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ORIGINAL ARTICLE

Mechanisms for the Enhancement of Caproic Acid and H_2 Production in *Ruminococcaceae* Bacterium CPB6 by Fe(II) and Mg(II): Growth and Gene Transcription Analyses

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Abstract
The production of caproic acid (CA) and hydrogen gas (H_2) from organic wastewater is economically attractive. The *Ruminococcaceae* bacterium CPB6 has demonstrated potential for CA production from lactate-containing wastewater. However, our understanding of the effects of Fe^{2+} and Mg^{2+} on the growth and metabolism of strain CPB6 remains limited. Therefore, this study aims to investigate the impact of Fe^{2+} and Mg^{2+} on CA and H_2 production, as well as on the expression of key genes involved in CA and H_2 biosynthesis pathway. The results indicate that Fe^{2+} positively affects cell proliferation and H_2 production while minimally impacting CA production. The highest levels of H_2 production were achieved with the addition of 200 mg/L Fe^{2+}. Conversely, Mg^{2+} significantly enhances CA and H_2 production, with the optimal yield observed in a medium enriched with 300 mg/L Mg^{2+}. Reverse transcription quantitative PCR (RT-qPCR) analysis reveals that Fe^{2+} promotes the expression of the hydrogenase gene, whereas Mg^{2+} has a negligible effect on hydrogenase expression. Notably, Fe^{2+} and Mg^{2+} inhibit the expression of key genes involved in CA synthesis. These findings suggest that Fe^{2+} enhances H_2 production by boosting cell biomass and the expression of the hydrogenase gene, whereas Mg^{2+} improves CA and H_2 production primarily by increasing cell biomass rather than influencing the expression of functional genes involved in CA biosynthesis.

Keywords Metal ions · Caproic acid · Biohydrogen · Transcription · Functional gene

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Introduction

Caproic acid (CA), a six-carbon medium-chain carboxylic acid, is a necessary ingredient for the production of alkanes and olefins in biofuels [1–3] and an antibacterial and anti-inflammatory agent in humans and livestock [4, 5], as well as a flavoring and feed additive [6, 7]. Traditionally, CA is obtained from fossil sources or vegetable oil through chemical processes [8, 9]. Recently, CA production through anaerobic fermentation has received more attention [10]. For example, the co-culture of rumen microorganisms with *Clostridium kluyveri* has shown promise in promoting CA production [11]. Nzeteu et al. showed a sustainable production of CA through a mixed culture utilizing butyric acid as the electron acceptor and lactic acid as the electron donor [12]. Dong et al. demonstrated the efficient generation of CA through anaerobic fermentation of organic waste [13]. These findings highlight the potential for resource recovery and organic waste treatment. Anaerobic fermentation also yields a significant amount of hydrogen gas (H_2), providing dual benefits of clean energy production and high-value compound generation [14]. H_2 is considered a favorable alternative to fossil fuels due to its lack of greenhouse gas emissions [15, 16]. Conventional methods for H_2 production, such as electrolysis and steam reforming, have drawbacks in terms of CO_2 emissions and high costs [17, 18]. Biohydrogen derived from organic matter offers a cost-effective and environmentally beneficial alternative [19, 20]. Currently, biohydrogen can be obtained through various methods, including photosynthetic fermentation, anaerobic fermentation, and microbial electrolysis [21–23]. Among these methods, anaerobic fermentation for biohydrogen production holds greater promise. This process allows for the generation of hydrogen from inexpensive organic substrates such as wastewater, mono-, di-, and tri-saccharides, as well as cheese whey and vegetable fruit wastes [24–26].

Metal ions play a significant role in microbial growth and metabolism. For example, Mg^{2+} and Ni^{2+} ions have been found to enhance the biomass and H_2 production of *Rhodobacter sphaeroides* strain MDC6521 [27]. Similarly, Mg^{2+} and Fe^{2+} are major factors influencing hydrogen production in *Ethanoligenens harbinense* [28]. Notably, certain metal ions, including Cr, Cu, and Zn, inhibit the activity of hydrogen-generating bacteria in sludge [29]. Furthermore, Fe^{2+} and Ni^{2+} have been shown to enhance H_2 and ethanol production by influencing hydrogenase activity and biomass in anaerobic continuous flow stirred reactors [30].

The *Ruminococcaceae* bacterium CPB6 is an anaerobic mesophilic bacterium known for its high CA production from lactate-containing wastewater with lactate as an electron donor and short chain carboxylic acids as electron acceptors [31]. However, the effects of metal ions on the growth and metabolism of strain CPB6 remain uncertain. In this study, we investigated the impact of Mg^{2+} and Fe^{2+} on the production of CA and H_2 in strain CPB6 through batch fermentation. Additionally, we examined the expression of genes encoding key enzyme responsible for the synthesis of CA and hydrogen, which encompassed acetoacetyl-CoA thiolase (Thl), butyryl-CoA: acetate CoA transferase (Cat), butyryl-CoA dehydrogenase (Bcd), and [FeFe]-hydrogenase (HydE), using RT-qPCR.

Materials and Methods

Bacterial Culture and Media

Strain CPB6 was isolated and preserved in our laboratory. The phylogenic analysis based on 16 S rRNA sequences and the whole genome revealed that strain CPB6 belongs to a

novel clade (genus) within the family *Ruminococcaceae*; therefore, it has been tentatively named as *Ruminococcaceae* bacterium CPB6 [6]. The CM medium composition per liter was as follows: 10 g glucose, 15 g sodium lactate, 5 g peptone, 0.5 g K_2HPO_4, 3 g yeast extract, 3 g NaCl, 5 g sodium acetate, 1 g NH_4Cl, 0.1 g $MgSO_4{\cdot}7H_2O$, 5 g beef powder, 1 mL vitamin solution, 1 mL trace element solution, 0.5 ml Na-resazurin solution, 0.5 g L-Cysteine-HCl-H_2O, with a pH of 6.5 ± 0.1. The anaerobic flasks were flushed with high-purity N_2 (99.99%) for 5 min, sealed with rubber stoppers, and autoclaved at 115 ℃ for 20 min. Vitamins were added after autoclaving, and the preparation of vitamin and trace element solutions followed the instructions in Wang et al. [31]. A glycerol stock of strain CPB6 stored at −80 ℃ was fully thawed and transferred to the CM medium for incubation at 37 ℃ for 48 h. The strain underwent three subcultures before being used for subsequent experiments.

Experimental Design

A 100 mL anaerobic flask with 50 mL of CM medium was supplemented with different concentrations of $FeSO_4{\cdot}7H_2O$ or $MgSO_4{\cdot}7H_2O$ to investigate their effects on the growth and metabolism of strain CPB6. The Fe^{2+} concentrations tested were 0, 100, 200, 300, 400, and 500 mg/L, respectively. The Mg^{2+} concentrations tested were 0, 100, 200, 300, 500, 700, and 900 mg/L, respectively. Strain CPB6 was incubated at 37 ℃ for 24 h as seed inoculum ($OD_{600\,nm} = 0.8 \sim 1.0$) for batch experiments. An inoculum concentration of 10% was used for each assay, and the gas produced was collected by draining (Fig. 1 illustrates the experimental setup).

Analysis Methods

The concentrations of volatile fatty acids, ethanol, and glucose in the culture broth were measured by an HPLC system (Agilent 1260 Infinity, USA) equipped with a differential refraction detector (RID) and a Hi-Plex H column (300 × 6.5 mm). The chromatographic column was operated with a mobile phase of 5 mM H_2SO_4 at 55 ℃. The fermentation broth was first centrifuged at 12,000 rpm for 2 min, and the supernatant was subsequently filtrated through a 0.22 μm filter (Millipore Corp, Bedford, MA) before HPLC analysis. Gas components analysis was performed using a gas chromatograph (Agilent 7890B, USA). A gas sample of 3 ~ 5 mL was taken with a syringe and manually injected into the gas chromatograph for analysis. The dry weight of the bacteria (biomass) was determined by

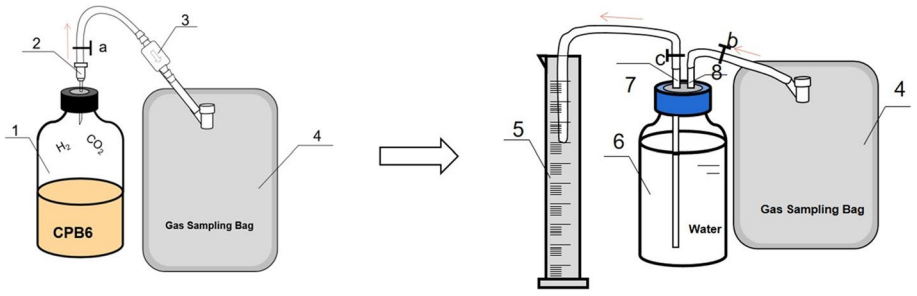

Fig. 1 Experimental setup [1]. anaerobic bottle, [2] venting syringe, [3] check valve, [4] gas sampling bag, [5] measuring cylinder, [6] gas collection bottle, [7] outlet pipe, [8] gas inlet pipe. a, b, and c are pipe jigs

drying the precipitate obtained from 1 mL of bacterial liquid culture to a constant weight in an oven (24 h at 80 ℃). The pH of the bacterial culture was measured using a pH meter (Mettler-Toledo Instruments Ltd.).

RNA Extraction and cDNA Synthesis

The total RNA was extracted from bacterial cultures by the UNIQ-10 Columnar Trizol Total RNA Extraction Kit according to the manufacturer's instructions. The quality of the RNA samples was assessed through 1.5% agarose gel electrophoresis. Reverse transcription was performed by using 1.5 μg of the total RNA. In the ice bath, the following reagents were added to the nuclease-free PCR tube: 1 μL of dNTP Mix (final concentration of 0.5 mM), 1 μL of Random Primer p(dN)6 (100 pmol), and 14.5 μL of DEPC water. The mixture was mixed and centrifuged for 3 ~ 5 s.

The reaction mixture was then incubated for 5 min at 65 ℃ in a warm bath, followed by 2 min at 0 ℃ in an ice bath, and finally centrifuged for 3 ~ 5 s. After submerging the tubes in an ice bath, the following reagents were added: 1 μL of Maxima Reverse Transcriptase (200 U), 0.5 μL of Thermo Scientific RiboLock RNase Inhibitor (20 U), and 4 μL of 5x RT buffer. The reagents were thoroughly mixed and centrifuged for 3 ~ 5 s. Reverse transcription was performed using a PCR machine, with incubation periods of 10 min at 25 ℃, 30 min at 50 ℃, and 5 min at 85 ℃. The resulting solution was stored at −20 ℃.

Reverse Transcription-Quantitative PCR (RT-qPCR)

Gene expression was quantified using SYBR Green I real-time fluorescence quantitative PCR. Primers targeting the functional and reference genes were designed using Primer Premier 5.0 software. The primer sequences are shown in Table 1. The fluorescent PCR device used was a LightCycler 480 II device (Roche, Rotkreuz, Switzerland). A 10 μL reaction system was employed, consisting of 5 μL of 2x SybrGreen qPCR master mix, 0.2 μL each of the 10 M upstream and downstream primers, 3.6 μL of ddH_20, and 1 μL of the template (cDNA). The amplification conditions were as follows: 3 min at 95 ℃ for pre-denaturation, 45 cycles of 15 s at 95 ℃ (denaturation), and 30 s at 60 ℃ (annealing/extension).

The transcription level of key genes including *thl*, *bcd*, *cat*, and *hydE* was determined according to the $2^{-(\Delta\Delta Ct)}$ method, with the *recA* as a reference gene for the normalization of

Table 1 Primer sequences for target and housekeeping genes

	Gene	Primer	Sequence
Housekeeping gene	*recA*	*recA*-F	5'-GGCGAAGGTATTTCCCATG-3'
		recA-R	5'-CCGAAGCACGAGGAGAAAT-3'
Target gene	*thl*	*thl*-F	5'-GCAGGTATCCCGATTAGCAC-3'
		thl-R	5'-GCAGATAAGGAGCGTTGGAC-3'
	cat	*cat*-F	5'-ACAGGTTCCGAGCGTCACTA-3'
		cat-R	5'-GAAACCTGGCACATTGCTACA-3'
	hydE	*hydE*-F	5'-CAGATGTCCTTTGACCACCG-3'
		hydE-R	5'-GGAACGGCGTGTCCTTGT-3'
	bcd	*Bcd*-F	5'-CAAGGGCTTCAAGGTCGC-3'
		Bcd-R	5'-GCTGATACGTCTGCCAAACTG-3'

gene expression levels, where $\Delta\Delta Ct = \Delta Ct(Ct_{target} - Ct_{recA})_{treat} - \Delta Ct\ (Ct_{target} - Ct_{recA})_{control}$ [32, 33]. In this experiment, the control group did not receive any metal ions.

Statistical Analysis

Statistical analysis of the results was conducted using SPSS software (version 26, IBM), with independent samples *t*-test for comparisons between two groups and ANOVA test for comparative analysis between multiple groups. Pearson correlation between CA or H_2 production and biomass or gene expression was analyzed and visualized using GraphPad Prism software (version 8). A positive correlation is indicated when the correlation coefficient is greater than 0, whereas a negative correlation is indicated when the coefficient is less than 0. The magnitude of the absolute value of the correlation coefficient represents the strength of the correlation. A *P*-value greater than 0.05 indicates a lack of statistical significance, while a *P*-value less than 0.05 suggests statistical significance.

Results

Effect of Fe^{2+} on the Growth and Metabolism of Strain CPB6

As shown in Fig. 2a, the biomass of strain CPB6 initially increased and then decreased with increasing Fe^{2+} concentration. At a concentration of 200 mg/L Fe^{2+}, the cell biomass reached the maximum of 1.43 g/L, significantly higher than the control without Fe^{2+} ($P < 0.05$). The pH exhibited a decline as Fe^{2+} concentration increased. Similarly, H_2 yield showed an initial increase followed by a decrease with increasing Fe^{2+} concentration, peaking at 1.88 ml H_2/mL medium at a concentration of 200 mg/L Fe^{2+}, significantly higher than the control group without Fe^{2+} ($P < 0.05$, Fig. 2b). The trend in H_2 production was consistent with cell biomass. However, the addition of Fe^{2+} had little effect on CA yield (Fig. 2b).

Effect of Mg^{2+} on the Growth and Metabolism of CPB6

According to Fig. 3a, the addition of Mg^{2+} exerted a noteworthy and positive influence on the enhancement of cellular proliferation. The biomass of the cells supplemented with Mg^{2+} was consistently higher than the control without Mg^{2+} ($P<0.05$). At a concentration of 300 mg/L Mg^{2+}, the cell biomass reached a maximum of 1.53 g/L. However, it was observed that the pH tended to drop as the Mg^{2+} concentration increased, especially when the Mg^{2+} concentration exceeded 200 mg/L. The trend in CA and H_2 production followed a similar pattern as the cell biomass. The maximum yields of CA and H_2 were 6446.04 mg/L and 1.31 mL/mL medium, respectively, at a concentration of 300 mg/L Mg^{2+} (Fig. 3b). Mg^{2+} effectively increased cell biomass and H_2 production within a range of concentrations (200 to 700 mg/L), as well as promoting CA production. These results demonstrate that the addition of Mg^{2+} significantly enhanced the growth of the CPB6 bacterium, contributing the higher CA and H_2 production.

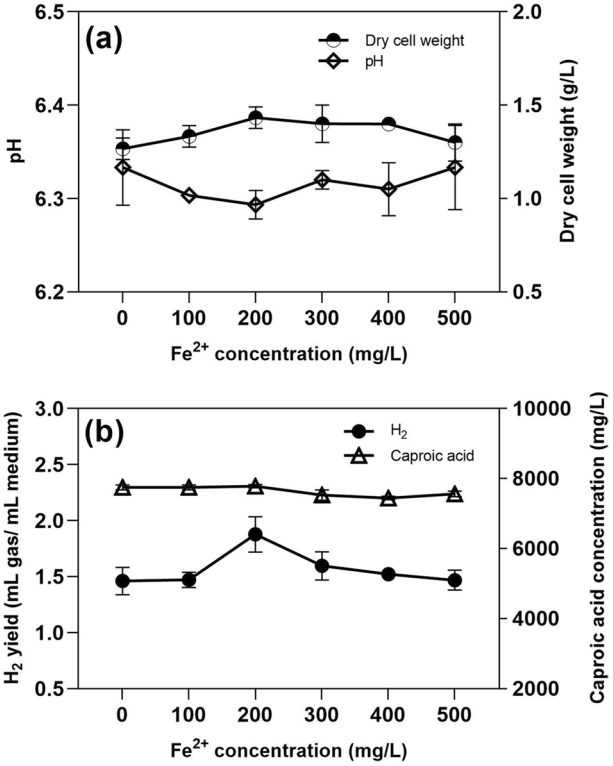

Fig. 2 Effect of Fe^{2+} on the growth and metabolism of strain CPB6. **a** Cell biomass and pH; **b** the production of H_2 and caproic acid

Effects of Fe^{2+} and Mg^{2+} on the Expression of Key Genes

The addition of Fe^{2+} led to a significant upregulation of the *hydE* gene, increasing its expression by 1 to 3.5 times compared to the control. The maximum gene expression was observed at a concentration of 200 mg/L Fe^{2+} (Fig. 4a). Conversely, Fe^{2+} had negligible effects on the expression of the *bcd* and *cat* genes. However, it exhibited a partial inhibitory effect on *thl* expression. In contrast, Mg^{2+} had minimal impact on the expression of *hydE* and *bcd* genes but strongly suppressed the expression of *cat* and *thl* genes (Fig. 4b). These findings indicate distinct effects of Fe^{2+} and Mg^{2+} on the expression of genes involved in CA and H_2 production.

Correlation Between Metabolites and Gene Expression and Biomass

H_2 production exhibited a strong positive correlation with the *hydE* expression ($r = 0.56$, $p < 0.05$, Fig. 5a) and cell biomass ($r = 0.64$, $p < 0.05$, Fig. 5b) in the presence of Fe^{2+}. However, when Mg^{2+} was added, H_2 production only positively correlated with cell biomass ($r = 0.67$, $p < 0.05$, Fig. 5e). CA production showed a weak negative correlation with cell biomass ($r = -0.38$, $p > 0.05$, Fig. 5c) when Fe^{2+} was added, but a significant positive correlation with cell biomass ($r = 0.46$, $p < 0.05$, Fig. 5f) when Mg^{2+} was added. These results suggest that Fe^{2+} primarily increased H_2 production by enhancing the activity

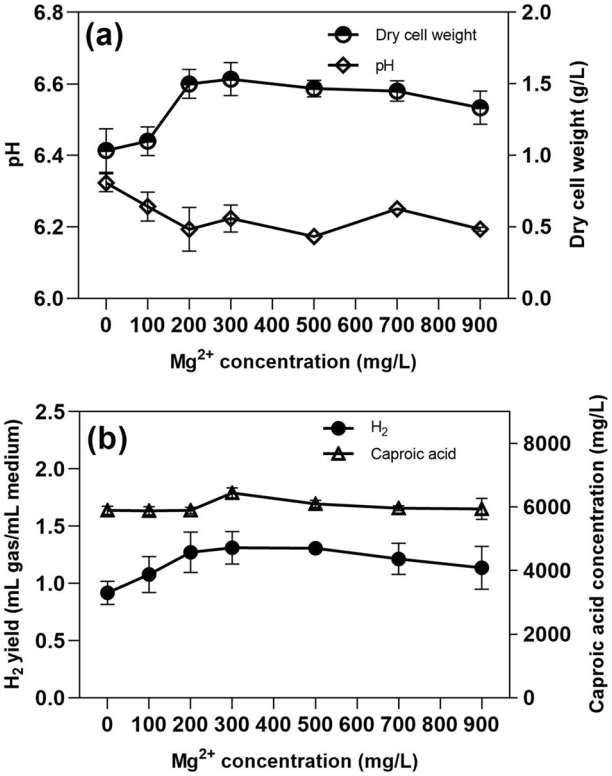

Fig. 3 Effect of Mg^{2+} on the growth and metabolism of strain CPB6. **a** Cell biomass and pH; **b** the production of H_2 and caproic acid

of hydrogenase and promoting cell growth. In contrast, Mg^{2+} increased both H_2 and CA production by stimulating cell growth. However, the mechanism underlying the negative correlation between CA production and carbon chain elongation genes under the influence of Fe^{2+} and Mg^{2+} requires further investigation.

Discussion

Iron plays a crucial role in microbial growth and metabolism. Previous study has demonstrated that Fe^{2+} significantly enhances hydrogenase activity, H_2 yield, and butyric acid synthesis in a membrane bioreactor [34]. Additionally, Fe^{2+} has been found to increase photosynthetic bacterial activity, biomass, and ATP generation by regulating the activities of succinate and NADH dehydrogenase [35]. Similar results have been observed in *E. harbinense* [28]. In this study, we observed that the addition of Fe^{2+} promoted cell growth, H_2 production, and hydrogenase gene expression, which is consistent with previous findings. Hydrogenases are important enzymes that catalyze the reduction of protons to produce hydrogen, and their activity relies on iron as an essential cofactor. Hydrogenases can be categorized as [NiFe]-, [FeFe]-, and [Fe]- hydrogenases depending on the kind of metal ion in the catalytic site [36]. These enzymes are Fe-dependent, with iron serving as a crucial cofactor for their catalytic activity. Iron is involved in the catalytic process of hydrogen oxidation or production in hydrogenases [37]. The hydrogenase in the strain CPB6 belongs

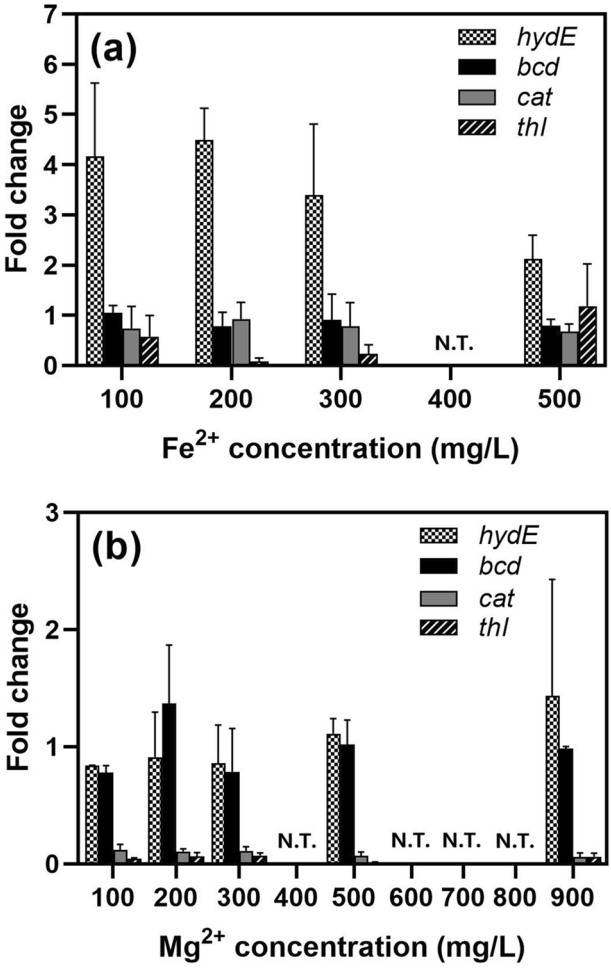

Fig. 4 Effect of Fe^{2+} (**a**) and Mg^{2+} (**b**) on the expression of key genes involved in H_2 and CA biosynthesis in strain CPB6. A value greater than 1 indicates upregulation of gene expression compared to control, while less than 1 indicates downregulation. N.T., not tested

to [FeFe]-hydrogenases group, which contains a unique iron-sulfur cluster responsible for catalytic activity [38, 39]. Fe^{2+} is likely to enhance H_2 production in strain CPB6 by increasing hydrogenase activity and influencing the catalytic process of hydrogen oxidation or production [40]. However, the addition of Fe^{2+} did not improve CA production, potentially attributed to the downregulation of *thl* gene. This gene is responsible for catalyzing the condensation of two acetyl-CoA molecules into acetoacetyl-CoA, which serves as the initial enzymatic step in CA biosynthesis [41].

Mg^{2+} is an activator for various kinases and synthases, as elucidated by previous studies [42]. Many kinases and cofactors of synthetic enzymes involved in the glycolytic process, such as hexokinase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase [40]. Consequently, Mg^{2+} plays a role in the glycolytic process by promoting the generation of pyruvate and NADH, ultimately leading to H_2 production through subsequent oxidation. Mg^{2+} is also involved in cellular processes such as cell wall and membrane composition [43] and regulation of ion channels [44]. The study by Hakobyan et al. illuminates the potential of Mg^{2+} to enhance hydrogen production in *Rhodobacter sphaeroides*, attributing

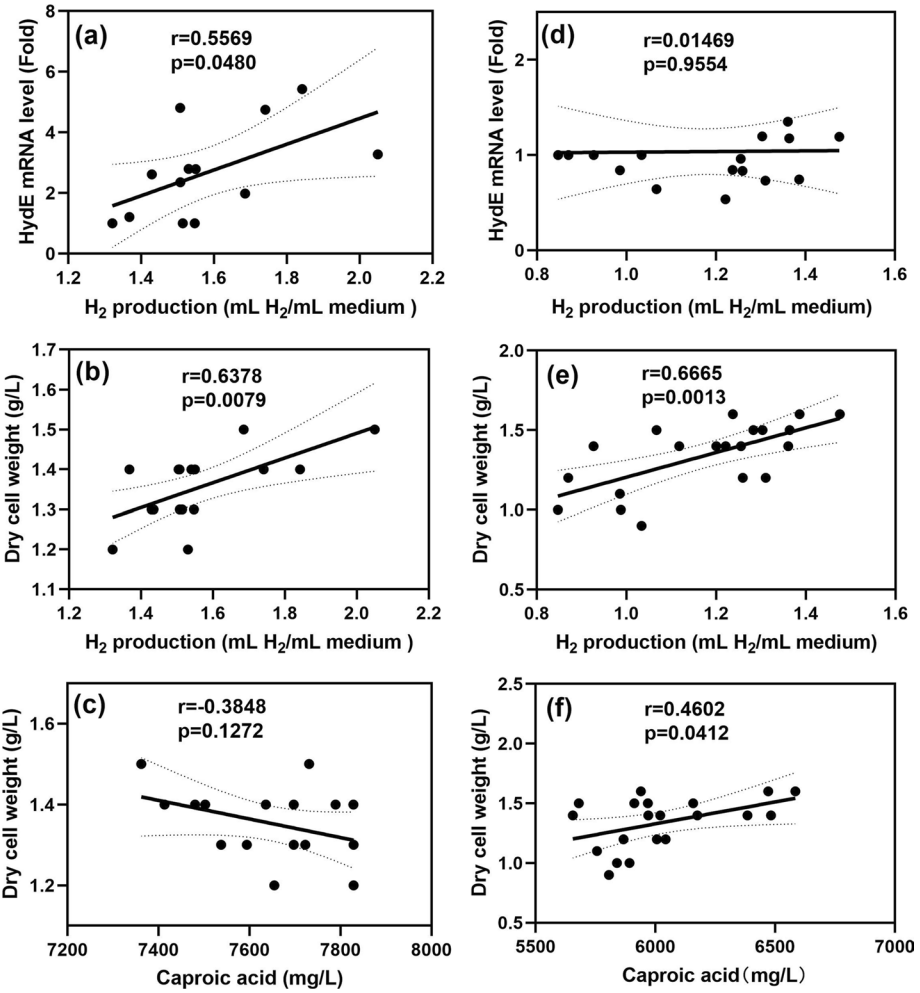

Fig. 5 Correlation analysis between metabolites and gene expression and biomass. The correlation between H_2 production and *hydE* expression (**a**) and biomass (**b**), as well as the relationship between CA production and biomass (**c**) in the presence of Fe^{2+}. The correlation between H_2 production and *hydE* expression (**d**) and biomass (**e**), as well as the relationship between CA production and biomass (**f**) in the presence of Mg^{2+}

this enhancement to the role of Mg^{2+} in improving cell growth and electron transfer [27]. Similarly, Zhao et al. demonstrate that the addition of 100 mg/L Mg^{2+} stimulates biohydrogen production and cell growth in *Clostridium beijerinckii*, albeit with a negative impact on the expression of the hydrogenase gene [45]. In our study, we observed a significant suppression of *thl* and *cat* expression in response to Mg^{2+}. However, the expression of *hydE* and *bcd* in strain CPB6 remained unaffected by the presence of Mg^{2+}. Notably, we have also observed a significant increase in both CA and H_2 production at Mg^{2+} concentrations of 300 mg/L. Although both Fe^{2+} and Mg^{2+}caused downregulation in the expression of *thl*, *cat*, and *bcd* genes involved in CA biosynthesis, the culture of CPB6 did not experience a decline in CA production. This can likely be attributed to the compensatory effect of increased cell biomass, which counterbalances the adverse effects of gene downregulation.

Conclusion

Previous studies have demonstrated the positive impact of metal ions on H_2 production and biomass in hydrogen-producing bacteria. However, this study reveals distinct effects of Fe^{2+} and Mg^{2+} on the production of CA and H_2 in the strain CPB6. Notably, Fe^{2+} and Mg^{2+} exert a greater influence on H_2 production compared to CA production. These findings suggest that Fe^{2+} and Mg^{2+} may operate through different pathways to influence H^2 and CA generation in strain CPB6. The careful optimization of Mg^{2+} and Fe^{2+} combination holds promise for achieving an optimal balance between cell growth and gene expression, thereby enhancing the production of both CA and H_2 by strain CPB6. However, further investigation is necessary to elucidate the co-regulatory effects of Mg^{2+} and Fe^{2+} on strain CPB6 and determine their optimal addition ratios.

Author Contribution Guihong Xie: writing—original draft preparation; Duo Huang and Xuemei Duan: partial data collection; Jun Liu and Siqi Yuan: data analysis and review; Yong Tao: conceiving, designing, review and editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability The data and materials presented in this study are available on request from the corresponding author.

Declarations

Ethical Approval This manuscript is a microbial fermentation study, not designed for human or animal experimentation, and therefore does not require ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate Human subjects were not designed for this study.

Consent for Publication This manuscript does not contain human study data.

Competing Interests The authors declare no competing interests.

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