Hydroxyapatite Fabrication for Enhancing Biohydrogen Production from Glucose Dark Fermentation

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ABSTRACT: Hydroxyapatite (HA) had the effect of maintaining the pH balance of the reaction system and promoting enzyme activity. In this work, hydroxyapatite was synthesized by coprecipitation and characterized for biohydrogen (bioH2) production from glucose. The highest bioH2 yield obtained was 182.33 ± 2.41 mL/g glucose, amended with an optimal dosage of 400 mg/L HA, which was a 55.80% higher bioH2 yield compared with the control group without any addition. The results indicated that HA facilitated the deterioration of organic substances and increased the concentration of soluble microbial products (SMPs). Microbial community analysis revealed that HA significantly increased the abundance of Firmicutes from 35.27% (0 mg/L, HA) to 76.41% (400 mg/L, HA), which played an essential role in bioH2 generation. In particular, the abundance of Clostridium sensu stricto 1 increased from 15.33% (0 mg/L HA) to 45.17% (400 mg/L HA) and became the dominant bacteria. The results also indicated that HA likely improves bioH2 production from organic wastewater in practice.

1. INTRODUCTION

Energy is the basis for society’s progress and mankind’s existence and is mainly derived from fossil fuels.1 The limitations of fossil energy sources and the environmental problems associated with fossil energy combustion are driving an increasing interest in the use of biofuels such as biogas (bioH2) and ethanol (EtOH) as alternative energy sources for transportation.2 Furthermore, bioH2 has a higher energy mass content compared to other fuels and can be produced from renewable substrates such as straw, waste sludge, and high concentrations of chemical oxygen demand (COD).3 Various approaches have been used to fabricate bioH2, including photoderived and dark fermentations. Dark fermentation is considered an efficient process and provides an attractive and environmentally friendly method for bioH2 production from renewable sources.4 BioH2 generation has been achieved through different routes involving obligate and facultative anaerobes. Dedicated anaerobic bacteria (e.g., Clostridium and Bacteroidetes) produce volatile fatty acids (VFAs) and bioH2 through glucose degradation. However, dark fermentation is kinetically and thermodynamically limited. Although fermentation provides rapid bioH2 production, accumulation of VFAs and high concentrations of byproducts such as EtOH during fermentation can inhibit bioH2 production, resulting in a lower rate of organic matter conversion to H2.5

To resolve the above issues, many techniques have been proposed, including optimization of process conditions (e.g., pH, organic load, and temperature) and various additives, including activated carbon (AC), biochar (BC), iron(III) oxide (Fe3O4), and magnetite, which help buffer the effects of VFA accumulation.6 Furthermore, the presence of trace elements

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[e.g., Fe$^{2+}$, Ni$^{2+}$, Fe$^{3+}$, and nanoparticles (NPs)] can bring about changes in enzymatic activity and promote the production of extracellular polymers (EPS), thus maintaining sludge granulation. Calcium ions (Ca$^{2+}$) are mediators that promote EPS production and influence bioH$_2$ production by facilitating electron transfer and altering EPS composition. Alkaline CaO$_2$ increases the activity of relevant enzymes and facilitates the release of organic matter from waste sludge. As EPS dissolves, the large amounts of enzymes contained in EPS are also released into the liquid phase, increasing the opportunity for contact with the substrate. The interaction of divalent cations (e.g., Ca$^{2+}$) with negatively charged material on EPS leads to the release of key enzymes, resulting in the formation of EPS substrates to promote hydrogen production. The presence of CO$_3$$^-$$^-$ alkalinity mitigates inhibition of the acidic environment by oxygen-containing groups. Furthermore, the hydroxide ion (OH$^-$) can disrupt the breakdown of refractory organic matter and release suitable substrates for biofuel production.

In addition, hydroxyapatite (HA) NPs free of byproducts, hazardous substances, and impurities were prepared by a coprecipitation method. The synthesized HA NPs with high crystallinity have a high specific surface area and low ion release. The HA obtained after drying and heat treatment can remove water vapor, NH$_3$, and CO$_2$ from the precipitate, thus giving hydroxyapatite better biological activity, bioactivity, and biostability. The Ca$^{2+}$ obtained by dissociation in alkaline solution can be used as the calcium source of HA.

Additionally, the Ca$^{2+}$ released from HA reacts with soluble phosphorus (PO$_4^{3-}$) to form a Ca$_6$(PO$_4$)$_2$ precipitate; importantly, this enables the reuse of fertilizer from digestate. HA has the potential to be used for dark fermentation, and its importance, this enables the reuse of fertilizer from digestate.

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In addition, hydroxyapatite has a hexagonal structure comprising a phosphate tetrahedron and a Ca$^{2+}$ site surrounded by OH$^-$$^-$$^-$. As a basic metal oxide rich in oxygen groups, it is rarely used in anaerobic dark fermentation. Moreover, HA can be produced from wastes such as phosphogypsum and shellfish, and its crystal structure unit is usually represented as Ca$_{10}$(PO$_4$)$_6$(OH)$_2$. As a new environmentally functional substance, HA has received much attention owing to its biocompatibility. It is often used as an adsorbent to remove heavy metals and dye wastewater from polluted bodies of water.

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Nonetheless, whether the Ca$^{2+}$, PO$_4^{3-}$, and OH$^-$ in HA can alter EPS and microbial communities needs to be further investigated to understand the mechanism of dark fermentation. Therefore, studies on the properties of HA in dark fermentation for bioH$_2$ production, effects of bioH$_2$ production, and the relationship between HA and microbial communities need further investigation. The aims of this study were (1) to prepare and characterize HA; (2) to study how HA dosage impacts bioH$_2$ production during dark fermentation; (3) to determine the metabolic pathways and changes in soluble microbial products (SMPs) amended with HA; (4) to explore the results of HA addition on microbial flora in accordance with the phylum, genus, and heat map; and (5) to assess the effects of HA on EPS using the excitation–emission matrix (EEM).

2. MATERIAls AND METHODS

2.1. Fabrication of HA and Characteristics of Inoculated Sludge. Aqueous Ca(NO$_3$)$_2$·4H$_2$O (100 mL, 1 M) and (NH$_4$)$_2$HPO$_4$ (100 mL, 0.6 M) were prepared separately according to stoichiometry and were slowly mixed by magnetic stirring. The resulting mixture was stirred vigorously for an additional 30 min, and the pH was maintained at 11. The reaction mixture was then dried at 90 °C and stirred for 3 h. After the reaction was completed and cooled, the solution was strained and washed with water and anhydrous EtOH to neutral, and the filtered sample was dried overnight at 80 °C. The sample was then ground and sintered at 10 °C/min and 900 °C for 3 h. Subsequently, the sintered sample was ground and sieved to obtain HA.

The original seed sludge was derived from an up-flow anaerobic sludge bioreactor (UASB) for handling citric acid sewage. To access the dominant fermentation microorganisms, the gathered anaerobic sludge was mixed using glucose (0.5 g) and then cultured for 30 days at 37 °C. Before conducting the bioH$_2$ generation experiments, the sludge was presoaked at 95 °C for 90 min to restrain the growth of methanogens and to concentrate hydrogen-producing bacteria (HPB). Subsequently, the heated sample was allowed to cool to approximately 37 °C and was incubated for 48 h. The inoculated sludge after the culture had the main parameters including total solids (TS), total organic carbon (TOC), and pH of 7.80 ± 1.67 wt %, 4612.00 ± 195.82 mg/L, and 7.0 ± 0.3, respectively.

One-way analysis of variance (ANOVA) was analyzed to test for significant statistical variations ($p < 0.05$) between the properties of various doses of HA using Origin 2021. All tests were conducted in triplicate.

2.2. Design for Batch Fermentation. BioH$_2$ production batch experiments were conducted in serum vials with a working volume of 500 mL. Seven gradient levels (20, 100, 200, 400, 800, 1200, and 1600 mg/L) of HA were used to study bioH$_2$ production, and the group without HA was used as a control. The glucose (10 g/L) and peptone (0.3 g/L) as feed were added in the reactors, and they were flushed using nitrogen for about 5 min to ensure an anaerobic environment. Prior to the experiments, the pH of the solution was adjusted to 7.0 ± 0.3.

The reactor was subjected to dark fermentation at 37 °C. The fermentation samples were collected every 6 h, and cumulative hydrogen production (CHP) was recorded once every 3 h. SMPs (VFAs and EtOH) were measured with a Shimadzu gas chromatograph along with a flame ionization detector and column (GC-2010). The samples and gases used were collected and sampled as described in a previous study. The hydrogen production rate (HPR) was also calculated based on the standard temperature and pressure (101.3 kPa, 25 °C). The H$_2$ yield was calculated according to the standard temperature and pressure with eq 1:

$$V_{\text{hydrogen}}(\text{mL, STP}) = V_{\text{hydrogen}}(\text{mL, T}) \times \frac{273}{273 + T} \times \frac{101325 - W}{101325}$$

where $T = 25$ °C, and $W$ is the H$_2$O vapor pressure at 25 °C (Pa).

2.3. Characteristics of HA. The crystal structure of HA was observed using X-ray diffraction (XRD, Bruker, AXS), and measurement curves were recorded at 10° and 80° (2θ). A Brunauer–Emmett–Teller analyzer (BET, Micromeritics, ASAP 2020) was used to assess the pore size, pore volume, and specific area of HA. The structure and crystalline form of
HA were observed by transmission electron microscopy (TEM, JEM 2100).

2.4. Collection of Liquid Samples and Analysis. BioH2 production, expressed in mL/g of glucose, was calculated as the volume of bioH2 produced per gram of glucose added to the reactor. The samples were passed through a 0.45 μm filtration membrane, and the concentrations of Ca and P in the filtered samples were assayed with inductively coupled plasma (ICP, Optima). The VFAs, pH, 16S rRNA, and EEM were tested in the same way. 16S rRNA gene sequencing was performed using Illumina MiSeq high-throughput sequencing technology for the reactors at the end of H2 production, and the sequences were resolved at the genus level to analyze HPB diversity. Further, the sludge with HA samples was processed and collected after fermentation. The interactions between HA and HPB were revealed by scanning electron microscopy (SEM, SU8010) and transmission electron microscopy (TEM, H-7650).

2.5. Significance Test, COD Mass Balance, And Kinetic Modeling. It is critical to assess process kinetics related to anaerobic fermentation reactor design, influencing factors, bioH2 production potential, and SMPs. Therefore, to determine whether the influence of different dosages of HA on bioH2 fermentation potential as well as SMPs was statistically significant, ANOVA was performed using Origin 2021 based on the bioH2 production and SMPs in dark fermentation. According to initial and final COD values as well as equivalent COD for the H2 produced (8 g COD/g H2), the COD mass balances were calculated, which were based on eq. 2.

\[
\text{COD mass balance} = \frac{\text{final COD} + \text{COD}_{\text{H2}}}{\text{initial COD}} \tag{2}
\]

\[
P(t) = P_m \exp\left(-\exp\left(\frac{R_m e^{\lambda(t-t)}}{P_n}\right) \right) + 1 \tag{3}
\]

where \(P_m\) is MHP (mL H2/g glucose), \(R_m\) is the maximum H2 production rate (mL H2/(g glucose h)), \(P(t)\) is CHP (mL H2/(g glucose h)) at time \(t\) (h), \(e = 2.718\), and \(\lambda\) is the lag time (h). Moreover, the \(\lambda\), \(P_m\), \(R_m\), and \(R^2\) were evaluated using Origin 2021.

3. RESULTS AND DISCUSSION

3.1. Fabrication of HA. The crystal architecture and surface area of HA are shown in the Supporting Information (Figures S1 and S2). The synthetic HA diffraction pattern was in agreement with that of standard HA (powder diffraction file; PDF, 09-0432). The peaks observed at 2θ angles of 25.8°, 28.1°, 29.0°, 31.7°, 32.2°, 32.8°, 34.0°, and 39.7° can be identified as the (002), (102), (210), (211), (112), (300), (202), and (310) reflections of the HA lattice. TEM images show that HA containing calcium and phosphorus was prepared.

3.2. BioH2 Yield Affected by HA. The bioH2 and VFAs can be generated by adding glucose to a mixed culture bioreactor. Figure 1 shows the effects of pretreatment on bioH2 production: (1) CHP for anaerobic dark fermentation...
Table 1. Kinetic Parameters of BioH₂ Yield from Dark Fermentation Modified with Hydroxyapatite (HA)

| HA (mg/L) | 0    | 20   | 100  | 200  | 400  | 800  | 1200 | 1600 |
|----------|------|------|------|------|------|------|------|------|
| \( P_\text{av} \) (mL/g) | 117.73 | 146.57 | 158.43 | 166.73 | 183.99 | 162.34 | 160.89 | 155.19 |
| \( R_\text{n} \) (mL/(g h)) | 16.46 | 14.02 | 14.57 | 15.59 | 16.92 | 14.93 | 15.39 | 15.41 |
| \( \lambda \) (h) | 6.03 | 6.12 | 5.98 | 6.4 | 6.33 | 6.33 | 6.48 | 6.24 |
| \( R^2 \) (%) | 99.68 | 99.87 | 99.84 | 99.84 | 99.84 | 99.82 | 99.81 | 99.77 |
| COD balance (%) | 87 ± 0.01 | 91 ± 0.02 | 91 ± 0.03 | 92 ± 0.03 | 92 ± 0.05 | 89 ± 0.07 | 87 ± 0.05 | 87 ± 0.06 |
| final pH | 5.05 ± 0.10 | 4.97 ± 0.13 | 5.10 ± 0.11 | 4.97 ± 0.20 | 4.93 ± 0.03 | 4.95 ± 0.10 | 4.88 ± 0.06 | 4.84 ± 0.01 |

reactions amended with HA and (2) HPR exhibited under the dark fermentation reaction with HA.

The bioH₂ yield was found to change with the addition of different doses of HA (Figure 1a,b). With the addition of seven gradients of HA, the average bioH₂ yields were all higher than those of the control reactor. BioH₂ production in the control without HA was 117.03 ± 2.31 mL/g glucose, and the HMP of 182.33 ± 2.41 mL/g glucose could be observed with the inclusion of 400 HA mg/L in the fermentation reaction, which was 55.80% greater than that of the control. The corresponding maximum HPR increased by 38.91%. Several studies have shown that the addition of metal oxides (e.g., CaO and CaO₂) can effectively increase bioH₂ productivity, and similar findings have been obtained with HA. This indicated an enhancement of the quantity and quality of short-chain fatty acid production from waste activated sludge using CaO₂ as an additive. HA can maintain the granulation of sludge. The optimal amount of metal ions could alter the morphology of the granular sludge and improve sludge activity, which in turn increases bioH₂. Our results indicated that the efficiency of bioH₂ generation was improved with the addition of HA (<400 mg/L). A similar observation was obtained in the fermentation of Ca²⁺ granular sludge for bioH₂ production. The affinity of HA for microorganisms allows a high enrichment of anaerobic microorganisms, which can provide better conditions for bioH₂ production. However, high concentrations of additives can lead to oxidative stress and cell membrane rupture. High doses of HA (>400 mg/L) can thus disrupt the granular sludge structure and create an unfavorable environment for bioH₂ operation. The CHP in all reaction groups with HA addition was higher than that in the control group but at different rates of bioH₂ production (Figure 1a). Bacterial cell surfaces and EPS are usually negatively charged. With the addition of HA, the Ca²⁺ in HA can connect to the negative charge on EPS, thus forming an EPS substrate and maintaining cellular morphology. The increased EPS secretion promotes the release of hydrogenase-producing enzymes and increases contact with substrates, thus increasing the production of BioH₂.

Ca²⁺ increases bacterial abundance by reducing electrostatic repulsion between negatively charged mixed culture bacteria (MCB). The results indicated that moderate amounts of HA increase the rate of bioH₂ production from dark fermentation, which could be boosted by enzyme activity, optimization of EPS, and microbial community structure.

3.3. Model-Based Kinetics and Metabolism for BioH₂ Generation. As shown in Figure 1a, the bioH₂ yield data from anaerobic fermentation were fitted using the modified Gompertz equation. In this study, a one-way ANOVA was performed on data such as H₂ yield under dark fermentation experiments by Origin 2021 to determine whether the effect of HA on dark fermentation was statistically significant. The \( p \)-values for all fermentation indicators could be obtained at less than 0.05, indicating that the addition of HA had a significant effect on H₂ yield and SMPs. The kinetic factors \( \lambda \), \( R_\text{av} \), \( P_\text{av} \), COD balance, and final pH values are shown in Table 1. The kinetics and analysis of variance can accurately reflect the HA dark fermentation H₂ production. With the increase in HA concentration, there is an adaptation phase for microorganisms. The highest \( P_\text{av} \) value fitted using the modified Gompertz model was 183.99 mL/g glucose. Its lag time range for fermentation was 5.98–6.48 h. However, there was an adaptation phase for the microorganisms as the HA concentration increased, and there was no significant change in the lag time of the dark fermentation bioH₂ production system. The reliability of these data was demonstrated by the fact that COD balance was maintained at 90% during dark fermentation. At the end of dark fermentation for bioH₂ production, the final pH dropped to 4.8–5.0 in all reactors. This small change in pH provided opportunities for bioH₂ production bacteria to acclimate to the new environment, had few suppressive effects on the microorganisms, and did not limit bioH₂ production in this study. The pH values of most production systems were reported to be relatively stable and close to the end pH.

3.4. Impact of HA on BioH₂ Fermentation Pathways. SMPs are the main components of bioH₂ production from anaerobically fermented organic matter and can be used to evaluate the degradation efficiency of SMPs. Dark fermentation for bioH₂ production has been conducted using glucose as a substrate, with EtOH, butyrate (HBut), acetate (HAc), and propionate (HPr) as the main products. Figure 1c depicts the variation in the yield of SMPs in the reactor, which correlates with the biological evolution of bioH₂. During dark fermentation, most of the glucose was decomposed, and the accumulation of SMPs varied. Addition of HA significantly promoted the release of soluble metabolites from anaerobically fermented organic matter (\( p < 0.05 \)) (Figure 1c,d). The SMP yield changed with increasing HA content. The optimal dose of HA was 400 mg/L, and the respective concentrations of EtOH, HBut, HAc, and HPr were 335.09 ± 5.16, 3474.72 ± 89.78, 2082.53 ± 76.65, and 207.80 ± 8.58 mg/L (Figure 1c). The concentration of SMPs was 6100.14 ± 109.02 mg/L with the addition of HA, which was 48.35% higher than that of the control.

The change in SMPs produced by dark fermentation in the presence of 400 mg/L HA is presented in Figure 1d. The degradation of glucose was consistent with the production of SMPs over a fermentation time of 6–30 h. Statistical analysis demonstrated \( p \)-values less than 0.05, suggesting that the bioH₂ generation reactor with added HA had a significant effect on SMPs, HAc, HPr, HBut, and EtOH concentrations. The results showed that anaerobic fermentation reactors with HA addition could improve the bioH₂ production rate of the dark fermentation process by providing HA to promote microbial growth and improve microbial activity. In this study, the HAc
and HBu were the dominant acids, suggesting that HAc and HBu production was associated with the dominant HPB groups, Firmicutes and Clostridium butyricum. As described in eqs 4 and 5, Clostridium butyricum was more adapted to bioH2 yield from HAc- and HBu-type fermentations.31

\[
\begin{align*}
\text{C}_6\text{H}_2\text{O}_6 + 2\text{H}_2\text{O} & \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2 \\
\Delta G_0 & = -206 \text{ KJ/mol} \\
\text{C}_6\text{H}_2\text{O}_6 & \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2 \\
\Delta G_0 & = -254 \text{ KJ/mol}
\end{align*}
\]

The reason is that the HBu-type pathway has lower Gibbs free energy and is thermodynamically more favorable for bioH2 yield by anaerobic colonies. The molar ratio of HAc to HBu (A/B) was strongly correlated with bioH2 yield from HAc- and HBu-type fermentations.31 The molar ratio of HAc to HBu (A/B) was strongly correlated with bioH2 yield from HAc- and HBu-type fermentations.31

When HA was enriched from 0 to 400 mg/L, A/B decreased from 0.66 to 0.60. Thus, HA addition could enhance the HBu fermentation pathway by increasing the activity and abundance of Clostridium butyricum. This phenomenon suggests that the amount of HA added was related to bioH2 yield. The pH at the end of dark fermentation changed with different doses of HA (Table 1). The pH in all reactors eventually recovered and stabilized in the range 4.8–5.1, which could allow anaerobic fermentation for bioH2 production.31

3.5. Effects of HA on the Morphological Characteristics on HPB. To further investigate the interaction of HA with HPB, the surface of anaerobic bacteria was characterized by SEM with energy spectra for elements such as Ca, P, and C (Figure S3). It was observed that carbon was the basic element of the organisms and was essentially located in the same place on the MCB. In the presence of HA, Ca and P were intensively localized on the bacterial surface in the scan area, and HA was inferred to be attached to the anaerobic bacteria. A previous report also showed that high concentrations of Ca increased sludge flocculation with the MHP.33

Further, TEM imaging was performed on samples treated with HA after fermentation to observe the interaction between HA and HPB in the dark fermentation system (Figure S4). It was observed that large aggregates of HA were detected on the cell surface.20,30 Some HA enters the interior of cells and causes damage to the EPS components.34 The EPS produced by MCB could trap most of the HA, thus interfering with material transfer and affecting electron transport.35

HA particles are trapped by EPS and form a membranelike substance that acts as a physical barrier to protect HA, preventing HA particles from attaching to the cell surface or moving into the cell membrane. However, some HA remains capable of perforating the EPS barrier and the cell surface and thus accessing the cell.5 An addition of 400 mg/L HA revealed

![Figure 2. Final concentrations of Ca and P from the dark fermentation system amended with hydroxyapatite (HA): (a) Ca and (b) P (percent releases of Ca and P have been labeled separately on the graph).](image)

![Figure 3. Variation of extracellular polymers (EPS) with hydroxyapatite (HA) dose: (a) 0 mg/L HA and (b) 400 mg/L HA.](image)
that most cells preserved their proper structure after intrusion, whereas the remainder exhibited destruction of their membranes and intracellular constituents. These characteristics showed variation in the tolerance of cells to HA particles, which affected the morphology of the cell surface and internal structure.

3.6. Effects of HA on the EPS. Figure 2 illustrates the variation of Ca and P elements in the dark fermentation system using HA. The ICP results revealed that the ion concentration increased with increasing HA concentration. With optimal HA (400 mg/L) addition, the concentrations of elements Ca and P reached 796.67 ± 5.77 and 349.67 ± 1.53 mg/L, respectively. The increase in ion concentration during dark fermentation was due to the increased dissolution of HA owing to weak acid conditions. Additionally, the Ca concentration was higher than the P concentration in all reactors. This indicates that the Ca²⁺ released from HA stimulates the activity of hydrolases and proteases.

The influence of HA on the EPS of MCB was evaluated using EEM spectrometry (Figure 3). EEM luminescence spectroscopy can capture specific fluorescence features to differentiate between fluorescent soluble and organic substances in the liquid stage. In this work, EEM was used to characterize extracellular polymers in sludge. Based on previous studies, the soluble organic matter liberated from the fermentation broth can be classified into five types depending on the emission excitation wavelengths (Table 2). In the fermentation reactor with HA addition, it was observed that the intensity of the fluorescence response of the experimental group with HA addition in zone IV was stronger than that in the control group, corresponding to the results of the SMP assay. The fluorescence intensity in the V region was lower than that of the control, and humic acid-like substances were reduced, accelerating their degradation. This may be because Ca²⁺ interacts with EPS to promote the release of hydrolases and proteases, facilitating the hydrolysis of polysaccharides and proteins. Further, the oxygen-rich groups in HA bind to EPS (−COOH, −OH, and −C=O) and weaken the inhibition of anaerobic fermentation. Thus, the presence of HA improved the biodegradability of organic matter and reduced the content of refractory organics. It improves the molecular structure, making the substrate more readily available for microbial uptake and providing a more biodegradable substrate for subsequent fermentation, thus facilitating dark fermentation for bioH₂ production. The remaining dissolved P could also be directly used without treatment as a phosphate fertilizer to promote plant growth.

3.7. Effects of HA on the Microbial Community. Microbial communities were profiled using high-throughput sequencing to reveal the mechanism by which HA improves bioH₂ fermentation (Figure 4). The results showed that HA addition changed the diversity and abundance of microbial communities. The Simpson diversity of samples with M1 (control, 0 mg/L HA) and M2 (400 mg/L HA) was 0.924 and 0.793, respectively. This result indicates that the addition of HA to dark fermentation reduced bacterial diversity. The bioH₂ producing phase is accomplished by faster dividing eubacteria, which can distinguish microbial abundance in a shorter period of time. The variation in community structure by phylum and genus is shown in Figure 4. The phylum distribution of MCB in the sludge samples for the control and test groups with 400 mg/L HA is also illustrated. In control trials, Firmicutes, Bacteroidetes, Chloroflexi, Patescibacteria, and Spirochaetae were the main bacterial

Table 2. Fluorescence Region Distribution of the EEM

| region | Em (nm) | Ex (nm) | substances               |
|--------|---------|---------|--------------------------|
| I      | 200–330 | 200–250 | tyrosine-like protein    |
| II     | 330–380 | 200–250 | tryptophan-like protein  |
| III    | 380–500 | 200–250 | fulvic-acid-like organics|
| IV     | 200–380 | 250–280 | soluble microbial byproducts |
| V      | 380–500 | 250–400 | humic-acid-like organics |

Figure 4. Evolution of the microbial consortium: (a) phylum and (b) genus. M1, 0 mg/L HA; M2, 400 mg/L HA.
phyla, accounting for 35.27%, 39.13%, 6.70%, 5.74%, and 3.49%, respectively, of the total bacterial sequences (Figure 4a). In contrast, in the experiment with 400 mg/L HA, the bacterial community structure comprised Firmicutes, Bacteroidetes, Chloroflexi including Chlorofluorocarbons, and Patescibacteria, accounting for 76.41%, 16.09%, 2.39%, 1.46%, and 0.53%, respectively. Firmicutes and Bacteroidetes remained the dominant bacteria, which is in agreement with previous research. Chloroflexi in the microbiological community during dark fermentation are thought to influence the previous community structure and suppress the development of Proteobacteria and Bacteroidetes. Firmicutes and Bacteroidetes, the main phylogenetic groups, indicated that these phyla have a significant influence on the transformation of organic substances. These results revealed that changes in the abundance of communities were mainly caused by changes in the above-mentioned microorganisms treated with HA. Addition of HA greatly increased the relative abundance of Firmicutes from 35.27% to 76.41%. The results showed that HA increased the enrichment of Firmicutes, which could boost the bioH₂ yield. Firmicutes can produce cellulases, proteases, and many other extracellular enzymes and are specifically associated with the degradation of organic substances, bioH₂ production, and the formation of acids. The results indicate that HPB could effectively decompose glucose and generate bioH₂. The reactor with HA facilitated the enrichment and growth of Firmicutes. In particular, the proportion of Clostridium sensu stricto 1 showed an increase from 15.33% to 45.17% (Figure 4b), which was the most significant change between M1 (control, 0 mg/L HA) and M2 (400 mg/L HA). Clostridium is a typical HPB that produces bioH₂ from diverse organic substrates such as hemicellulose, cellulose, starch, sucrose, and glucose. Under analogous pretreatment terms, Clostridium has also been found as the key species in other dark fermentations, such as in bioH₂ production from mixed cultures pretreated with radiation and acid. The structure of the microbial community evolved in bioH₂ generation revealed that Clostridium sensu stricto 1 was directly related to bioH₂ production. Bacteroidetes, Proteobacteria, and Chloroflexi species were present symbiotically in the community, whereas Firmicutes evolved independently and rarely interacted with other bacteria, degrading complex organic matter into small molecules. This partly facilitated its successful increase in dark fermentation for bioH₂ production, gradually gaining dominance in the microbial community.

Further, a higher proportion of bacterial were found to belong to Firmicutes, whose abundance accounts for 72%. In the present study, a high percentage of HBu was achieved by the addition of HA. Clostridium butyricum also increased significantly, as shown in Figure 5. In fact, Clostridium butyricum produced only HBu. Therefore, HBu as a product of Clostridium butyricum indicated that H₂ production is more adapted to HBu-type fermentation in the presence of HA. Therefore, Firmicutes, Clostridium sensu stricto 1, and Clostridium butyricum might be closely related to the production of HBu during dark fermentation. Therefore, enrichment of Firmicutes and Clostridium is probably the primary cause for enhanced bioH₂ production by HA addition.

Although HA could be employed to enable high bioH₂ yields, the probable mechanisms are mostly related to HA...
concentration and microbial diversity. Its possible mechanisms are as follows: (1) Ca and P elements enhance the activities of HPB and optimize the microbial community structure, and (2) HA enriches the dominant HPB with Firmicutes (76.41%) and Clostridium sensu stricto I (45.17%) at an optimal concentration of 400 mg/L, which enhances bioH2 production. Future efforts are required to assess the sustainability and feasibility of modified HA-mixed fermentation for bioH2 production from complex organic compounds.

4. CONCLUSION
The effects of different doses of HA on bioH2 were investigated using dark fermentation. The results showed that the highest bioH2 yield was obtained with 400 mg/L of HA addition, which was 55.80% higher than that of the control group. HA addition enhanced the bioH2 yield of dark fermentation and increased the abundance of HPB with Firmicutes and Clostridium sensu stricto I being dominant. HA contributes to HBu-type fermentation, maintains pH balance, and promotes enzyme activity. However, excess HA (>800 mg/L) reduced the bioH2 yield owing to the higher Ca2+ concentration released from HA.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00059.
Additional figures including hydroxyapatite material characterization and interaction of added hydroxyapatite with fermented sludge (PDF)

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Notes
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ABBREVIATIONS
HA, hydroxyapatite; bioH2, biohydrogen; H2, hydrogen; SMPs, soluble microbial products; EtOH, ethanol; COD, chemical oxygen demand; VFs, volatile fatty acids; AC, activated carbon; BC, biochar; Fe2O3, iron(III) oxide; NPs, nanoparticles; EPS, extracellular polymers; Ca2+, calcium ions; –OH, hydroxyl; PO4 3−, phosphorus; SMPs, soluble microbial products; EEM, excitation–emission matrix; UASB, up-flow anaerobic sludge bioreactor; HPB, hydrogen-producing bacteria; TS, total solids; TOC, total organic carbon; ANOVA, analysis of variance; XRD, X-ray diffraction; BET, Brunauer–Emmett–Teller; TEM, transmission electron microscopy; ICP, inductively coupled plasma; SEM, scanning electron microscopy; TEM, transmission electron microscopy; MHP/MHY, maximum hydrogen production; CHP, cumulative hydrogen production; HPR, hydrogen production rate; MCB, mixed culture bacteria; HAc, acetic; HPr, propionate; HBu, butyrate

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