Effect of Graphene Oxide and Biochar as Redox Mediators Addition on Photo-fermentation Biohydrogen Production

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The addition of graphene oxide promotes electron transfer between microorganisms in a photo-fermentative biohydrogen production system, while biochar improves the efficiency of hydrogen production by buffering the pH. In order to improve the efficiency of biohydrogen production, the effects of two redox mediators (ROMs), biochar and graphene oxide, at different concentrations on photo-fermentation biohydrogen production were studied. The results showed that the addition of graphene oxide and biochar decreased the redox potential (ORP) of the system. The lowest ORP was -286 mV (graphene oxide) and -290 mV (biochar), which represent that the reducing power of fermentation broth increased. When the addition of graphene oxide was 150 mg/L, the cumulative biohydrogen production reached the maximum of 404 mL, which was 46.3% higher than that of the control group without graphene oxide; When biochar was added at 1 g/L, the cumulative biohydrogen production reached the maximum of 383 mL, which was 45.9% higher than that of the control group. At the same time, the cumulative biohydrogen production was fitted by Gompertz equation, indicating that the kinetic parameters were very suitable to describe the effect of the addition of graphene oxide and biochar on the biohydrogen production from corn stalks by photo-fermentation.

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INTRODUCTION

The world's energy is dominated by non-renewable energy such as oil, coal, and natural gas, which causes environmental problems such as the greenhouse effect and energy depletion (Liu *et al*. 2023). The substitution of non-renewable energy sources is imperative. Biohydrogen energy is a renewable energy with great development potential because of its clean, pollution-free nature, along with its high calorific value (Sadeq *et al*. 2024). The common ways of hydrogen production include water electrolysis, hydrogen production from fossil energy, and biohydrogen production. The cost of hydrogen production by water electrolysis is high, which has limited its large-scale production, and hydrogen production from fossil energy gives rise to serious environmental pollution problems (Chai *et al.* 2021). By contrast, biohydrogen production has been widely studied because of its low energy consumption, low greenhouse gas emissions, and easy access to raw material (Zhang *et al.* 2024). Photo-fermentative biohydrogen production, as one of the main types of biohydrogen production, is a process in which photo-fermentative bacteria (*e.g.*, purple non-sulfur bacteria, green sulfur bacteria, *etc*.) convert organic matter into biohydrogen under light conditions, relying on the catalytic action of nitrogen-fixing enzymes or hydrogenases. Compared with dark-fermentation biohydrogen production, photo-fermentation biohydrogen production had stronger practicability due to the fact that all kinds of agricultural waste and organic wastewater can be used as fermentation substrates (Akhlaghi and Najafpour-Darzi 2020). Moreover, it is possible to improve the efficiency of biohydrogen production by adjusting light intensity, substrate concentration, pH value, and adding catalyst (Zhang *et al.* 2022). The titanium dioxide photocatalysts added in biohydrogen production, also increased the growth of bacteria, and the utilization of acidic by-products by bacteria were accelerated, and which increased the amount of biohydrogen production (Tahir *et al.* 2022).

Redox mediators (ROMs) function as electron shuttles, facilitating electron transfer in redox reactions. Biochar as a kind of ROM has attracted the attention of many scientists because its aromatic and quinone structures produced redox activity, Biochar's quinone functional group acts as an electron shuttles whose reduction potential matches that of the photo-fermentative bacteria, thus facilitating transmembrane electron transfer (Dai *et al.* 2016). Biochar as a kind of ROM has attracted the attention of many scientists because its aromatic and quinone structures produced redox activity (Dai *et al.* 2016). Biochar is a carbon-rich material processed from lignocellulosic biomass, animal manure, or municipal solid waste (Qian *et al.* 2015). The addition of biochar to anaerobic digestion of municipal solid waste improves hydrogen production with a maximum hydrogen production of 906.4 ± 10.1 mL, while the addition of biochar prevents ammonia inhibition (Sharma and Melkania 2017). During anaerobic methanogenesis of propionate and butyrate, the addition of biochar promotes biofilm formation and alleviates acid inhibition; specifically, the biomethane productivity increases by 16 to 25% (Zhao *et al.* 2016). Trace minerals contained in the biochar could be used as nutrients to replace expensive additives; the metallic constituents in biochar (Ca, Mg, Fe, Mn, and Zn) serve as essential micronutrients that stimulate microbial growth and metabolic activity, thereby enhancing hydrogenase/nitrogenase-mediated biohydrogen production in photo-fermentative systems (Sun *et al.* 2020). They play a pH buffering role to reduce the acid inhibition in the microbial fermentation system (Sunyoto *et al.* 2016). In a simulated two-stage anaerobic digestion of carbohydrates, biochar addition increased the maximum yield of biohydrogen by 31% a methane by 41.6% (Sunyoto *et al.* 2016).

Graphene oxide has strong redox ability to promote direct and indirect electron transfer between organisms (Vemuri *et al.* 2022). As ROMs, graphene oxide has unique physical and chemical properties, including high conductivity, large surface area, good mechanical strength and special biological antibacterial properties, which allows graphene oxide to be used in the field of biotechnology (Nguyen *et al.* 2017). Biomethane (1.0 g/L) has been produced by adding graphene oxide to anaerobic digestion, which promotes the generation of biomethane to 695.0 ± 9.1 mL/g; that is, the generation rate of biomethane was found to increase by 25.0% (Lin *et al.* 2017). The addition of graphene oxide promotes direct interspecies electron transfer between the microorganism and the substrate during the bioreaction, with less electron loss (Lin *et al.* 2017). The addition of graphene oxide stimulates biohydrogen production but does not affect the metabolic pathway of bacteria (Vemuri *et al.* 2022). Using colloidal wastewater as fermentation substrate, adding magnetite and graphene oxide nanocomposites into the fermentation, the biohydrogen production increases by 42% (Mostafa *et al.* 2016).

The research on ROMs of biochar and graphite oxide has focused on anaerobic digestion for methane and dark-fermentation for biohydrogen production, while the effect of ROMs on photo-fermentation biohydrogen production has not been reported. This study investigated the effect of ROMs on photo-fermentation biohydrogen production based on the biocatalytic function and electron transfer function of redox mediators.

EXPERIMENTAL

Materials

Mixed photo-fermentative bacteria HAU-M1 were cultivated by Henan Agricultural University, consisting of *Rhodospirillum rubrum* (27%), *Rhodopseudomonas palustris* (28%), *Rhodobacter capsulatus* (25%), *Rhodobacter sphaeroides* (9%), and *Rhodobacter capsulatus* (11%) (Zhang *et al.* 2020b). Photo-fermentative bacteria growth medium consisted of 0.5 g/L NH_4Cl, 1 g/L $NaHCO_3$, 0.5 g/L yeast extract, 0.1 g/L K_2HPO_4, 2 g/L CH_3COONa, 0.1 g/L $MgSO_4$, and 1 g/L NaCl (Zhang *et al.* 2020b). Biohydrogen production medium of photo-fermentative bacteria consisted of 0.4 g/L NH_4Cl, 0.2 g/L $MgCl_2$, 0.1 g/L yeast extract, 0.5 g/L KH_2PO_4, 2 g/L NaCl, and 3.56 g/L sodium glutamate (Zhang *et al.* 2020b).

The corn stalks were taken from Zhengzhou, Henan Province. They were washed with water, dried in an oven (Beijing Zhongxi yuanda Technology Co., Ltd., China) for 24 h, and crushed in a crusher (Hanbo Mechanical and Electrical Co., Ltd., China). After sieving with a 60-mesh sieve, the corn stalks were stored in a sealed bag. Corn stover biochar (Henan Dongtai Carbon Materials Co., Ltd., China) was used and sieved through a 60-mesh sieve. The particle size of graphene oxide (Kaisa New Materials Co., Ltd., China) was 85-mesh. The elemental analysis of graphene oxide was 42.73% C, 51.59% O, 2.51% H, and 2.17% S. Corn stover biochar was produced by slow pyrolysis under anaerobic conditions. The collected corn stover biochar was cooled to room temperature, pulverized and filtered through a 60-mesh sieve. The major elements were 51.49% C, 3.23% H, 1.72% N, and 1.1% S (Zhu *et al* 2021).

Methods

A 150 mL conical flask was used as a reaction vessel. Corn stalks (5 g) and citric acid-sodium citrate buffer (0.05 M, pH = 4.8) of 100 mL were added to the reaction vessel. Then ROMs were weighed with a balance (Jiangyin Precision Balance Instrument Co., Ltd., China), and were placed in the conical flask. The addition amount of graphene oxide ROMs was set to 50, 150, 250, 350, and 450 mg/L, respectively, while biochar was added at 1, 2, 3, 4, 5 g/L, respectively. For photo-fermentation fermentation to produce biohydrogen, 2 mL of cellulase (Novozymes Biotechnology, Denmark) and 45 mL of HAU-M1 mixed photo-fermentative bacteria in logarithmic phase (volume fraction of 30%) were put into the reaction vessels, with biohydrogen producing medium. The pH of the solution was adjusted to 7.0 with NaOH or H_2SO_4 (3 mol/L). Finally, the conical flasks were sealed with rubber stoppers and placed in a constant temperature incubator (Jiangsu Kexi Instrument Co., Ltd., China) at 30 °C and incandescent lamps with light intensity of 3000 Lx. The experimental group without the addition of graphene oxide and biochar was set as the control group. Three parallel experiments were set up for each experimental group. The produced biohydrogen was collected by gas collection bags and sampled every 12 h for determination.

The biohydrogen content was determined by gas chromatography (Agilent, 6820 GC-14B, USA). Using nitrogen as the carrier gas and 99.99% high-purity hydrogen as the standard gas, the analysis was performed on a 5Å molecular sieve packed column. The column temperature, injection port temperature, and detector temperature were maintained at 80, 100, and 150 °C, respectively. The pH and redox potential (ORP) of the fermentation broth was determined by a pH meter equipped with Mettler Toledo LE 438 probes (PHS-3C, Shanghai, China) and a portable redox potentiometer (Sanxin, SX712, China), respectively.

The modified Gompertz model was used as an analytical tool (Lu *et al.* 2020). The Gompertz equation is suited for photo-fermentation biohydrogen production as a function of fermentation time, allowing for a more intuitive analysis of data. The model was used to fit the change of cumulative biohydrogen production in the process of photo-fermentation biological biohydrogen production. The improved Gompertz model can be formulated as follows,

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

where $P(t)$, P_m, Rm, λ, t, and e represent the cumulative biohydrogen production, the final total biohydrogen production, the maximum biohydrogen production rate, the biohydrogen production delay period, the fermentation time, and the constant 2.718, respectively.

RESULTS AND DISCUSSION

Effect of Graphene Oxide and Biochar as Redox Mediators on Biohydrogen Production by Photo-Fermentation

Figure 1 shows the effect of graphene oxide and biochar as redox mediators on the cumulative hydrogen production and hydrogen production rate of photo-fermentation. It can be seen from Fig. 1(a) that the cumulative biohydrogen production increased first and then decreased with the increase of graphene oxide addition. When the addition of graphene oxide was 150 mg/L, the cumulative biohydrogen production reached the maximum of 404 mL, which was 46.3% higher than that of 276 mL without graphene oxide. This was attributed to the fact that the addition of graphene oxide promoted the direct interspecies electron transfer between biohydrogen-producing microorganisms and substrates, which was favorable for maintaining the biohydrogen production activity of the photo-fermentative bacteria, thus promoting the increase of cumulative biohydrogen production (Huang *et al*. 2022). When the addition of graphene oxide was 250 mg/L, the increase of the addition of graphene oxide still promoted the biohydrogen production by photo-fermentation, but the cumulative biohydrogen production was lower than the fermentation system with the addition of 150 mg/L. The further increase of graphene oxide addition (350 and 450 mg/L) had a negative impact on photo-fermentation biohydrogen production, and which showed the cumulative biohydrogen production were much lower than that of the control group. That was to say, graphene oxide had a saturation inhibition effect on the electron transfer between photo-fermentation biohydrogen-producing bacteria and fermentation substrates, and too much graphene oxide was not conducive to biohydrogen production (Yang *et al.* 2024). Figure 1(b) shows that when the addition amount of biochar was 1 g/L, the cumulative biohydrogen production by photo-fermentation was 383 mL, which was 45.6% higher than that of control group (263 mL).

With the increase of biochar addition (2 to 4 g/L), the cumulative biohydrogen production of photo-fermentation biohydrogen production decreased gradually, but all were higher than control group. When the addition of biochar was 5 g/L, the cumulative biohydrogen production was significantly less than control group. The reason was that the excessive accumulation of biochar in the biohydrogen production container blocked light projection, resulting in the reduction of light energy conversion efficiency that photo-fermentative bacteria used (Zhang *et al.* 2020a). The cumulative biohydrogen production of 150 mg/L graphene oxide was 404 mL while the cumulative biohydrogen production was only 383 mL with the addition of 1 g/L biochar. Therefore, the appropriate addition of graphene oxide and biochar could increase the cumulative biohydrogen production of photo-fermentation, and the effect of graphene oxide was more significant than that of biochar.

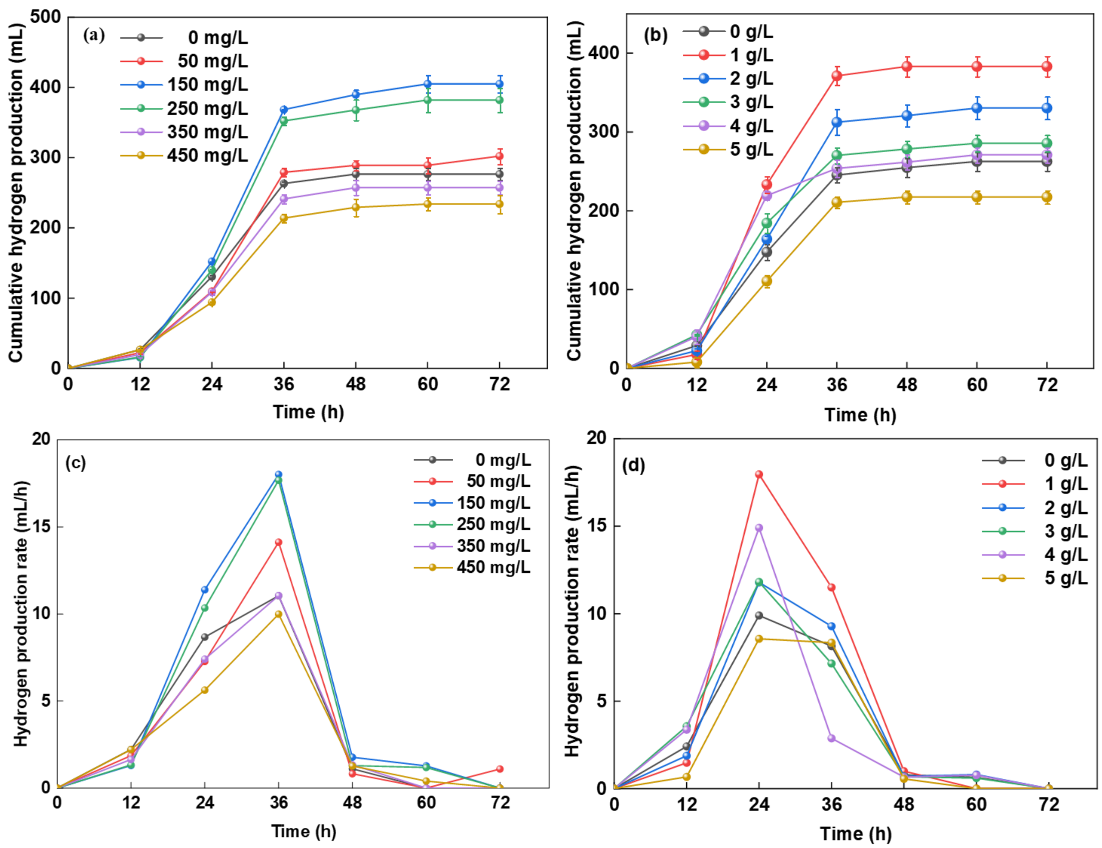

Fig. 1. Effect of graphene oxide and biochar as redox mediators on photo-fermentation biohydrogen production, (a) cumulative biohydrogen production with graphene oxide addition; (b) cumulative biohydrogen production with biochar addition; (c) biohydrogen production rate of graphene oxide addition; (d) biohydrogen production rate of biochar addition

Biohydrogen production rate is an important index to evaluate the performance of biological biohydrogen production. The effects of graphene oxide and biochar on the biohydrogen production rate of photo-fermentation are shown in Fig. 1(c, d). In Fig. 1(c), the biohydrogen production rate of 150 mg/L and 250 mg/L graphene oxide was rapidly increased to 11.3 mL/h and 10.3 mL/h within the beginning 24 h, which was 31% and 19% higher than the control group, respectively. When the biohydrogen production reaction was carried out for 36 h, the biohydrogen production rate of all groups appeared to peak. Figure 1(d) shows the biohydrogen production rate with 1 g/L biochar reached the maximum of

17.96 mL/h at 24 h, which was 82% higher than the control group (the rate of 9.9 mL/h). The biohydrogen production rate with 4 g/L biochar was also higher than control group, and it decreased sharply at 36 h, so the overall biohydrogen production was lower. Moreover, biohydrogen production rate of biochar addition with 5 g/L was the lowest. The process of biological hydrogen production showed that the rate of biological hydrogen production decreased sharply after 36 h of adding biochar, which may be due to the basic exhaustion of organic matter decomposed by microorganisms, or the inactivation or even death of photosynthetic bacteria due to the low pH value. This is basically consistent with Wang's study on microorganisms (Wang *et al.* 2011). That was to say, the appropriate addition of graphene oxide and biochar ROMs tended to improve the biohydrogen production rate of photo-fermentative bacteria.

Effect of Graphene Oxide and Biochar as Redox Mediators on ORP of Photo-Fermentation Biohydrogen Production

The effect of graphene oxide and biochar as redox mediators addition on the ORP of the photo-fermentation biohydrogen production are shown in Fig. 2. The ORP of fermentation broth was often used as an important parameter in biochemical reactions, reflecting the level of free electrons that gained or lost in water or solution. The lower the ORP, the greater the reduction capacity. The ORP value of the fermentation broth was negative, which indicated that the fermentation broth had reducing ability and could promote biohydrogen production. On the contrary, the oxidation was stronger. The ORP of the fermentation broth showed a trend of first decreasing and then increasing with increase of graphene oxide and biochar ROMs. In Fig. 2(a), when the addition of graphene oxide was 250 mg/L and the reaction time was 12 h, and the lowest ORP was -274 mV. Compared with the control, the ORP values of biohydrogen production systems with graphene oxide were lower, which was attributed to the stronger direct interspecies electron transfer between microorganisms and corn stalks (Vemuri *et al.* 2022). Within 24 to 72 h, the ORP of the biohydrogen production system all increased, which was due to the gradual decrease of the reducing power. The reduction capacity of the fermentation broth was consistent with the cumulative biohydrogen production Fig. 1(a).

Figure 2(b) shows that the ORP of each biohydrogen production system was low at 12 to 24 h, and the ORP values of the experimental groups with biochar addition were significantly lower than that of the control group. The ORP of the biohydrogen system increased from 36 to 60 hours due to the hydrogen production depleting the reducing power of the hydrogen system, resulting in an increase in ORP, whereas the ORP of the biohydrogen system with the addition of biochar showed a slower decreasing trend compared to the control. The results showed that the appropriate addition of graphene oxide and biochar could create a strong reducing environment for photo-fermentation, obtain a lower ORP, enhance the ability of H^+ to obtain electrons, and improve the biohydrogen production rate (Yang *et al.* 2024).

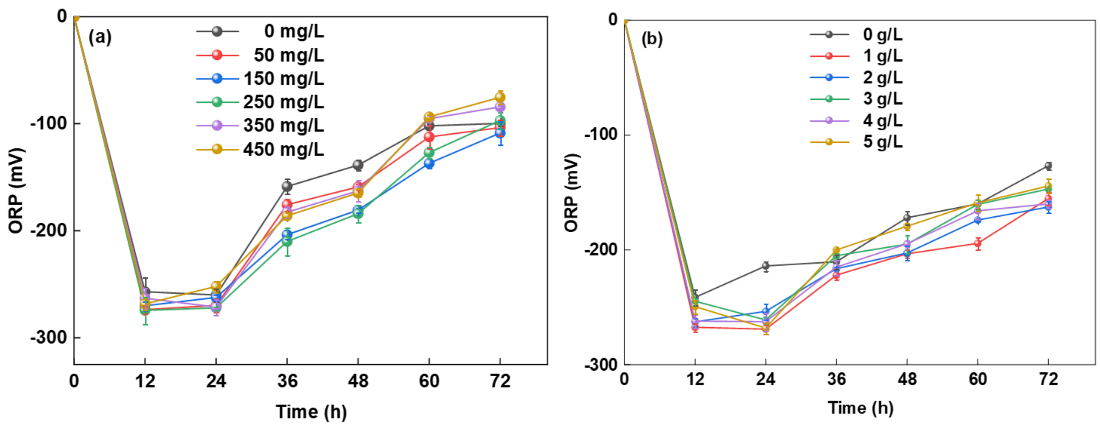

Fig. 2. Effect of graphene oxide and biochar as redox mediators on ORP, (a) graphene oxide; (b) biochar

Effect of Graphene Oxide and Biochar as Redox Mediators on pH of Photo-Fermentation Biohydrogen Production

Figure 3 shows the effect of ROMs addition on the pH change in the biohydrogen production process by photo-fermentation. It can be seen from Fig. 3(a) that the pH value of the fermentation broth of the biohydrogen production system with the addition of graphene oxide decreased greatly from 7 to less than 5.9 before 12 h. Meanwhile, the pH of the biohydrogen production system with the addition of biochar at 12 h were maintained at about 6.0 Fig. 3(b), which was slightly higher than that of the graphene oxide addition. The pH of the fermentation broth with the addition of 150 mg/L graphene oxide decreased the most to 5.92 (Fig. 3a). In the initial stage of photo-fermentation biological biohydrogen production, photo-fermentative bacteria converted reducing sugars from corn stalks hydrolysis into biohydrogen, acetic acid and butyric acid, which resulted in the decrease of pH due to the accumulation of acidic by-products of biohydrogen production (Guo *et al.* 2020). With the progress of biohydrogen production, part of acidic by-products was consumed by photo-fermentative bacteria metabolism and converted into biohydrogen (Guo *et al.* 2020), which led to the increase of pH (Fig. 3). At 36 to 60 h of photo-fermentation biohydrogen production, the pH value of the biohydrogen production system with biochar addition showed an obvious increasing trend, and was higher than that of the control group Fig. 3(b), which may also be the buffering effect of biochar on the pH value of the fermentation solution (Sunyoto *et al.* 2016).

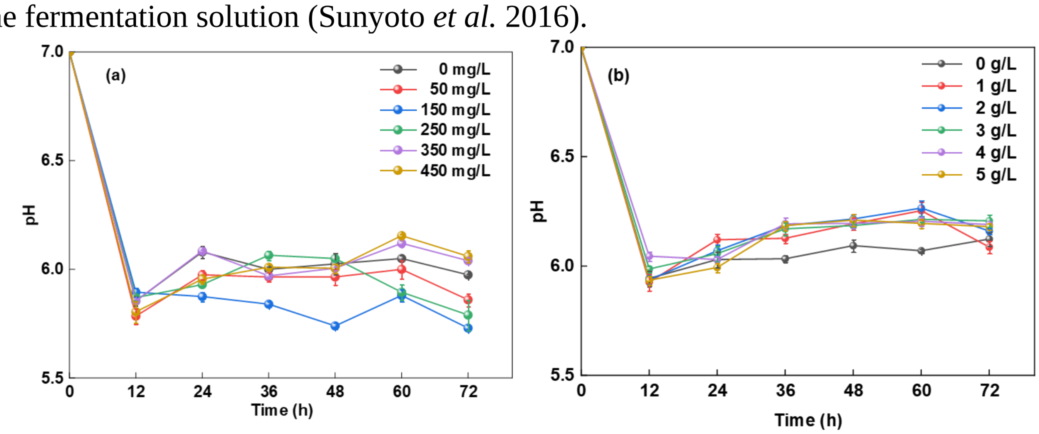

Fig. 3. Effect of graphene oxide and biochar as redox mediators addition on pH, (a) graphene oxide; (b) biochar

Kinetic Analysis of Photo-fermentation Biohydrogen Production by Adding ROMs

To clarify the biohydrogen production mechanism of photo-fermentative bacteria, the experimental data of photo-fermentation biohydrogen production with addition of ROMs was fitted with the Gompertz model. Table 1 shows the change of kinetic parameters of photo-fermentation biohydrogen production with the addition of graphene oxide and biochar. The maximum potential cumulative biohydrogen production of the experimental group (addition of graphene oxide) and the control group was almost the same as the actual cumulative biohydrogen production. The delay time (λ) of biohydrogen production of the experimental group (19.83 h, 18.77 h, 19.31 h, 18.66 h, 18.28 h) were longer than that of the control group (13.88 h), indicating that the addition of graphene oxide put of the delay time of photo-fermentation biohydrogen production. The R_m value represents the maximum biohydrogen production rate of photo-fermentation calculated by kinetic simulation. The maximum hydrogen production rate calculated in the kinetic fitted R_m values was higher than experimental data.

Table 1. Kinetic Parameters of Photo-Fermentation Biohydrogen Production

Concentration (g/L)	P_m (mL)	R_m (mL/h)	λ (h)	R^2
Graphene oxide				
0	282.69	11.28	13.88	0.99162
0.05	293.89	17.75	19.83	0.99449
0.15	401.65	25.23	18.77	0.99836
0.25	378.47	25.01	19.31	0.99809
0.35	257.76	14.42	18.66	0.99295
0.45	233.40	12.28	18.28	0.98914
Biochar				
0	265.22	10.08	11.92	0.99626
1	387.02	20.37	11.51	0.99906
2	333.01	12.18	12.68	0.99569
3	287.28	11.55	9.40	0.99893
4	266.46	16.18	9.98	0.99867
5	217.62	12.50	18.54	0.99894

For biohydrogen production with biochar added, the maximum potential cumulative biohydrogen production of 387 mL was reached with 1 g/L of biochar, and the potential capacity decreased with the increase of biochar concentration. Biohydrogen production rate calculated by the model for the control group (10.08 mL/h) was basically consistent with experimental result, while the calculated biohydrogen production rates of the biochar addition were different with the actual biohydrogen production rate. But the coefficients of determination (R^2) were greater than 0. 99, which indicated that Gompertz equation could well describe biological fermentation with biochar addition.

Comparative Analysis of the Effect of Additives on Photo-Fermentation Biohydrogen Production

Table 2 shows some comparisons between graphene oxide studied in this paper and some other additives studied in other papers. According to Table 2, the additives for promoting photo-fermentation biohydrogen production were mainly some metals, metal ions, metal compounds, and biomass materials. Additives such as $CaTiO_3$, $BaTiO_3$, and $MgTiO_3$ was respectively put in photo-fermentation biohydrogen production with 8, 7, and

3 g/L, and the cumulative biohydrogen production increased by 32.8%, 24.1%, and 48.3%, respectively (Yang *et al.* 2023). Basalt fiber as a low-cost additive was added to biohydrogen production by photo-fermentation, when the addition of basalt fiber was 1.5 g/L, the cumulative biohydrogen yield reached 323 mL, which represents an increase by 15.74%. Defoamer and Fe^{2+} addition also showed enhancement of biohydrogen production by photo-fermentation. Compared with above studies ($CaTiO_3$, $BaTiO_3$, $MgTiO_3$, and basalt fiber addition), the addition of graphene oxide and biochar also showed very good performance, and in which the cumulative hydrogen production was as high as 404 mL with 150 mg/L graphene oxide addition, and compared with biochar, the graphene oxide additions were much lower, which promoted the hydrogen production even better.

Table 2. Comparison between Results in this Study and Other Additive Papers

Additive	Addition Amount	Volume (mL)	Cumulative Biohydrogen Production (mL)	Biohydrogen Production Rate (mL/h)	References
Graphene oxide	150 mg	150	404	11.13	This study
Biochar	1 g	150	330	17.96	This study
$CaTiO_3$	8 g	150	310	19.61	Yang *et al.* (2023)
$BaTiO_3$	7 g	150	299	12.35	Yang *et al.* (2023)
$MgTiO_3$	3 g	150	347	15.20	Yang *et al.* (2023)
Defoamer	0.125 mL/L	200	359	19.67	Wang *et al.* (2022)
Fe^{2+}	2500μ mol/L	200	351		Lu *et al.* (2022)
Basalt fiber	1.5 g/L	180	323		He *et al.* (2021)

Future Research Directions

In the future, the authors' work may focus on studying a series of biochar and graphene oxide products, as well as the impact of graphene oxide and biochar with different chemical compositions and preparation procedures on the effect of photo-fermentation biological hydrogen production.

CONCLUSIONS

1. Both graphene oxide of 150 mg/L and biochar of 1 g/L significantly enhanced the cumulative biohydrogen production of photo-fermentation, and the biohydrogen production of graphene oxide as additive was higher than that of biochar. The cumulative biohydrogen production reached the maximum of 404 mL with graphene oxide of 150 mg/L, which was 46.3% higher than that of the control group.
2. The addition of graphene oxide and biochar reduced the redox potential of photo-fermentation biohydrogen production, and the biochar showed a buffer capacity for the pH of the fermentation broth. At the same time, the Gompertz equation described photo-fermentation biohydrogen production by adding graphene oxide and biochar was well.

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