2].

Although optimization of dark fermentation and development of green-synthesized nanoparticles have been widely studied, limited research has explored the combined application of water hyacinth-derived Fe_3O_4 nanoparticles and biochar-supported iron particles for improving biohydrogen production. This highlights a clear research gap and supports the novelty of integrating invasive weed-based nanomaterials with dark fermentation systems to enhance hydrogen yield in a sustainable and cost-effective manner.

III. MATERIALS AND SUBSTRATE PREPARATION

Fresh water hyacinth (*Eichhornia crassipes*) was collected from Ukkadam Lake, Coimbatore, Tamil Nadu, India. The biomass was washed thoroughly with tap water to remove impurities and the roots were removed. The stems and leaves were sun-dried for 2–4 days and further oven-dried at 105°C until constant weight. The dried biomass was ground into fine powder and stored in airtight containers. Juice shop wastewater was collected from a nearby outlet and filtered using muslin cloth to remove coarse solids. The filtrate was used directly as a co-substrate in fermentation trials. Alkali pretreatment was performed using 2% NaOH to enhance biodegradability and sugar release. The dried water hyacinth powder was treated at $90\text{--}100^\circ\text{C}$ for 1 hour with continuous stirring at 150 rpm. After cooling, the slurry was filtered and repeatedly washed with tap water followed by distilled water until the filtrate pH reached 7.0. The neutralized biomass was oven-dried at 60°C , crushed into uniform powder, and stored for further analysis.

A. PREPARATION OF ENHANCEMENT MATERIALS

Iron oxide nanoparticles were synthesized through a green synthesis approach using water hyacinth leaf extract. Leaves

were washed, cut, boiled in distilled water, and filtered. A mixed iron salt solution containing FeCl_3 and FeSO_4 was prepared, and the plant extract was added gradually under continuous stirring. Nanoparticle formation was confirmed by color change and precipitate formation. The precipitate was filtered, washed with distilled water, and oven-dried at $60\text{--}70^\circ\text{C}$ for storage. Water hyacinth biochar was produced by pyrolysis of dried biomass under oxygen-limited conditions at $500\text{--}550^\circ\text{C}$. After cooling, the biochar was crushed, sieved, and stored. Iron-loaded biochar (Fe-biochar) was prepared by impregnating biochar in a mixed FeCl_3 and FeSO_4 solution under continuous stirring. The material was filtered, washed to remove excess salts, and dried at $60\text{--}70^\circ\text{C}$.

B. CHARACTERIZATION TECHNIQUES

Raw and alkali-pretreated water hyacinth biomass were characterized for moisture content, total solids (TS), volatile solids (VS), and pH. Moisture content and total solids were determined by oven drying 1 g of biomass sample at 105°C until constant weight. Volatile solids were determined by igniting oven-dried samples at 550°C for 1 hour using a muffle furnace. For inoculum VS analysis, 50 mL of sludge sample was dried at 105°C and ignited at 550°C for 1 hour. pH was measured using a digital pH meter. FTIR analysis was performed for raw biomass, alkali-pretreated biomass, iron oxide nanoparticles, and Fe-biochar to identify functional groups and confirm Fe–O bonding. Spectra were recorded in the range of $4000\text{--}400\text{ cm}^{-1}$.

C. INOCULUM PREPARATION

Activated sludge inoculum was collected from the Sequencing Batch Reactor (SBR) unit of Nanjundapuram Sewage Treatment Plant, Coimbatore. To suppress methanogens and enrich hydrogen-producing bacteria, the sludge was heat-shocked at 90°C for 15 minutes. The inoculum was cooled and used immediately, while remaining sludge was stored at 4°C .

D. DARK FERMENTATION EXPERIMENT AND GAS ANALYSIS

Batch dark fermentation was carried out in a 500 mL side-arm conical flask with a working volume of 250 mL. The reactor was sealed with a rubber stopper and connected to a gas collection system through the side arm. Temperature was maintained using a fabricated Styrofoam incubation chamber equipped with a 100 W U-shaped heating element connected to a thermostat, maintaining $35 \pm 2^\circ\text{C}$. Aluminum foil lining was used to reduce heat loss, and a side opening was provided for tubing connection. The fermentation medium consisted of alkali-pretreated water hyacinth powder mixed with juice shop wastewater. The initial pH was maintained at 5.5–6.0 without external pH adjustment. Gas produced was collected through a rubber tube connected to the side arm. Water displacement was used in Trial 1, Trial 3, and Trial 4, while gas sampling bags were used in Trial 2. Hydrogen presence was preliminarily confirmed using the flame (pop) test, where collected gas was

TABLE I
EXPERIMENTAL TRIALS AND OPERATING CONDITIONS FOR DARK FERMENTATION

Trial	Biomass (g)	Inoculum (mL)	Wastewater (mL)	Additive	Dosage (g)	Gas Collection	Objective
Trial 1 (Control – Low Load)	1	50	200	Nil	Nil	Water displacement	Confirm stable fermentation and initial hydrogen generation
Trial 2 (Control – High Load)	5	100	150	Nil	Nil	Gas sampling bag	Evaluate performance under higher substrate loading
Trial 3 (Fe-Biochar)	6	260	133	Fe-biochar	0.4	Water displacement	Study Fe-biochar effect on hydrogen production and stability
Trial 4 (WH-NP)	6	260	133	WH nanoparticles	0.4	Water displacement	Compare WH-NP performance with Fe-biochar

released near a flame source and the characteristic pop sound indicated hydrogen. Volatile fatty acid (VFA) analysis was performed on filtered fermented broth samples using standard titration method to assess acidogenic activity, which is closely associated with hydrogen-producing pathways.

E. EXPERIMENTAL TRIALS

1) *SUBSTRATE-TO-INOCULUM (S/I) RATIO ON VS BASIS*: The substrate-to-inoculum ratio was calculated on a VS basis to maintain fermentation stability. The S/I ratio was maintained within the range of 0.5–2.0 to avoid inhibition due to excess acid accumulation. Table I shows the trial conducted. $S/I \text{ Ratio} = (\text{Mass of Substrate (VS)}) / (\text{Mass of Inoculum (VS)})$ • Mass of substrate (VS) = dry substrate mass × VS fraction • Mass of inoculum (VS) = inoculum volume × sludge VS concentration (g/L)

2) *TRIALS CONDUCTED*: A total of four experimental trials were conducted under batch dark fermentation conditions. The first two trials were carried out to optimize substrate loading and inoculum volume, while the third and fourth trials were performed to evaluate the influence of enhancement additives (green-synthesized nanoparticles and iron-loaded biochar).

IV. RESULT AND DISCUSSION

A. SUBSTRATE, INOCULUM CHARACTERIZATION AND PH ANALYSIS

The physicochemical characteristics of water hyacinth biomass and inoculum were analyzed to understand their suitability for dark fermentation. Moisture content of the biomass was found to be 0.7%, while total solids were 99.3%, indicating effective drying of the substrate. The volatile solids content was 81.7%, confirming the presence of a high biodegradable organic fraction favorable for hydrogen production. The inoculum sludge showed a volatile solids concentration of 10.12

g/L, indicating sufficient microbial biomass availability for fermentation.

The pH of different components was also analyzed. Juice shop wastewater showed an acidic pH of 4.69, while alkali-pretreated biomass had a pH of 6.52. The inoculum sludge showed a near-neutral pH of 7.5, and the mixed fermentation medium had an initial pH of 5.78. This slightly acidic pH range is favorable for hydrogen-producing bacteria and helps suppress methanogenic activity, supporting selective dark fermentation.

B. FTIR ANALYSIS OF BIOMASS AND FE-BIOCHAR

FTIR analysis was carried out to evaluate structural changes in raw and alkali-pretreated water hyacinth biomass. Raw biomass showed characteristic peaks at approximately 3400 cm^{-1} (O–H stretching), 2920 cm^{-1} (C–H stretching), 1730 cm^{-1} (C=O stretching of hemicellulose), and 1510–1600 cm^{-1} (aromatic C=C stretching of lignin), confirming the presence of lignocellulosic components. After NaOH pretreatment, the reduction in the 1730 cm^{-1} peak indicated hemicellulose removal, while reduced intensity in lignin-associated peaks around 1510 cm^{-1} suggested partial delignification. The increased intensity of the broad O–H peak near 3400 cm^{-1} confirmed the exposure of hydroxyl groups and loosening of biomass structure. These observations indicate that alkali pretreatment effectively disrupted the lignin–hemicellulose matrix and improved cellulose accessibility, supporting enhanced fermentation performance. FTIR analysis of iron-loaded biochar further confirmed the presence of oxygen-containing functional groups such as –OH, C=O, and C–O, which provide active binding sites for iron species. Variations in peak intensity and band patterns compared to raw biochar indicate successful iron impregnation. The results confirm the formation of a stable Fe-biochar composite, making it suitable as an enhancement additive for dark fermentation.

TABLE II
PHYSICOCHEMICAL CHARACTERISTICS OF WATER HYACINTH BIOMASS AND INOCULUM

Parameter	Sample	Result
Moisture Content (%)	Water hyacinth biomass	0.7%
Total Solids (TS %)	Water hyacinth biomass	99.3%
Volatile Solids (VS %)	Water hyacinth biomass	81.7%
Volatile Solids (VS g/L)	Inoculum (sludge)	10.12 g/L

TABLE III
PH VALUES OF SUBSTRATE COMPONENTS AND MIXED FERMENTATION MEDIUM

Component	pH
Juice shop wastewater	4.69
Water hyacinth (pretreated)	6.52
Sludge	7.50
Mixed fermentation medium	5.78

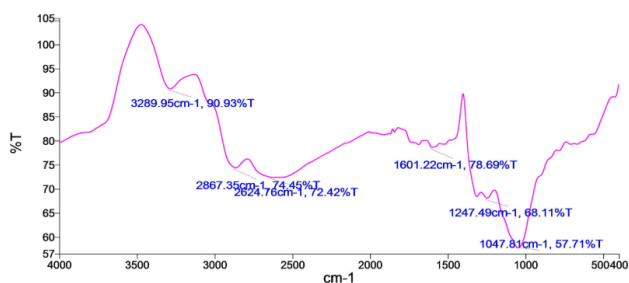


Fig. 1. FTIR spectrum of raw water hyacinth

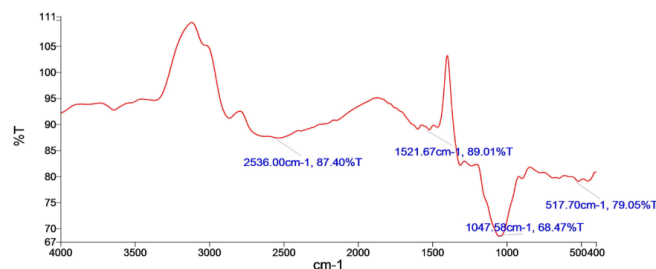


Fig. 2. FTIR spectrum of alkali pretreated water hyacinth

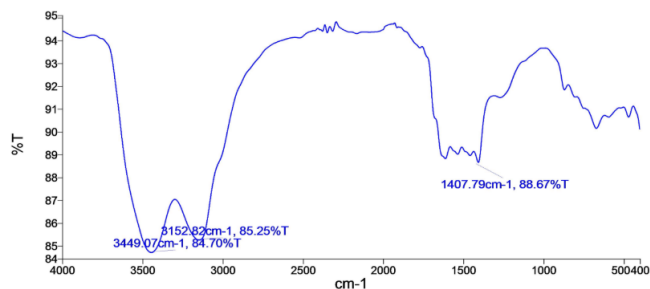


Fig. 3. FTIR spectrum of iron-loaded water hyacinth Biochar

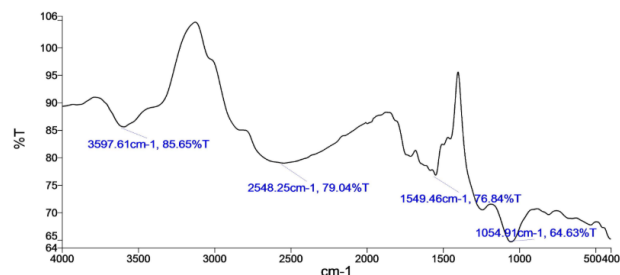


Fig. 4. FTIR spectrum of iron-loaded water hyacinth Nanoparticle

C. SEM AND XRD ANALYSIS OF IRON-LOADED BIOCHAR

SEM analysis of iron-loaded water hyacinth biochar confirmed the successful deposition of iron particles on the biochar surface. The SEM micrographs showed a rough, irregular, and highly porous carbon structure with fractured layers, which is characteristic of pyrolyzed biochar. Bright granular clusters were observed on the surface, indicating the presence of iron/iron oxide particles, since heavier elements appear brighter in SEM images. The iron particles were distributed over the biochar matrix with some degree of agglomeration, which is commonly due to magnetic attraction and particle clustering during synthesis. The porous morphology and iron particle anchoring provide enhanced surface area, improved microbial attachment sites, and better electron transfer capability, supporting the effectiveness of iron-loaded biochar in enhancing biohydrogen production during dark fermentation.

The XRD analysis of iron-loaded biochar showed sharp peaks, confirming the presence of crystalline Fe_3O_4 phases.

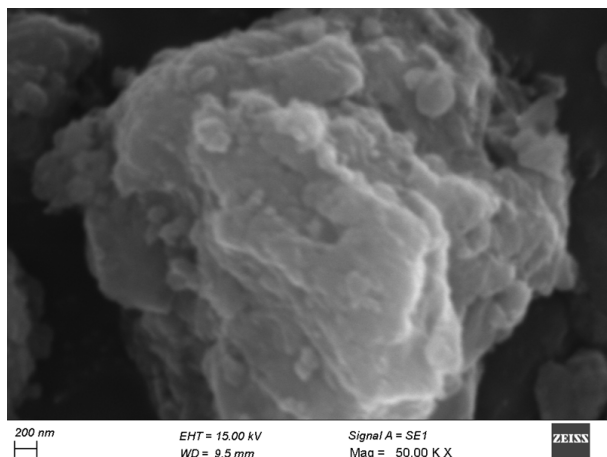


Fig. 5. SEM micrograph of Fe_3O_4 nanoparticles

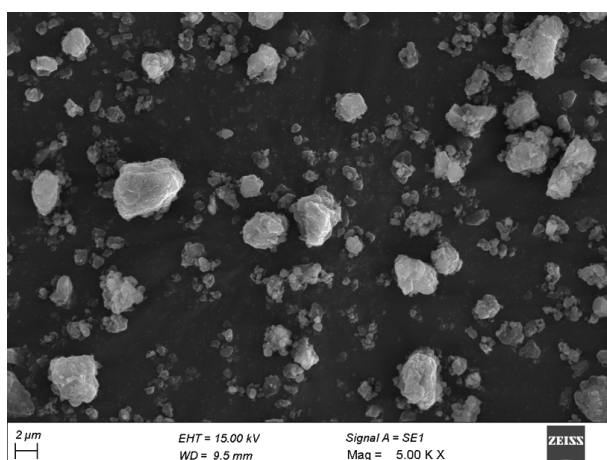


Fig. 6. SEM micrograph of Fe-biochar showing porous structure and iron deposition

These peaks validate the successful incorporation of iron nanoparticles derived from water hyacinth. The crystalline iron oxides enhance electron conductivity and microbial hydrogenase activity in dark fermentation. Biochar provides a porous support, dispersing iron particles and adsorbing inhibitory metabolites. Together, Fe_3O_4 nanoparticles and biochar-supported iron create a synergistic catalytic system that boosts biohydrogen yield.

D. SUBSTRATE-TO-INOCULUM (S/I) RATIO

The substrate-to-inoculum (S/I) ratio was calculated on a volatile solids (VS) basis to ensure stable microbial activity and avoid inhibition during fermentation. All trials were maintained close to the recommended S/I range of 0.5–2.0, ensuring stable fermentation performance.

E. BIOGAS PRODUCTION

Biogas production was monitored for all trials during the first 24 hours of dark fermentation. In Trial 1 (1 g substrate), gas evolution was measured using the water displacement method, and a biogas volume of approximately 70–80 mL

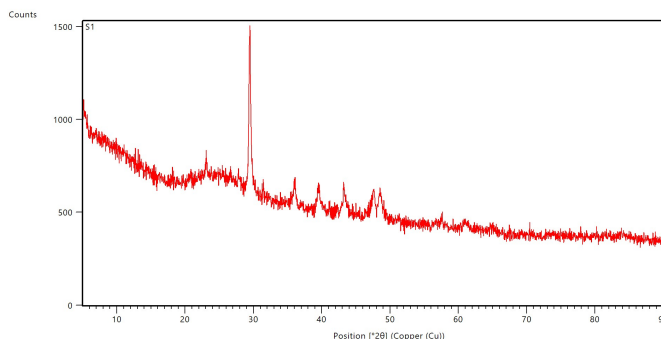


Fig. 7. XRD pattern of Fe-biochar confirming Fe_3O_4 crystalline peaks

was recorded, indicating active fermentation. In Trial 2 (5 g substrate), biogas production increased to around 95–100 mL within 24 hours. The addition of enhancement materials



Fig. 8. Dark fermentation setup with water displacement method



Fig. 9. Dark fermentation setup with gas collection bag (35–37 °C)

significantly improved gas generation. In Trial 3 (Fe-biochar particles), the highest biogas volume of approximately 150

TABLE IV
SUBSTRATE-TO-INOCULUM (S/I) RATIO CALCULATION FOR EXPERIMENTAL TRIALS

Trial	Substrate (g)	VS Fraction	Substrate VS (g)	Inoculum (mL)	Inoculum VS (g/mL)	Inoculum VS (g)	S/I Ratio
Trial 1	1	0.817	0.817	50	0.01012	0.506	1.61
Trial 2	5	0.817	4.085	200	0.01012	2.024	2.02
Trial 3 (Fe-Biochar)	6	0.817	4.902	260	0.01012	2.631	1.86
Trial 4 (WH-NP)	6	0.817	4.902	260	0.01012	2.631	1.86

mL was observed within 24 hours. Similarly, Trial 4 (green-synthesized water hyacinth nanoparticles) produced around 140 mL of biogas in 24 hours. Overall, Fe-biochar showed the maximum enhancement in biogas production compared to the control trials.

F. SUBSTRATE UTILIZATION AND COD ANALYSIS

COD analysis was performed to evaluate substrate degradation during fermentation. COD of the fermented sample was calculated using titration with a blank FAS value of 1.9 mL, resulting in a COD value of approximately 608 mg/L. The reduction in COD along with biogas evolution confirms effective substrate utilization during fermentation. The higher biogas volumes observed in Trial 3 (Fe-biochar) and Trial 4 (nanoparticles) compared to control trials indicate improved conversion of organic matter due to the presence of enhancement additives, resulting in better fermentation efficiency.

V. CONCLUSION

This study successfully demonstrated enhanced biohydrogen production from alkali-pretreated water hyacinth using juice shop wastewater as a co-substrate under batch dark fermentation. FTIR analysis confirmed effective lignocellulosic breakdown after NaOH pretreatment and validated successful iron impregnation onto biochar. SEM analysis confirmed the porous surface morphology of Fe-biochar and the successful deposition of iron particles, while XRD analysis confirmed the presence of crystalline Fe_3O_4 phases in the composite.

Among all experimental trials, Fe-biochar exhibited the highest improvement in gas generation, producing 150 mL within 24 hours, followed by water hyacinth nanoparticles with 140 mL, compared to the lower outputs of Trial 1 (70–80 mL) and Trial 2 (95–100 mL). The reduced COD value (608 mg/L) further indicated efficient substrate degradation and microbial utilization. Overall, Fe-biochar proved to be the most effective additive, highlighting its strong potential as a low-cost and efficient enhancement material for improving dark fermentation-based biohydrogen production from invasive water hyacinth biomass.

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