The Impact of Fe^{2+} and Na^{+} Concentrations on Hydrogen Production with Three Different Fermenter Bacteria

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Batch experiments were conducted to investigate the effects of Fe^{2+} and Na^{+} on the hydrogen (H_2) production performance from three different metabolic type hydrogen-producing bacterial strains. The appropriate amount of Fe^{2+} significantly promoted the H_2 production of all three hydrogen-producing bacteria. The combination of H_2 production and liquid products showed that Fe^{2+} was more suitable for the H_2 production and metabolism of *E. harbinense* ZGX4. When the Fe^{2+} concentration was 0.05 g/L, the H_2 production and liquid products concentrations were 2170 mL/L-medium and 6530 mg/L, respectively. Na^{+} enhanced the H_2 production of *E. harbinense* ZGX4 and *C. butyricum* 1.209 but inhibited the H_2 production of *E. cloacae* 1.2022. Na^{+} made *C. butyricum* 1.209 exhibit the best H_2 production and metabolic performance when the Na^{+} concentration was 2 g/L, while the H_2 production, and liquid products concentration were 2460 mL/L-medium and 5350 mg/L, respectively. At the end of the experiment, it was found that the addition of Fe^{2+} could change the type of fermentation in *C. butyricum* 1.209. Therefore, further exploration of the effects of other metal ions on model hydrogen-producing strains has great potential for achieving high hydrogen production rates, among other things.

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INTRODUCTION

Biological hydrogen (H_2) production by fermentation is a key technology for H_2 production due to its advantages of relatively high H_2 production capacity, abundant raw material resources, low carbon footprint, and environmental friendliness (Yin and Wang 2017; Łukajtis *et al*. 2018; Zhang *et al*. 2021a). Hydrogen-producing microorganisms in the fermentation system are the main actors in obtaining hydrogen, the target product. This is achieved by converting organic substrates into H_2 through the catalytic activity of two key enzymes hydrogenase and nitrogenase (Akhlaghi and Najafpour-Darzi 2020; Baeyens *et al*. 2020; Sivaramakrishnan *et al*. 2021). Research in this field focuses on how to effectively utilize hydrogen-producing fermentation bacteria and thereby improve the activity of hydrogen-producing enzymes to increase the efficiency and capacity of the reaction system.

Microorganisms are affected by various factors during hydrogen-producing fermentation, such as substrate, pH, temperature, and metal ions, among which metal ions

are the key factors affecting microbial H_2 production (Infantes *et al*. 2011; Tandon *et al*. 2018). The type and concentration of metal ions affect the growth of hydrogen-producing bacteria and the structure and function of hydrogen-producing enzymes, which affect the efficiency of H_2 production. Hydrogenase is a type of protein containing iron, nickel, and other metal elements that can catalyze the reversible oxidation of H_2. By adding metal ions as cofactors to the fermentation medium, the catalytic activity of hydrogenase can be effectively enhanced to improve the H_2 production capacity of microbial fermentation and the H_2 production rate of the reaction system (Bao *et al*. 2013; Srivastava *et al*. 2019). For example, Ni^{2+} and Fe^{2+} are cofactors of enzymes and necessary factors for H_2 synthesis (Baeyens *et al*. 2020). Zhao *et al*. (2017) investigated the effect of Fe^{2+} and Mg^{2+} on the H_2 production of *Ethanoligenens harbinense* and obtained the maximum H_2 production by adding 600 mg/L $MgCl_2{\cdot}6H_2O$ and 100 mg/L $FeSO_4{\cdot}7H_2O$ in the optimal medium. Sekoai and Daramola (2018) evaluated the effect of different concentrations of Fe^{2+}, Mg^{2+}, Ca^{2+}, and Ni^{2+} on H_2 production by fermentation using potato waste as a substrate, and 293 $mLH_2/g{\cdot}TVS$ (total volatile solids) was obtained. In addition, Mg^{2+}, Ca^{2+}, and Ni^{2+} also affect H_2 production (Sekoai and Daramola 2018). Boni *et al*. (2014) investigated the effect of ferrous ion concentration on H_2 production in organic waste fermentation. They found that a suitable ferrous ion concentration would stimulate H_2 production by hydrogen-producing bacteria. Regarding zinc ion, Zhang *et al*. (2021b) reported that increasing its concentration from 1 mg/L to 5 mg/L can effectively increase hydrogen production. At 2 mg/L Zn^{2+}, the maximum hydrogen production was 592 ± 13 mL. Salazar-Batres and Moreno-Andrade (2022) reported that when using glucose as a substrate, 2 mg Ni^{2+}/g $VS_{inoculum}$ generated the highest hydrogen production (774±7.3 mL H_2/L/d). In another study, the authors evaluated the effect of three different iron compounds (Fe_2O_3, $FeSO_4$, $FeCl_3$) on hydrogen production from sugar beet pulp. The authors observed that the addition of 0.1 gFe_2O_3/dm^3 in an intermittent experiment resulted in a maximum hydrogen production of more than 200 dm^3H_2/kgVS, which was twice that of the control (Cieciura-Włoch *et al*. 2020).

The studies mentioned above demonstrated the existence of a certain promoting effect of metal ions on the fermentation H_2 production of strains, but the effect of Fe^{2+} and Na^+ on the H_2 production from different types of fermentation strains has rarely been reported under the same cultural conditions. This study reveals the impact of metal ions on the hydrogen production of different fermentation strains. Fe is the main component of hydrogenase, which catalyzes the reduction of protons into hydrogen. Na^+ promotes H_2 production by promoting the growth of H_2-producing microorganisms. In this study, three different type of fermentation strains, namely *Ethanoligenens harbinense* ZGX4, *Clostridium butyricum* 1.209, and *Enterobacter cloacae* 1.2022, were used to probe into the effects of Fe^{2+} and Na^+ on H_2 production in the same cultural conditions. Batch experiments were applied to investigate cell growth, H_2 production, and liquid products. Finally, combining H_2 and liquid products production, Fe^{2+} was the most suitable for the H_2 production metabolism of the ethanol-type fermentative hydrogen-producing bacteria *E. harbinense* ZGX4. At the same time, Na^+ was found to be more suitable for butyric acid-type fermentative hydrogen-producing bacteria *C. butyricum* 1.209. This study has the potential to benefit the regulation and optimization of the fermentative process in biohydrogen production.

EXPERIMENTAL

Bacteria and Culture Condition

An ethanol-fermenting hydrogen-producing bacterium, *Ethanoligenens harbinense* ZGX4, was obtained from the State Key Laboratory of Urban Water Resources and Environment of Harbin Institute of Technology (Xing *et al*. 2006; Li *et al*. 2019). *Clostridium butyricum* 1.209 and *Enterobacter cloacae* 1.2022 were obtained from the Strain Preservation Center of the Chinese Academy of Sciences. The medium for cell growth and H_2 production was composed of glucose 20 g/L, peptone 4.0 g/L, beef paste 2.0 g/L, yeast juice 1.0 g/L, K_2HPO_4 1.5 g/L, $MgCl_2{\cdot}6H_2O$ 0.2 g/L, L-cysteine 0.5 g/L, vitamin solution (Table 1) 10 mL, trace element solution (Table 1) 10 mL, and resazurin (0.2% v/v) 1 mL. The concentrations of Fe^{2+} (from $FeSO_4{\cdot}7H_2O$) and Na^+ (from NaCl) were adjusted according to the fermentative experimental requirements, and the pH of the system was adjusted to 6 to 6.4 by 1 M KOH and 1 M HCl.

Table 1. Composition of Vitamin Solution and Trace Element Solution

Vitamin Solution	Content (g/L)	Trace Element Solution	Content (g/L)
Cobalt ammonium	0.01	$MnSO_4{\cdot}7H_2O$	0.01
Ascorbic acid	0.025	$ZnSO_4{\cdot}7H_2O$	0.05
Riboflavin	0.025	H_3BO_3	0.01
Citric acid	0.02	$N(CH_2COOH)_3$	4.5
Pyridoxal	0.05	$CaCl_2{\cdot}2H_2O$	0.01
Folic acid	0.01	Na_2MoO_4	0.01
P-aminobenzoic acid	0.01	$CoCl_2{\cdot}6H_2O$	0.2
Inositol	0.025	$AlK(SO_4)_2{\cdot}12H_2O$	0.01

Serum bottles with a total volume of 250 mL were used for the batch culture experiment, and the volume of solution was 100 mL. The mouths of the bottles were tightly corked to prevent gas leakage. N_2 was used to purge the whole system for 5 min and then the bottle was sealed quickly to ensure the entire system oxygen-free. Hydrogen was collected continually using a deformable gas bag, and the air pressure of the closed system was the same as atmospheric pressure. The serum bottles were placed in a constant temperature shaker and incubated at a speed of 130 rpm and a temperature of 35 ± 1 ℃. When the activated microbial cells grew to the mid-log stage, they were inoculated into each test vial with the same inoculum amount of 2% (v/v) per vial. Gas samples and liquid fermentation products were taken regularly. A three-way valve was used at the gas sampling port to maintain the sampled gas consistent at the gas concentration of the fermentation flask, and the H_2 content was determined.

Hydrogen Production Experiment Design

The effects of different concentrations of Fe^{2+} and Na^+ on three model hydrogen-producing strains in their growth and hydrogen production were compared and analyzed by batch culture experiments. The three strains have different optimal concentrations of Fe^{2+} and Na^+. In previous experiments, a wider range of metal ion concentrations (Fe^{2+} and Na^+) was selected to investigate their effect on hydrogen production in advance. By this means, a suitable concentration was determined for each metal ion that was selected for different bacteria in this study.

To investigate the effect of metal ions on the H_2 production of the three bacteria, preliminary experiments were carried out to determine the concentration range of Fe^{2+} and Na^+ by batch culture. For *E. harbinense* ZGX4, the concentrations of Fe^{2+} in the medium were 0, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5, and 1.0 g/L, according to the experimentally set gradient. For *C. butyricum* 1.209, Fe^{2+} concentrations were set at 0, 0.05, 0.1, 0.2, 0.25, 0.5, 1, 1.5, 2, and 4 g/L. For *E. cloacae* 1.2022, Fe^{2+} concentrations were set to 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.5, 0.75, and 1.0, 1.5, 2.0, and 4.0 g/L. Na^+ concentrations were set at 0, 0.5, 1, 1.5, 2, 2.5, and 3 g/L for *E. harbinense* ZGX4. For *C. butyricum* 1.209, the Na^+ concentrations were set at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 10 g/L. For *E. cloacae* 1.2022, the Na^+ concentrations were set at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 10.0 g/L. All operations were performed three times to ensure the reproducibility of the data, and the results were summarized as means with standard deviations.

Analysis Methods

Bacteria were diluted appropriately with physiological saline, and the optical density of the culture was measured at 600 nm. The fresh medium with the same dilution times was used as the control, and the dilution times multiplied by the optical density value of the sample was taken to be the true optical density value of the sample. A fermentation solution was taken and centrifuged at 4500 rpm to collect the bacteria. After suspending with normal saline and washing twice, the bacteria were dried at 105 ℃ to constant weight. An analytical balance was used to determine the dry weight of the cells (Song *et al*. 2013; Trchounian *et al*. 2016). Excel software was used to obtain the regression equation of absorbance and cell concentration: y=0.4229x-0.008, R^2=0.9936, where x is optical density and y is cell concentration. H_2 content was determined using a Shanghai Analytical Instrument Factory SC-II gas chromatograph, thermal conductivity cell detector, stainless steel column, column length and diameter of 2 m×φ5, carrier TDS-01, 60-80 mesh, using high-purity nitrogen as carrier, flow rate 70 mL/min, measured at room temperature, and injection volume 500 μL (Jitrwung and Yargeau 2015; Lamont *et al*. 2017). The liquid products (ethanol and volatile fatty acids) were determined by GC122 gas chromatograph of Shanghai Analytical Instrument Factory, with a stainless steel column, 2 m column length (5 mm inner diameter), carrier GDX103, 60 to 80 mesh, H_2 flame monitor, evaporation chamber 200 ℃, column temperature 190 ℃, detection chamber temperature 240 ℃, carrier gas nitrogen, flow rate 50 mL/min, H_2 flow rate 50 mL/min, and air flow rate 500 mL/min. Tests were carried out with 1 mL of culture solution, adding 1 to 2 drops of 6 M HCl, centrifuging at 5000 rpm for 15 min, and taking 2 μL of supernatant into the sample for detection (Wang *et al*. 2017).

RESULTS AND DISCUSSION

Effect of Fe^{2+} on the Growth of Three Hydrogen-Producing Bacteria

Fe^{2+} is one of the most important metal ions for microorganisms' growth and H_2 production. Duran Padilla *et al.* (2014) studied the growth of the hydrogen-producing strain *Clostridium acetobutylicum* ATCC 824 and found that adding Fe^{2+} (20 mg/L) enhanced its growth (Durán-Padilla *et al*. 2014).

As shown in Fig. 1, the bacterial concentration of *E. harbinense* ZGX4 was the highest when the Fe^{2+} concentration was 0.05 to 0.1 g/L. The bacterial concentration was

significantly lower when the Fe^{2+} concentration was greater than 0.1 g/L or less than 0.05 g/L. These findings showed that both low and high concentrations of Fe^{2+} had a negative effect on the growth of *E. harbinense* ZGX4, and when the concentration was 0.05 g/L, the maximum bacterial concentration was 0.53 g/L. For *C. butyricum* 1.209, the bacterial concentration fluctuated less with the change in Fe^{2+} concentration, and the growth was better. When the Fe^{2+} concentration was 0.25 g/L, the bacteria reached the maximum concentration value of 0.52 g/L. For *E. cloacae* 1.2022, with the increase of Fe^{2+} concentration, the bacterial concentration showed a trend to increase and then decrease and reached the maximum (0.32 g/L) at the Fe^{2+} of 0.25 g/L, and its average biomass was the lowest among the three strains.

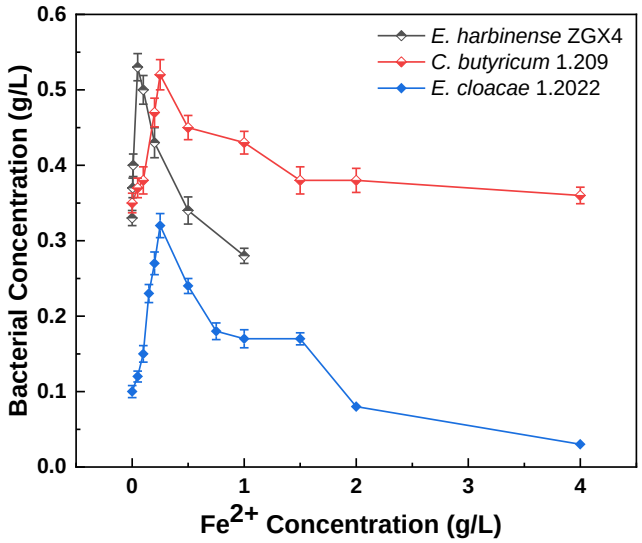

Fig. 1. Effect of Fe^{2+} on the growth of three bacteria

Effect of Fe^{2+} on H_2 Production by Three Hydrogen-Producing Bacteria

Figure 2 shows changes in H_2 content and H_2 production with Fe^{2+} concentration from the three bacteria. For *E. harbinense* ZGX4, when the Fe^{2+} concentration was less than 0.1 g/L, the H_2 content had little difference and it reached a maximum of 72.7%(v/v) when Fe^{2+} was 0.1 g/L. Its H_2 production fluctuated less with the change in Fe^{2+} concentration. When the Fe^{2+} concentration was at 0.05 g/L, the H_2 production reached the maximum of 2170 mL/L-medium (19500 mL H_2/mol glucose). Below or above this concentration, the H_2 production decreased. The concentration of 0.05 to 0.1 g/L was the most suitable Fe^{2+} concentration range for *E. harbinense* ZGX4 to produce H_2. Zhang *et al*. (2015) investigated the effect of temperature, pH, Mg^{2+}, and Fe^{2+} on the H_2 production of *Ethanoligenens harbinense* YUAN-3, and this strain was the same as the one in this study. Moreover, when $MgCl_2 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ were both 300 mg/L and under the optimal other conditions, the maximum H_2 yield of 2140 mL/L could be obtained, which was similar to the results of this study (Zhang *et al*. 2015). For *C. butyricum* 1.209, H_2 content showed a trend of increase and then decrease in the process of increasing Fe^{2+} concentration, reaching a maximum value of 64.5% when Fe^{2+} concentration was 0.2 g/L. H_2 production fluctuated greatly with the change in Fe^{2+} concentration. H_2 production was more sensitive to Fe^{2+}. When the Fe^{2+} concentration was 0.2 to 0.25 g/L, H_2 production was the highest. When the Fe^{2+} concentration was 0.2 g/L, H_2 production reached a maximum value of 2540 mL/L-medium (22900 mL H_2/mol glucose). Above or below this

concentration, H_2 production decreased. When Fe^{2+} concentration exceeded 1.0 g/L, H_2 content and H_2 production were low. H_2 production was completed within 20 hours, indicating that Fe^{2+} at this concentration (1.0 g/L) had an inhibitory effect on the H_2 production of *C. butyricum* 1.209. The most suitable Fe^{2+} concentration for H_2 production was 0.2 to 0.25 g/L. For *E. cloacae* 1.2022, when the Fe^{2+} concentration was less than 0.25g/L, H_2 contents showed little difference, fluctuating around 20%. When the Fe^{2+} concentration was 0.5 g/L, H_2 content reached the maximum value of 27.5%. H_2 production reached the maximum value of 785 mL/L- medium (7070 mL H_2/mol glucose) with a Fe^{2+} concentration of 0.75 g/L. Compared with the above two bacteria, the H_2 production of this bacterium was lower, and the fluctuation was the smallest. The Fe^{2+} concentration range corresponding to the optimal H_2 production lagged, which indicated that *E. cloacae* 1.2022 was less affected by Fe^{2+} than *E. harbinense* ZGX4 and *C. butyricum* 1.209, and the suitable Fe^{2+} concentration for H_2 production was 0.5 to 1 g/L.

Except for a few points, the changes in the growth of the three bacteria were consistent with the changes in H_2 production, indicating that within a certain range, H_2 production increased with the growth of bacteria and that bacterial growth was positively correlated with the H_2 production.

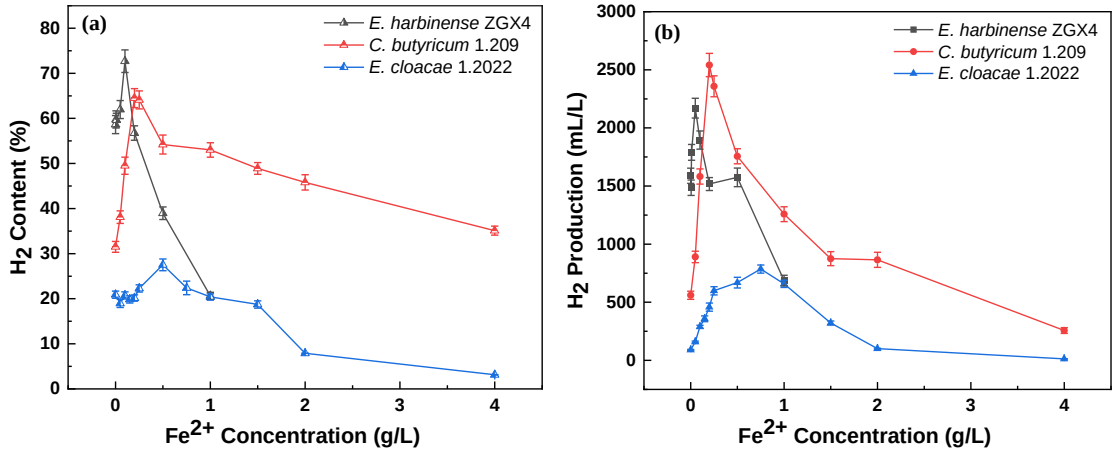

Fig. 2. Effect of different Fe^{2+} concentrations on H_2 production. (a) H_2 content (b) H_2 production

Effect of Fe^{2+} on the Liquid Products of Three Bacteria

With the change in metal ion concentration, the liquid products of the three bacteria were mainly ethanol and volatile fatty acids (VFAs). The VFAs were mainly acetic acid, propionic acid, and butyric acid. Ethanol can be used as a liquid fuel and chemical feedstock, while VFAs have applications in the industrial food and pharmaceutical industries and are considered renewable carbon sources (Agnihotri *et al*. 2022; Yasser Farouk *et al*. 2022). Therefore, the hydrogen-producing bacteria can produce valuable liquid products while fermenting and producing H_2.

In terms of liquid products (Fig. 3), the effect of Fe^{2+} concentration on the liquid products of *E. harbinense* ZGX4 showed good consistency with H_2 production. The amount of total liquid products reflects the bacterial group metabolism of the H_2 production operating system. Its specific composition and proportion will reflect the metabolic type of H_2 production operating system. The main types of liquid products were ethanol and acetic acid, which was a typical ethanol-type fermentation. The overall amount of liquid products reached its maximum when Fe^{2+} was 0.05 g/L, which was 6530 mg/L, the highest among

the three bacteria (Fig. 3(a)). The effect of Fe^{2+} on the liquid products of *C. butyricum* 1.209 was relatively stable with minor fluctuation. The total amount of liquid products reached a maximum value of 4850 mg/L when the concentration was 0.25 g/L. However, with the increase of Fe^{2+}, ethanol production increased, and butyric acid fermentation was replaced by mixed acid fermentation. The main liquid products were ethanol, acetic acid, and butyric acid, indicating that adding Fe^{2+} to the fermentation system can alter the fermentation type (Fig. 3(b)). The conversion of bacterial fermentation mechanism to ethanol-type fermentation had better H_2 production capacity (Wang *et al*. 2018). The main liquid products of *E. cloacae 1.2022* were ethanol, acetic acid, and formic acid, which are characteristic of mixed acid fermentation (Chen *et al*. 2021b). The lowest total amount of liquid products of the three bacteria were obtained, whereas when Fe^{2+} was 0.75 g/L, the highest total amount of liquid products was observed, 2205.1 mg/L.

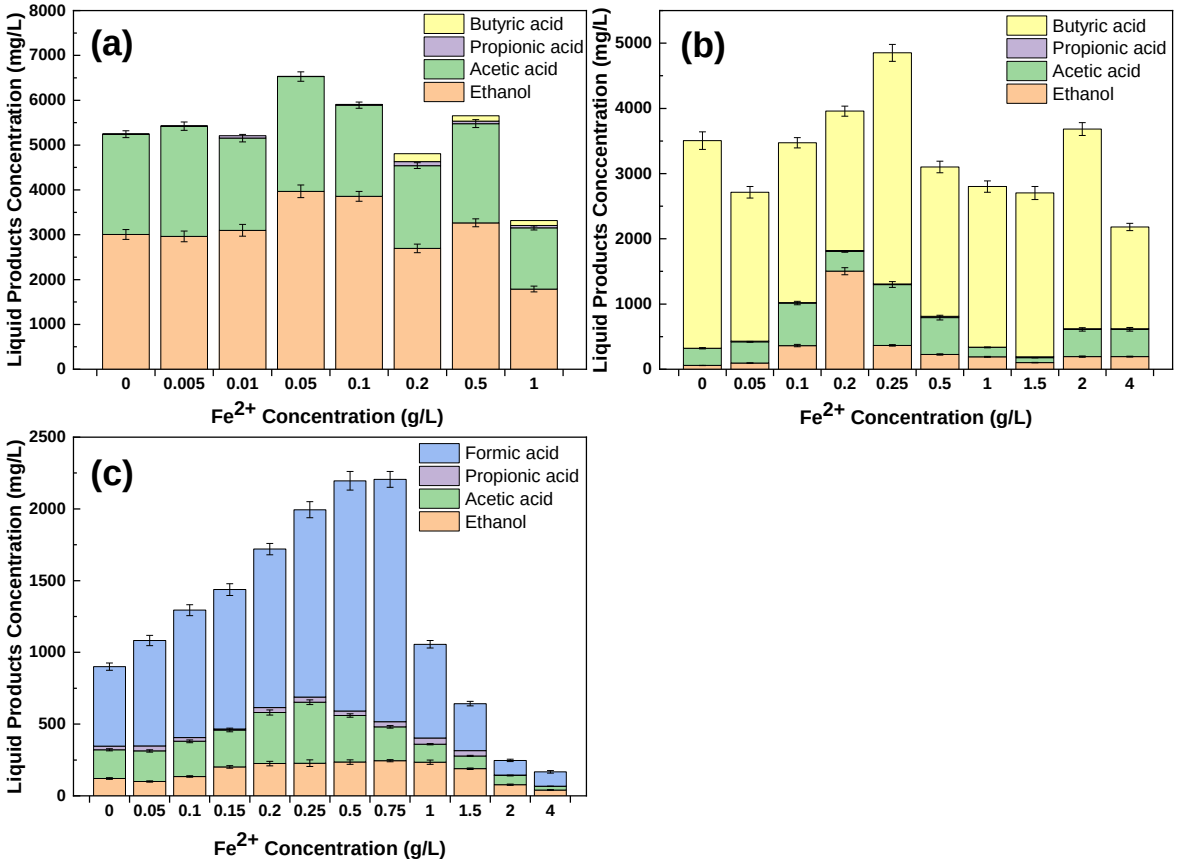

Fig. 3. Effect of different Fe^{2+} concentrations on the liquid products for three bacteria. (a) *E. harbinense* ZGX4 (b) *C. butyricum* 1.209 (c) *E. cloacae* 1.2022

In summary, the appropriate concentration of Fe^{2+} promotes H_2 production in the three bacteria, which may be due to the participation of Fe^{2+} in the synthesis of ferredoxin, an essential coenzyme in microbial H_2 production metabolism that activates catalase (Gad El-Rab *et al*. 2018; Morra *et al*. 2014). The proper concentration of Fe^{2+} can improve hydrogenase activity in hydrogen production (Zhang *et al*. 2017). A study on H_2 production from sludge in the presence of Fe^{2+} revealed the mechanism of enhanced H_2 production by Fe^{2+} (Yin and Wang 2021). Compared with *E. cloacae* 1.2022, *E. harbinense* ZGX4 and *C. butyricum* 1.209 had better H_2 production performance with the addition of Fe^{2+}.

However, Fe^{2+} changed the fermentation type of *C. butyricum* 1.209. The optimal H_2 production of *E. harbinense* ZGX4 and *C. butyricum* 1.209 was similar. When the optimal gas production was reached, *E. harbinense* ZGX4 required less Fe^{2+} while producing the highest total amount of liquid products. *E. harbinense* ZGX4 generates more H_2 production and has the highest total amount of liquid products when Fe^{2+} is added.

Effect of Na^+ on the Growth of Three Hydrogen-Producing Bacteria

Sodium salt is an important medium component in the expanded culture of hydrogen-producing microorganisms. However, sodium salt's effect on hydrogen-producing fermentation bacteria has been the subject of few studies (Junghare *et al*. 2012). Therefore, it is necessary to study the effect of sodium salt on hydrogen-producing fermentation bacteria. NaCl addition improves H_2 production and promotes cell growth. This will eliminate sodium ions' inhibitory effect in the medium preparation process.

As shown in Fig. 4, the growth amount of *E. harbinense* ZGX4 was the highest when Na^+ concentration was 1.0 to 1.5 g/L. The growth amount was relatively low when Na^+ concentration exceeded 1.5 g/L or was less than 1.0 g/L. This showed that both low and high concentrations of Na^+ inhibited the growth of *E. harbinense* ZGX4. The maximum increase was 0.485 g/L when the concentration was 1.0 g/L. For *C. butyricum* 1.209, the growth amount of *C. butyricum* 1.209 was higher when the Na^+ concentration was 2.0 to 3.0 g/L, reaching a maximum value of 0.511 g/L when Na^+ concentration was 3.0 g/L. However, when Na^+ concentration exceeded 3.0 g/L or was less than 2.0 g/L, the growth amount was lower, indicating that the low and high concentrations of Na^+ would inhibit the growth of *C. butyricum* 1.209. For *E. cloacae* 1.2022, the growth of *E. cloacae* 1.2022 was higher when Na^+ concentration was 1.5 to 2.0 g/L. When Na^+ concentration was 1.5 g/L, the growth amount reached a maximum value of 0.38 g/L (Fig. 4).

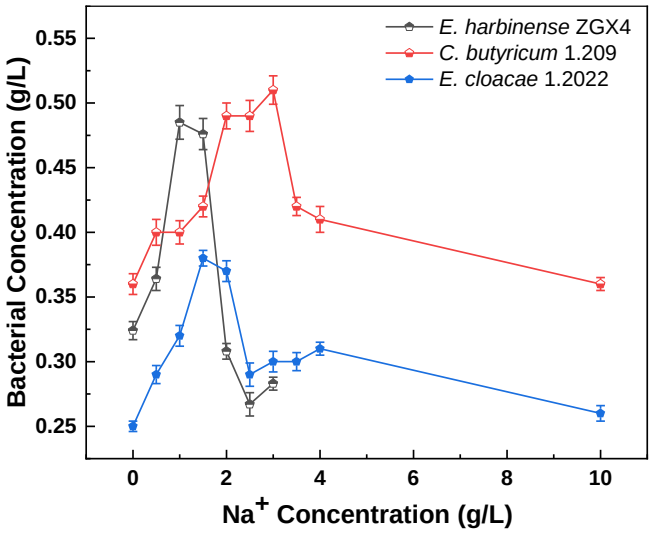

Fig. 4. Effect of Na^+ on the growth of three bacteria

Effect of Na^+ on the H_2 Production of Three Bacteria

Figure 5 shows these three hydrogen-producing bacteria were more sensitive to Fe^{2+} than Na^+. The optimal Na^+ concentration range for H_2 production lagged behind that of Fe^{2+}. For *E. harbinense* ZGX4, H_2 content ranged between 55% and 65% with a small range, reaching a maximum Na^+ concentration of 1.5 g/L. When Na^+ concentration was

higher than 1.5 g/L, H_2 production decreased with the increase of Na^+ concentration. When Na^+ concentration was higher than 1.5 g/L or lower than 1.0 g/L, H_2 production declined. The fluctuation of H_2 production was slight. When Na^+ concentration was 1.5 g/L, it reached a maximum value of 1510 mL/L-medium (13600 mL H_2/mol glucose), and 1.0 to 1.5 g/L was the most suitable Na^+ concentration range of *E. harbinense* ZGX4 for H_2 production. For *C. butyricum* 1.209, the change in H_2 content had a similar trend to H_2 production. When Na^+ concentration was 2.0 g/L, it reached a maximum value of 65.2%. When Na^+ concentration was lower or higher than 2.0 g/L, H_2 production was low. When Na^+ concentration was 2.0 g/L, H_2 production reached the maximum value of 2460 mL/L-medium (22100 mL H_2/mol glucose). Among the three bacteria, its H_2 production was the highest. However, its fluctuation was significant and more sensitive to the change in Na^+ concentration. Its optimal H_2 production threshold was 2.0 to 2.5 g/L. *C. butyricum* 1.209 was *Clostridium*, and it has been reported that *Clostridium* is an ideal H_2 producer (Cai *et al*. 2021). For *E. cloacae* 1.2022, H_2 content reached the highest value of 27.0% only when Na^+ concentration was 1.5 g/L. The other concentrations had little impact, fluctuating between 21.9% and 23.3%. H_2 production decreased as Na^+ concentration increased. H_2 production in the control group was 72.9 mL/L-medium (656 mL H_2/mol glucose). However, when Na^+ concentration exceeded 1.5 g/L, H_2 production was significantly reduced, and the lowest was only 3.39 mL/L-medium. This showed that adding Na^+ did not promote H_2 production but inhibited H_2 production by fermentation of hydrogen-producing bacteria. H_2 production was the lowest among the three bacteria.

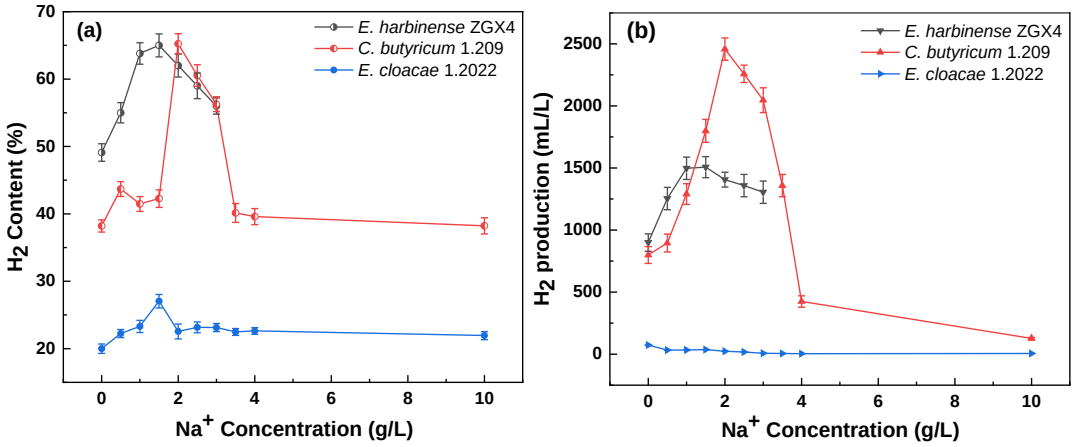

Fig. 5. Effects of different Na^+ concentrations on H_2 production. (a) H_2 content (b) H_2 production

Table 2. Hydrogen Production of Different Strains

Strains	Maximum H_2 Production	Reference
Clostridium butyricum INET1	2180 mL/L	Yin and Wang 2017
Photosynthetic bacteria	27.34±1.13 mL/g TS	Zhang *et al*. 2021
Enterobacter ludwigii IF2SW-B4	545±5 mL/L	Tandon *et al.* 2018
mixed bacteria	292.8 mL H2/g·TVS	Sekoai and Daramola 2018
E. harbinense ZGX4	2170.2 mL/L	This study
C. butyricum 1.209	2541.0 mL/L	This study
E. cloacae 1.2022	785.2 mL/L	This study

In this study, three model hydrogen-producing strains were used. The effects of Fe^{2+} and Na^+ on hydrogen production by three strains were investigated, and the highest hydrogen production was obtained. Compared with other strains (Table 2), these three strains also had higher H_2 production efficiency.

Effect of Na^+ on the Liquid Products of Three Bacteria

Ethanol and acetic acid were the main liquid products for *E. harbinense* ZGX4 with Na^+ concentration changes (Fig. 6).

The changing trend was consistent with the changing direction of H_2 production. When the concentration of Na^+ was 1.5 g/L, total liquid products reached a maximum of 4920 mg/L. For *C. butyricum* 1.209, the main kinds of liquid products were acetic acid and butyric acid. The change rule of the total amount of liquid products was consistent with the change rule of H_2 production when Na^+ concentration was higher than 3.0 g/L. It was significantly inhibited and fluctuated greatly due to the change in Na^+ concentration, reaching a maximum value of 5350 mg/L when the Na^+ concentration was 2.0 g/L. For *E. cloacae* 1.2022, combined with its liquid products, the main liquid products were ethanol, acetic acid, formic acid, and a small amount of propionic acid. The law was inconsistent with the changing direction of H_2 production when the concentration of Na^+ was higher than 2.0 g/L. Obvious inhibition occurred when the concentration of Na^+ was 2.0 g/L. The total of liquid products was the largest, at 3250 mg/L. The liquid products production was relatively high, but H_2 production was low, probably because the addition of Na^+ or the output of VFAs inhibited H_2 production of hydrogen-producing bacteria (Boni *et al*. 2014; Singh and Wahid 2015; Chen *et al*. 2021a). Average liquid product production was the lowest among the three strains.

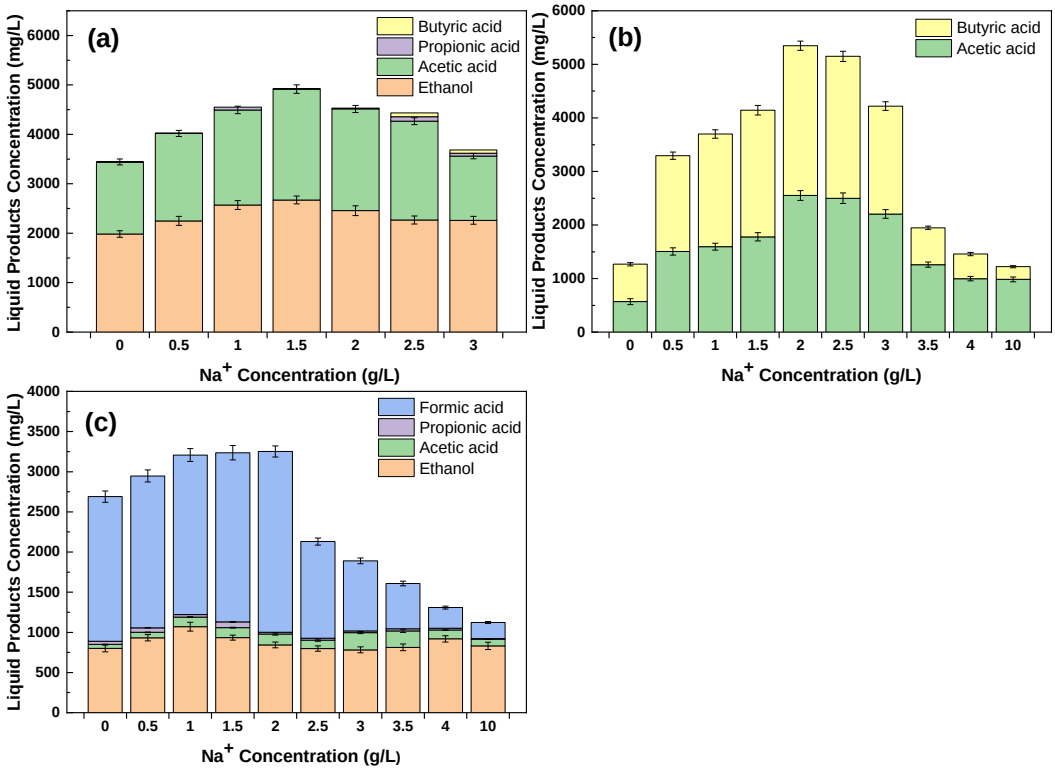

Fig. 6. Effect of different Na^+ concentrations on the liquid products for three bacteria. (a) *E. harbinense* ZGX4; (b) *C. butyricum* 1.209; (c) *E. cloacae* 1.2022

Overall, it was found that the appropriate Na^+ concentration could improve H_2 production of *E. harbinense* ZGX4 and *C. butyricum* 1.209. On the contrary, Na^+ could inhibit the hydrogen production of *E. cloacae* 1.2022, which was different from Fe^{2+}. The optimal H_2 production thresholds were relatively close for *E. harbinense* ZGX4 and *C. butyricum* 1.209. However, *C. butyricum* 1.209 had a higher hydrogen production. Comparing the best H_2 production, *C. butyricum* 1.209 was 38.7% higher than *E. harbinense* ZGX4, and within the optimal H_2 production range, it had higher liquid product production. Adding Na^+ would make *C. butyricum* 1.209 have the most increased H_2 and liquid products production.

CONCLUSIONS

1. The effects of Fe^{2+} and Na^+ on the H_2 production performance of the strains was investigated using three different fermentation types of hydrogen-producing strains. The results showed that different metal ions had various effects on the H_2 production performance of the strains.
2. Except for Na^+, which inhibited the H_2 production of *E. cloacae* 1.2022, Fe^{2+} and Na^+ positively impacted the growth and H_2 production metabolism of the three different fermentation types of strains.
3. Combining H_2 and liquid product production, Fe^{2+} was the most suitable for H_2 production metabolism of ethanol-type fermentative hydrogen-producing bacteria *E. harbinense* ZGX4. At the same time, Na^+ was found to be more suitable for butyric acid-type fermentative hydrogen-producing bacteria *C. butyricum* 1.209.
4. At the end of the experiment, it was found that adding Fe^{2+} could change the type of fermentation in *C. butyricum* 1.209.

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