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The Effect of Thermal and Alkaline Pretreatment in POME (Palm Oil Mill Effluent) as a Substrate for Dark Fermentation Process on Biohydrogen Production

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ABSTRACT

Palm Oil Mill Effluent (POME) is a promising substrate for biohydrogen production through dark fermentation, although pretreatment is often required to enhance hydrogenogenic activity. This study evaluated the effects of alkaline (NaOH) and thermal pretreatments on POME characteristics and hydrogen production. Fresh POME was sieved and adjusted to initial pH values of 6, 7, or 8 using 6 M NaOH, with or without heat shock treatment (100 °C for 60 min). Batch fermentation was conducted at 60 °C for 24 h, while pH, oxidation-reduction potential (ORP), and dissolved oxygen (DO) were monitored. Pretreatment shifted POME conditions from acidic and oxidative to neutral-alkaline and reductive environments, with several treatments achieving ORP values below −200 mV, indicating favorable anaerobic conditions. Refrigerated storage reduced pH and increased ORP, whereas NaOH addition increased pH and lowered ORP. Hydrogen production varied among treatments. The highest yield was obtained from fresh POME adjusted to pH 7, producing 0.03655 g H_2 with an ORP of approximately −514 mV. In contrast, stored POME subjected to pH 7 adjustment and heat shock produced substantially lower hydrogen yields. In a second batch, pH 8 treatments generated 0.01157–0.02002 g H_2, representing 2.4–3.1 times higher production than the controls, while pH 6 treatments showed the lowest yields. Overall, neutral-to-alkaline pH and strongly negative ORP were associated with improved biohydrogen production.

1. INTRODUCTION

The increasing demand for sustainable and renewable energy sources has led to growing interest in hydrogen as a clean fuel, due to its high energy content and zero carbon emissions during combustion (Osman *et al*., 2023). Among the available biological processes, dark fermentation has emerged as a promising route for biohydrogen production because of its ability to convert a wide range of organic wastes into hydrogen under anaerobic conditions. One such waste with high potential is Palm Oil Mill Effluent (POME), a liquid by-product from palm oil processing that is abundantly generated in Southeast Asia, particularly in Indonesia and Malaysia (Cabrol *et al*., 2017). POME is rich in carbohydrates, proteins, and volatile solids, making it a suitable substrate for microbial hydrogen production through dark fermentation (Gupta *et al*., 2024).

In dark fermentation, the organic compounds in POME serve as carbon and energy sources for acidogenic bacteria, which metabolize them to produce hydrogen, volatile fatty acids (VFAs), and carbon dioxide. The hydrogen yield depends largely on the metabolic pathways involved. The acetate pathway theoretically yields 4 mol H_2 per mol glucose, while the butyrate pathway yields 2 mol H_2 per mol glucose (Cabrol *et al*., 2017). Experimental results, however, often

report yields in the range of 1–3 mol H_2/mol glucose, with the maximum reported value of ~4 mol H_2/mol glucose when acetate is the dominant by-product (Gupta *et al*., 2024). These values remain far below the theoretical maximum of 12 mol H_2/mol glucose predicted by stoichiometric conversion, underscoring the gap between theoretical and practical performance (Osman *et al*., 2023). This discrepancy is largely attributed to microbial competition, incomplete substrate utilization, and the diversion of electrons to reduced by-products such as butyrate and propionate rather than hydrogen (Gómez-Camacho *et al*., 2021).

Efforts to overcome these limitations have focused on optimizing fermentation conditions, reactor design, and microbial community structure. Among these strategies, pretreatment of substrates or inocula has been widely explored as an effective approach to enhance hydrogen production. Pretreatment methods can be classified into several categories: physical (heat shock, microwave, ultraviolet irradiation), mechanical (ultrasonication, high-shear mixing, filtration), chemical (acid, alkali, oxidative chemicals, enzymatic hydrolysis), and biological (substrate enrichment, inoculum manipulation, controlled aeration) (Bundhoo, 2015). These treatments aim to improve substrate biodegradability, increase the availability of fermentable sugars, and selectively enrich hydrogen-producing bacteria while suppressing hydrogen-consuming methanogens (Gómez-Camacho *et al*., 2021).

The principle underlying the effectiveness of pretreatment lies in the physiological differences between hydrogen-producing bacteria and methanogens. Clostridium species, as major hydrogen producers, are capable of forming endospores, allowing them to survive extreme conditions such as heat or pH fluctuations. In contrast, methanogens are non-sporulating and highly sensitive to stress conditions, making them more susceptible to pretreatment interventions (Barth *et al*., 2024). Consequently, heat shock pretreatment (HST) is commonly employed to suppress methanogens and enrich spore-forming hydrogen producers. Several studies have reported significant increases in hydrogen yield from POME after heat shock, with conditions around 80 °C for 15–30 minutes being optimal (Zainal *et al*., 2023).

In addition to heat shock, alkaline pretreatment has been recognized as a useful method to improve the hydrolysis of complex organic matter in POME. Alkali addition disrupts lignocellulosic structures, enhances solubilization of organic compounds, and increases the release of fermentable sugars (Primeia *et al*., 2025). When applied to POME, alkaline treatment has been shown to increase biodegradability and subsequently improve hydrogen yields in dark fermentation systems. Thus, combining thermal and chemical pretreatments has the potential to simultaneously enhance substrate availability and control microbial community composition, providing synergistic benefits for hydrogen production efficiency. Previous studies on POME pretreatment also show that thermal or hydrothermal conditioning can improve substrate conversion and biohydrogen performance when operational severity is optimized (Zainal *et al*., 2024).

Despite the promising results reported in the literature, the effectiveness of pretreatment strategies is strongly influenced by the characteristics of the substrate, including its organic content, inhibitory compounds, and microbial community composition. Therefore, careful evaluation of pretreatment methods is essential to ensure process feasibility and scalability. In the case of POME, thermal and alkaline pretreatments represent two practical approaches that can be feasibly applied in palm oil mill operations given the availability of heat and chemical resources. The applicability of pretreatment for POME is therefore highly dependent on the selected technique, treatment intensity, and the specific physicochemical characteristics of the effluent (Khadaroo *et al*., 2019).

Although alkaline and thermal pretreatments have been widely reported in dark fermentation studies, the response of POME remains substrate-specific because POME is a heterogeneous liquid-solid organic matrix containing residual oil, long-chain fatty acid fractions, suspended and colloidal organic matter, soluble carbohydrates and proteins, fine fibrous residues, and indigenous microbial communities. Therefore, the scientific significance of this study lies in evaluating how fresh and refrigerated POME respond to selected alkaline and thermal conditioning through changes in pH, ORP, DO, visual physicochemical characteristics, and hydrogen production. The pretreatment target in this study was not limited to a single component such as lignin or cellulose, but rather to the complex organic fractions of POME, especially residual oil/long-chain fatty acid fractions, colloidal organic matter, and fine fibrous residues that may influence hydrolysis, redox conditions, and hydrogenogenic activity. Specifically, this study aimed to: (1) evaluate the effects of NaOH-based pH adjustment and heat shock treatment on selected physicochemical indicators of POME, namely pH, ORP, and DO; (2) assess the corresponding hydrogen production during 24-hour thermophilic dark fermentation; and (3) provide a preliminary consideration of chemical and thermal inputs required for pretreatment relative to the observed increase in H_2 production.

2. MATERIALS AND METHODS

The substrate used in this study for the dark fermentation process was Palm Oil Mill Effluent (POME), collected from the wastewater outlet stream of PTPN VIII Cikasungka Palm Oil Mill, Bogor, West Java, Indonesia. The collected stream was no longer utilized in the crude palm oil (CPO) production line. The research was conducted using an exploratory experimental design. Batch dark fermentation experiments were carried out in modified Duran® reagent bottles (1,000 mL; DWK Life Sciences, Germany) that served as laboratory-scale bioreactor. Process monitoring was conducted using a hydrogen gas sensor module (ZE07-H_2; Zhengzhou Winsen Electronics Technology Co., Ltd., China), a pH meter (Extech pH-100; Extech Instruments/FLIR Systems, USA), an oxidation-reduction potential (ORP) meter (Lutron ORP-203; Lutron Electronic Enterprise Co., Ltd., Taiwan), and a dissolved oxygen (DO) meter (Lutron DO-5509, Taiwan), along with the required calibration kits and supporting measurement instrument.

2.1. Pretreatment of POME and Bioreactor Control

Raw Palm Oil Mill Effluent (POME) was sieved through a 10-mesh screen to separate the liquid fraction from empty fruit bunch (EFB) fibers and other coarse solids. Dark fermentation with untreated Raw POME served as the control, against which the effects of pretreatments were compared. The untreated POME, with an initial pH of approximately 4.2, was directly introduced into the bioreactors without further treatment.

For each batch fermentation run, 500 mL of POME was introduced into each 1,000 mL modified Duran® bottle, leaving approximately 500 mL headspace for gas accumulation and sampling. For the pretreatment experiments, POME was adjusted to different initial pH levels (6, 7, and 8) by adding 6 M NaOH solution dropwise while continuously mixing until the target pH was reached. In addition, a combined pretreatment was applied, consisting of pH neutralization to 7.0 followed by heat shock treatment (HST) at 100 °C for 60 minutes. The experimental runs were conducted as exploratory single-reactor tests for each treatment condition, with untreated POME used as the corresponding control within the same sample batch. Because different POME batches and storage durations were involved, the results were interpreted descriptively rather than as a complete factorial design.

Following each experimental run, the substrate was replaced with either Raw POME or stored POME (kept at 4 °C in a refrigerator) for subsequent repetitions. To ensure anaerobic conditions, entrapped air inside the bioreactor was purged by flushing with pure N_2 gas for 1.5 minutes at a flow rate of 20 NL/min prior to fermentation.

Dark fermentation was conducted for 24 hours under thermophilic conditions, maintaining the reactor temperature at 60 °C. In this study, the focus was placed on the utilization of indigenous hydrogen-producing bacteria naturally present in POME, without additional microbial inoculation. Samples were collected every 2 hours throughout the fermentation process to monitor changes in POME characteristics, particularly pH, dissolved oxygen (DO), and oxidation–reduction potential (ORP).

The experimental procedure followed this sequence: POME collection → 10-mesh sieving → fresh use or refrigerated storage at 4 °C → pH adjustment to 6, 7, or 8 using 6 M NaOH and/or HST at 100 °C for 60 min → reactor loading → N_2 purging → 24-hour thermophilic dark fermentation at 60 °C → periodic measurement of pH, DO, ORP, and H_2 concentration → calculation of cumulative H_2 production. This workflow was added as the experimental scheme to clarify the whole process and improve reproducibility.

2.2. Measurement and Analysis of H_2 Production

The hydrogen gas produced during dark fermentation was measured using a ZE07-H_2 hydrogen gas sensor module (Zhengzhou Winsen Electronics Technology Co., Ltd., China) connected to the reactor via a diaphragm gas pump and tubing system. Measurements were performed every 2 hours over the 24-hour fermentation period, with each measurement lasting for 3 minutes. Gas sampling was carried out through the outlet port located on the top of the bioreactor cap, where the pump operated at a flow rate of 0.4 L/min to draw headspace gas from the reactor. The extracted gas was directed through the sensor before being released into the atmosphere. The output from the sensor consisted of hydrogen concentration values, expressed in parts per million (ppm), representing the fraction of hydrogen in the total sampled gas over the specified measurement time. The cumulative hydrogen production obtained from dark fermentation was subsequently converted into mass units using Equation (1).

$$\sum m_g = [H_2] \times M_r \times V_m \times Q \times t \quad (1)$$

where: $\sum m_g$ is total hydrogen production (g), $[H_2]$ is hydrogen gas concentration (ppm), *Mr* is molecular weight of hydrogen gas (2.016 g/mol), V_m is molar volume of an ideal gas (22.4 L/mol, at STP), *Q* is pump flow rate (L/min), and *t* is sampling time (min).

Dimensional consistency of Equation (1) was checked by converting the hydrogen concentration from ppm into a volume fraction ($[H_2]\times10^{-6}$). The gas volume (L) passing through the sensor during each measurement is $Q \times t$, so the estimated hydrogen gas volume (L) is ($[H_2]\times10^{-6}$)(Q)(t). Dividing this volume by *Vm* (L/mol) gives the amount of hydrogen in mol, and multiplying by *Mr* (g/mol) gives the final hydrogen mass in gram. Therefore, the equation yields total hydrogen production in gram.

2.3. Statistical and Interpretative Approach

A two-way ANOVA was considered; however, inferential statistical analysis was not applied because the present study was conducted as an exploratory experiment involving limited replication, different POME batches, and different storage durations. Applying a factorial ANOVA under these conditions could overstate the statistical validity of the treatment effects. Therefore, the data were analyzed descriptively by comparing trends in pH, ORP, DO, and hydrogen production across treatment and control conditions. Consequently, claims in the Results and Discussion were revised to emphasize association and treatment-dependent trends rather than statistical causality.

3. RESULTS AND DISCUSSION

3.1. Initial Characteristics of POME as Substrate

A detailed compositional analysis of the initial POME substrate, including lignin, cellulose, hemicellulose, and individual sugars such as glucose, arabinose, fructose, xylose, and sucrose, was not conducted in the present exploratory study. Therefore, the discussion of pretreatment targets was limited to the observed physicochemical indicators (pH, ORP, DO, viscosity/color changes) and the general POME matrix reported in the literature. This limitation has been acknowledged, and future work should include direct compositional analysis to distinguish the contribution of lipid-derived fractions, soluble sugars, and fine lignocellulosic residues to hydrogen production.

The POME samples used in this study were collected during the October–November milling season, when fresh fruit bunches (FFB) were processed into crude palm oil (CPO). Sampling was performed at the wastewater outlet channel, ensuring that the substrate was fresh and had not undergone sedimentation. Fresh POME consisted of a mixture of residual oil, sludge, and water. Two different sample batches were collected, leading to variations in initial physicochemical properties. The first batch was used for Tests 1–3, while the second batch was used for Tests 4–5. Fresh samples (Tests 1 and 4) were processed immediately, whereas Tests 2, 3, and 5 used POME stored at 4 °C to suppress microbial degradation. Control runs with untreated POME were performed in parallel to assess the effects of pretreatments. Initial values of pH, oxidation–reduction potential (ORP), and dissolved oxygen (DO) are shown in Table 1.

Storage altered the physicochemical properties of POME despite refrigeration. A decrease in pH was observed across controls, from 4.60 (Test 1) to 4.49 (Test 2, 11 days) and 4.48 (Test 3, 22 days). Similarly, the second batch decreased from 4.28 (fresh, Test 4) to 3.89 (Test 5, 6 days). These reductions suggest residual fermentation and organic acid formation continued during storage, even at 4 °C, although microbial activity was minimized by dormancy (Vongvichiankul *et al*., 2017).

Simultaneously, ORP values shifted toward more positive (oxidative) states, from –409 mV (Test 1) to –100 mV (Test 2) and –1 mV (Test 3). A similar pattern occurred in the second batch (–266 mV to –122 mV). This can be attributed to increased proton (H^+) concentration at lower pH, which promotes electron release, as well as elevated dissolved oxygen acting as an electron acceptor. Liu *et al*. (2016) defined ORP > –50 mV as indicative of the aerobic processes, whereas anaerobic fermentation requires ORP < –200 mV. These shifts explain why hydrogen yields decreased with longer storage in the first batch.

Table 1. Initial values of pH, ORP and DO POME parameters

Test	Storage Duration	pH	ORP (mV)	DO (mg/L)	Type of Pre-treatment
1	Fresh	7.00	-514	0.5	pH-7
		4.60	-409	0.5	control
		7.00	-330	0.5	pH-7 & Heat Shock
2	11 days	7.00	-247	1.0	pH-7
		4.49	-100	1.3	control
		7.00	-267	0.3	pH-7 & Heat Shock
3	22 days	7.00	-19	0.5	pH-7
		4.48	-1	0.6	control
		7.00	-154	0.1	pH-7 & Heat Shock
4	Fresh	6.00	-269	0.3	pH-6
		4.28	-266	0.4	control
		8.00	-369	0.2	pH-8
5	6 days	6.00	-278	0.3	pH-6
		3.89	-122	0.4	control
		8.00	-395	0.2	pH-8

The addition of 6 M NaOH to POME shifted the medium from acidic conditions toward neutral-to-alkaline conditions (pH 6-8), and this adjustment was accompanied by lower ORP values (Figure 1). This pH-ORP relationship should be interpreted cautiously because ORP is not controlled by pH alone. ORP reflects the combined influence of dissolved oxygen, active redox couples, substrate composition, and microbial metabolic activity. In this context, NaOH addition likely changed the acid-base condition of the medium and affected pH-dependent redox equilibria, while the decrease in DO and the development of anaerobic metabolism may also have contributed to the lower ORP values. Therefore, the observed reduction in ORP is better interpreted as a treatment-associated redox shift rather than a direct electron-donating effect of hydroxide ions.

ORP values are widely used as operational indicators in anaerobic bioreactors. In general, ORP below approximately -200 mV indicates conditions favorable for strict anaerobic metabolism, while higher or less negative ORP values may indicate oxygen intrusion, lower reducing activity, or changes in the dominant redox reactions. Previous studies have identified ORP around -284 +/- 33 mV as favorable during the acidogenesis phase for hydrogen formation (Vongvichiankul *et al*., 2017). In the present study, the fresh POME adjusted to pH 7 or 8 showed relatively negative ORP values and higher hydrogen production than several control conditions. However, this pattern is interpreted as an association within the present dataset, not as proof of direct causation. Recent reviews further emphasize that redox

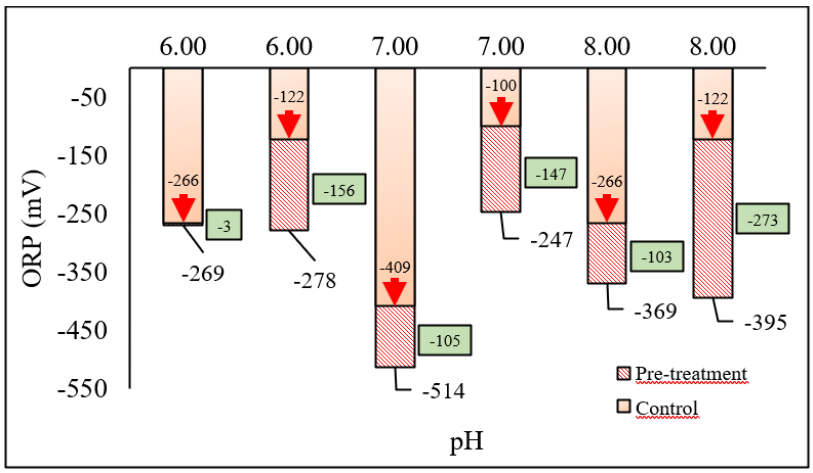

Figure 1. Effect of NaOH-based pH adjustment on pH and ORP. The green box indicates the selected pH adjustment condition; pH values are placed above the corresponding condition to improve readability.

potential influences hydrogenase activity, metabolic flux distribution, and the dominance of hydrogen-producing bacteria during dark fermentation (Sim *et al*., 2023).

In contrast, POME samples that underwent storage exhibited ORP shifts toward more positive or less reductive values. This transition was associated with decreased pH and, in some cases, increased dissolved oxygen, suggesting that acid accumulation, oxygen diffusion during handling/storage, and reduced anaerobic microbial activity may all have contributed to the observed redox changes. Such oxidative drift may reduce the suitability of POME for hydrogenogenic fermentation because obligate anaerobic hydrogen-producing bacteria generally require a sufficiently reductive environment. As noted by Hongo *et al*. (1972), aerobic bacteria initiate growth at positive ORP values, while strict anaerobes generally require more negative ORP conditions.

3.2. Alkaline and Thermal Pretreatment Effects on POME

Alkaline pretreatment with 6 M NaOH may improve the accessibility of complex organic matter contained in POME by altering colloidal and suspended organic fractions. However, because detailed compositional analysis was not conducted, the interpretation should not be limited to lignocellulosic degradation alone. The likely pretreatment targets include residual oil and long-chain fatty acid fractions, soluble and colloidal organics, and fine fibrous residues that passed through the 10-mesh screening step. When combined with thermal treatment (100 °C), the pretreatment may have promoted solubilization of organic matter; nevertheless, this mechanism requires confirmation through direct measurements such as soluble COD, carbohydrate, protein, lipid, and VFA analyses.

In this study, alkaline (NaOH 6 M) and thermal pretreatment were associated with visible changes in the substrate, including darkening and increased apparent viscosity. These observations suggest possible physicochemical transformations, but they should be interpreted as hypotheses because viscosity, salinity, phenolics, and melanoidin compounds were not directly quantified.

One possible transformation during alkaline treatment is saponification, in which triglycerides and residual oil fractions in POME may react under alkaline conditions to form glycerol and fatty acid salts. This mechanism is plausible because raw POME may contain residual oil and lipid-derived compounds; however, the present study did not directly measure triglyceride, glycerol, or long-chain fatty acid concentrations. Therefore, saponification is discussed as a possible mechanism rather than a confirmed reaction pathway in this experiment.

$$CH_2COOR_1 - CHCOOR_2 - CH_2COOR_3 + 3\ NaOH \rightarrow 3\ RCOONa + C_3H_8O_3 \quad (2)$$

If saponification occurred, the formation of glycerol and fatty acid salts could have affected the physical properties of the substrate and potentially influenced hydrolysis and mass transfer. However, because glycerol and viscosity were not quantitatively measured, the observed increase in thickness should be treated as a qualitative indication that requires further verification.

The strongest visual thickening was observed at pH 8, where the highest NaOH dosage was applied. This observation suggests a possible trade-off between alkaline conditioning and changes in substrate physical properties. Nevertheless, direct viscosity and salinity measurements are needed before concluding that mass-transfer limitation or osmotic stress occurred.

Alongside glycerol accumulation, fatty acids released by hydrolysis underwent neutralization with NaOH, forming ionic salts such as sodium oleate and sodium palmitate (Equation 3):

$$C_{16}H_{32}O_2 + NaOH \rightarrow C_{16}H_{31}O_2Na + H_2O \quad (3)$$

The accumulation of sodium salts may influence microbial activity at elevated concentrations by increasing osmotic pressure and affecting enzyme function. However, since sodium concentration and salinity were not measured in this study, this interpretation is presented as a possible explanation supported by previous literature rather than direct evidence from the present experiment (Kim *et al*., 2009; Lee *et al*., 2012). This consideration is important because inhibition in dark fermentative hydrogen production may arise from sodium-related osmotic stress, LCFA accumulation, altered enzyme activity, and shifts in volatile fatty acid pathways (Chen *et al*., 2021).

Moreover, increased salinity has been linked to reductions in the C/N ratio of the substrate, a critical parameter in maintaining balanced microbial metabolism. Low C/N ratios limit nitrogen assimilation and can alter metabolic flux, further reducing hydrogen yield (Lee *et al*., 2019). In extreme cases, these shifts may promote the accumulation of inhibitory metabolites, compounding the negative effects of salinity on hydrogen production.

Taken together, these observations suggest a potential dual challenge: alkaline pretreatment may improve substrate conditioning and redox suitability, but excessive alkaline dosage may also alter physical and ionic properties of the medium. Further measurements of soluble COD, VFA profile, viscosity, sodium concentration, phenolics, and microbial community structure are required to verify these mechanisms and determine the optimum dosage.

Visual observations indicated that POME underwent a distinct color transformation from dark brown to nearly black as NaOH was added and the medium shifted toward alkaline conditions (Figure 2). This darkening trend has also been reported by Chou *et al*. (2010), who observed similar results under combined pretreatments at pH 13 with heat shock at 80 °C. Several possible mechanisms may contribute to this phenomenon, including changes in suspended solids, partial solubilization of organic matter, release of phenolic-like compounds, or non-enzymatic browning reactions. However, these mechanisms were not directly measured in the present study.

Another possible explanation is the formation of melanoidin-like polymers through non-enzymatic Maillard-type reactions between sugars and amino compounds under alkaline and thermal conditions. Because melanoidin and phenolic compounds were not quantified, this interpretation should be viewed as a hypothesis that may help explain the observed color change rather than definitive evidence of inhibitory compound formation.

The intensity of such color-forming reactions is generally influenced by pH, temperature, substrate composition, and reaction time. In this study, the observed darkening at higher alkaline dosage indicates that organic restructuring may have occurred. Future studies should quantitatively assess phenolics, melanoidin-like compounds, soluble COD, and inhibitory thresholds to verify whether these changes affect hydrogen-producing microbial communities.

pH 4.28 pH 6.00 pH 8.00

Figure 2. Colour change in POME due to pH

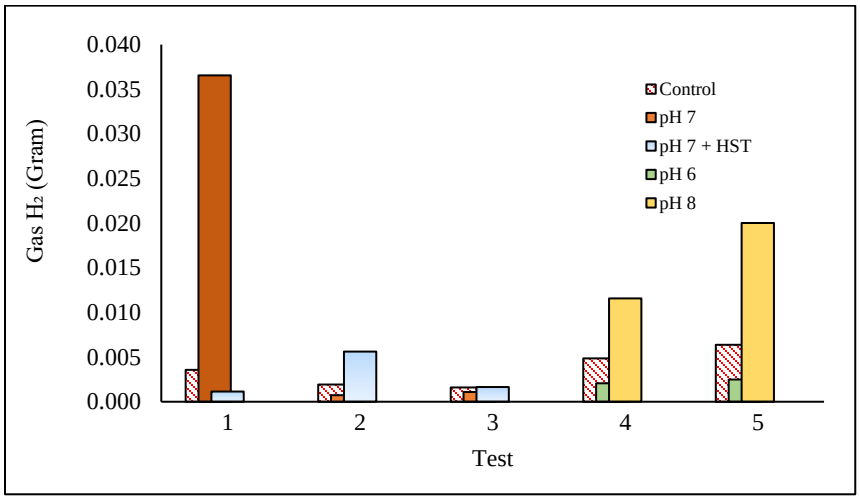

Figure 3. Total biohydrogen gas production over 24 h

3.3. Effect of Pretreatment on Biohydrogen Production

The results of the 24-hour dark fermentation process presented in Figure 3 show that the pretreatment conditions were associated with different levels of biohydrogen production. In Test 1 (fresh POME, pH 7), the ORP reached -514 mV, which was below the anaerobic threshold of -200 mV (Liu *et al*., 2016). Under this condition, the highest hydrogen yield was observed (0.03655 g), representing a ~10.2-fold increase over the control (0.00358 g). Because the present experiment was exploratory and limited in replication, this result is interpreted as a descriptive treatment-associated trend rather than a statistically confirmed effect. Similar studies have reported that reductive conditions during acidogenesis may support hydrogen production (Vongvichiankul *et al*., 2017).

In contrast, the combination of pH 7 and HST yielded 0.00562 g (Test 2) and 0.00165 g (Test 3). Relative increases over their respective controls were modest (2.9x and 1.0x), suggesting limited and inconsistent benefit under the present storage and treatment conditions. Test 2 (11 days storage, pH 7) exhibited a less negative ORP (-247 mV), while Test 3 (22 days storage, pH 7) shifted further to -19 mV. Although the pH was adjusted to 7, prolonged storage may have shifted the medium toward less favorable redox conditions due to residual fermentation, oxygen diffusion during handling/storage, and reduced anaerobic activity. The lower hydrogen production under HST should not be interpreted as direct evidence of damage to hydrogen-producing bacteria because microbial viability and community structure were not measured. Rather, the result indicates that the applied HST condition (100 °C, 60 min) did not consistently improve hydrogen production in this dataset. Milder HST conditions reported in the literature may therefore be worth evaluating in future work (Cabrol *et al*., 2017; Zainal *et al*., 2023).

For the second batch of samples, alkaline pretreatment at pH 8 yielded ORP values of -369 mV (Test 4, fresh) and -395 mV (Test 5, 6 days storage). These values were within a reductive range and were accompanied by higher hydrogen production (0.01157 g and 0.02002 g, respectively) than the corresponding controls (0.00486 g and 0.00638 g). Conversely, pretreatment at pH 6 gave ORP values around -269 mV (Test 4) and -278 mV (Test 5), with lower hydrogen production of 0.00207 g and 0.00249 g. These results suggest that neutral-to-alkaline pretreatment, particularly pH 7-8, may be more favorable than pH 6 under the tested conditions. However, without VFA profiles and microbial data, the dominance of acetate, butyrate, or propionate pathways cannot be confirmed directly. The control samples without pretreatment showed higher or less negative ORP values, which were generally associated with lower hydrogen production, indicating a correlation between redox condition and hydrogen output within the present dataset. Recent POME-based studies also indicate that initial pH strongly affects hydrogen generation, with pH regulation influencing substrate conversion and process performance in dark fermentation systems (Syaichurrozi *et al*., 2025).

Overall, the present dataset indicates an association between initial pH, ORP, and hydrogen production: more reductive ORP values tended to coincide with higher hydrogen output when the pH was maintained in the neutral-to-alkaline range. Nevertheless, ORP should be treated as an operational indicator rather than a sole causal factor, because it is influenced by dissolved oxygen, microbial activity, substrate composition, and redox-active compounds. These findings support the importance of jointly monitoring pH and ORP during pretreatment and startup phases, while further replicated experiments, VFA analysis, and microbial characterization are needed to confirm the underlying mechanisms.

3.4. Preliminary Pretreatment Input Consideration

From a process feasibility perspective, the observed increase in hydrogen production should be interpreted together with the additional chemical and thermal inputs required for pretreatment. In alkaline pretreatment, the main input is the NaOH solution used to adjust POME to the target pH. The chemical input can be estimated from $nNaOH = M \times V$, where nNaOH is the amount of NaOH added (mol), M is NaOH molarity (mol/L), and V is the NaOH solution volume (L). In thermal pretreatment, the main input is the heat required to increase substrate temperature, which can be approximated as $Q = m \times Cp \times \Delta T$, where Q is heat input (kJ), m is the mass of POME (kg), Cp is the specific heat capacity of the liquid substrate ($kJ \cdot kg^{-1} \cdot °C^{-1}$), and ΔT is the temperature increase (°C).

In the present laboratory-scale experiment, alkaline pretreatment, particularly pH 8 in the second batch, increased hydrogen production compared with the corresponding controls. However, a full cost-benefit or net energy analysis was not conducted because heat recovery, chemical cost, scale-up efficiency, and net usable energy from H_2 were not measured. Therefore, the results should be interpreted as preliminary evidence of process response rather than complete

process optimization. Future work should evaluate whether the incremental H_2 yield is sufficient to offset NaOH consumption and thermal energy input under realistic palm oil mill conditions.

4. CONCLUSIONS

The physicochemical characteristics of POME used as substrate were influenced by storage and pretreatment, as reflected by changes in pH, oxidation-reduction potential (ORP), and dissolved oxygen (DO). During storage at 4 °C, pH tended to decrease and ORP shifted toward less reductive values, indicating conditions that may be less favorable for hydrogenogenic fermentation. In contrast, NaOH addition increased the pH and was associated with more negative ORP values. Alkaline and thermal treatments also produced visible changes, including darkening and increased apparent viscosity; however, the related mechanisms, such as saponification, salt formation, or melanoidin-like compound formation, should be regarded as possible explanations because they were not directly quantified in this study.

Hydrogen production varied across the tested treatment conditions. In the first batch, the highest total H_2 was obtained at pH 7 with fresh POME (0.03655 g, Test 1). The combination of pH 7 + HST yielded 0.00562 g (Test 2) and 0.00165 g (Test 3), indicating that the applied HST condition did not consistently improve hydrogen production under the tested storage conditions. In the second batch, alkaline pretreatment at pH 8 produced higher hydrogen values than the corresponding controls, namely 0.01157 g (Test 4) and 0.02002 g (Test 5). Within the limited experimental dataset, pH 8 showed a favorable response, but this condition should not yet be generalized as the most robust strategy without further replication, statistical testing, microbial analysis, and energy/cost evaluation.

AUTHOR CONTRIBUTION STATEMENT

Author	C	M	So	Va	Fo	I	R	D	O	E	Vi	Su	P	Fu
ACF	✓	✓				✓		✓		✓	✓	✓		
BSP	✓	✓			✓				✓	✓	✓			✓
NWAU			✓	✓			✓			✓			✓	✓

C: Conceptualization
M: Methodology
So: Software
Va: Validation
Fo: Formal Analysis
I: Investigation
D: Data Curation
R: Resources
O: Writing - Original Draft
E: Writing - Review & Editing
Vi: Visualization
Su: Supervision
Fu: Funding Acquisition
P: Project Administration

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