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RESEARCH ARTICLE

Ferric citrate enhanced bioreduction of Cr(VI) by *Bacillus cereus* RCr in aqueous solutions: reduction performance and mechanisms

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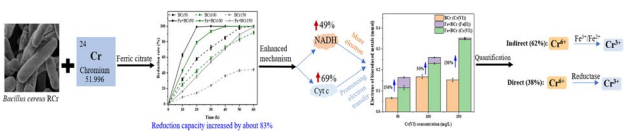
Abstract

The bioreduction characteristics and mechanisms of Cr(VI) onto *Bacillus cereus* RCr enhanced by ferric citrate were investigated. The optimum conditions were initial pH 9, temperature 40 °C, inoculation amount 4%, and glucose 3 g/L, respectively. The addition of 1.5 g/L ferric citrate increased the average reduction rate from 120.43 to 220.61 mg/(L•h) compared with the control (without ferric citrate). The binding capacity of Cr(III) on the cell surface increased to 21%, in which the precipitates were mainly CrO(OH), Cr_2O_3, and $FeCr_2O_4$. Cell membrane was the main site of reduction, related important functional groups: –COOH, C-H, $-NH_2$, C=C, and P-O. Fe(III) increased the yield of NADH and cytochrome c by approximately 48.51% and 68.63%, which significantly facilitated the electron generation and electron transfer, thus increasing the amount of electrons in the bioreduction of heavy metals by an average of 110%. Among the electrons obtained by Cr(VI), the proportion of indirect reduction mediated by Fe(III)/Fe(II) shuttle was 62% on average, whereas direct reduction mediated by reductase was 38%. These results may provide insights into the bioreduction process by bacteria enhanced by Fe(III) for detoxification of heavy metals with multiple valences, as an important step towards improving microbial remediation.

Graphical abstract



Keywords *Bacillus cereus* RCr · Cr(VI) reduction · Enhancement mechanisms · Reduction proportions

Introduction

Chromium (Cr) is widely used in tanning, electroplating, smelting and other industrial production, which primarily exists in the oxidation state of Cr(VI) and Cr(III) (Arishi

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and Mashhour 2021). Cr(VI) is a class A carcinogen designated by the WHO with strong migration abilities and solubility, which is harmful to health and the environment, thus its safety limit is 0.1 mg/L in aqueous solutions (Lin et al. 2022). The reduction of Cr(VI) to Cr(III) is recognized as the most effective method, which could be achieved by chemical, physical and biological methods (Acharyya et al. 2023). Among these methods, chemical remediation agents are quick to remediation, but easy to secondary pollution, biological remediation is environmentally friendly and stable but slow (Xia et al. 2019). The combination of biology and chemistry has the advantages of both while subtly avoiding the disadvantages (Yang et al. 2022).

In recent years, the researchers began to study chemical methods to promote microbial remediation, such as quinones (Zheng et al. 2023a), sulfur sources (Zhang et al. 2021), biochar (Huang et al. 2020), iron sources (Meng et al. 2022), and other substances that promote electron transfer, which act as electron shuttles and mediate chemical reduction. Among them, iron is the most common in nature and has excellent effects, which has attracted much attention recently. At first, it was considered that in the absence of AQDS, Fe(III) minerals alone could not accelerate the reduction due to the lack of Fe(II) from biological sources, Fe(III) minerals and AQDS synergistically accelerated the transfer of electrons from bacteria to Cr(VI), and reduction mechanism was divided into direct reduction and indirect reduction (Meng et al. 2018). Subsequent research revealed that *Shewanella oneidensis* MR-1 could chemically reduce Cr(VI) by transferring electrons to Fe(III) after adding $FeCl_3$•$6H_2O$ (Liu et al. 2019). Similarly, the addition of Fe(III) significantly improved the performance of aerobic reduction of Cr(VI) by *Sporosarcina saromensis* w5, and the removal rate was nearly complete, while the removal rate of Cr(VI) in the control group was only 61.6%, under anaerobic conditions, Fe(III) also significantly enhanced the reducing capacity (Huang et al. 2021). In addition, the other studies have indicated that ferric citrate promoted the generation and transfer of electrons by *S. oneidensis* MR-1, thus accelerating the biological reduction of Te(IV) (He et al. 2021). Recently, in practical application of adding $FeCl_3$•$6H_2O$, Fe(III) enhanced the reduction capacity of Cr(VI) in MFC-granular sludge coupling system by enhancing the system's redox capacity and electrochemical performance (Su et al. 2023). Therefore, Fe(III) has the potential to increase the reduction rate of bacteria and enhance Cr(VI) resistance. However, the relative role and relative contribution ratio of direct reduction and indirect reduction in the mechanism of Fe(III) promoting reduction by bacteria are still unclear, the effects of Fe(III) on bioreduction pathway such as electron generation and transmission has not been comprehensive, which leads to restriction of the development of regulatable Cr (VI) remediation.

The objectives of the research were (1) to determine the effects and extent of Fe(III) on the reduction of Cr(VI) in aqueous solutions by *Bacillus cereus* RCr; (2) to investigate the enhanced mechanism of Fe(III) on Cr(VI) reduction, the reducing site, and the distribution of Cr species; (3) to discuss the relative roles and relative contributions of direct bioreduction and indirect chemical reduction.

Materials and methods

Strain and chemicals

The strain was isolated from highly contaminated soil (total Cr content was approximately 6000 mg/kg) of a Cr salt-producing factory in Guangdong Province, China. Twenty-five grams of contaminated soil samples were taken from the soil 20 cm below the surface in 250-mL conical flasks, and 150 mL of deoxidized LB medium (pH = 8.5) was added, shaken, and cultured at 35 °C in an anaerobic glove box. After 2 days, the supernatant was taken for isolation and purification.

Four milliliters of Cr solution (50 mg/L, pH = 8.5) were added to the sterilized five centrifuge tubes, and 1 mL of supernatant was transferred to a No.1 centrifuge tube. After shaking well, 1 mL of the No.1 diluent was transferred to the No.2 centrifuge tube. The same operation was done in turn in other centrifuge tubes to form a diluent with a dilution factor of $10^{-1} \sim 10^{-5}$. The 10^{-5} diluent was evenly coated on the sterilized solid LB medium, and the plate was cultured at 35 °C for 1 day. The single colony was inoculated into the solid medium for line separation and purification, and the pure bacteria were inoculated into the LB medium containing Cr(VI) to test its capability to reduce Cr(VI). Finally, the most efficient reducing bacteria was obtained, and identified it by 16 s rRNA gene sequencing.

All reagents are analytically pure. The Cr(VI) stock solution (2 g/L) was prepared by dissolving 2.8291 g $K_2C_{r2}O_7$ in 500 mL of deionized water. The seed liquid was incubated anaerobically at 35℃ and static for 12 h (OD_{600}≈1.5), using sterile LB medium (pH = 8.5). All the containers used in the experiment were anaerobic bottles. After autoclaving, the bottles were immediately transferred to the sampling chamber of an anaerobic glove box, vacuum-pumped, and then replaced with high-purity nitrogen for three times. Then, the anaerobic bottles were transferred to the glove box, the configured medium was added and N_2 was injected to deoxygenate it, stock solution was added after cooling, and finally the seed liquid was inoculated and the lid with rubber plug was tightened. It was then put it into an anaerobic incubator for static culture.

Optimization of conditions

In order to optimize the conditions for the reduction of Cr(VI) by RCr, according to the previous research, the effects of interaction between temperature (35, 40, 45 °C), pH (8.0, 9.0, 10.0), glucose concentration (2, 3, 4 g/L), and inoculation amount (3%, 4%, 5%) on the reduction were studied by response surface methodology. The Box-Behnken design (BBD) was selected for four factors and three levels experimental designs; the reduction rate of Cr(VI) was took as the response value. The reaction time was 48 h, and an initial concentration of Cr(VI) was 50 mg/L.

Effects of Fe(III) on reduction of Cr(VI)

For the purpose of study, the effect of Fe(III) on reduction was that four reaction systems were set up, B: strain RCr, BF: strain RCr + ferric citrate, BCr: strain RCr + $K_2C_{r2}O_7$, Fe + BCr: strain RCr + $K_2C_{r2}O_7$ + ferric citrate. Under the optimum conditions, the effect of a 20-h reaction on reduction under different concentrations of ferric citrate was detected, the changes of reduction rate, OD_{600} and Fe(II) concentration of BCr, and Fe + BCr system with reaction time were detected. Samples were taken regularly, OD_{600} was determined by ultraviolet spectrophotometer, culture solution was filtered by a 0.22-μm filter membrane, Cr(VI) concentration was determined by 1, 5-diphenyl carbazole method (DPCI), total Cr was determined by atomic adsorption, and Fe(II) concentration was determined by o-phenanthroline spectrophotometry. Three parallel samples for each experiment are done. The formula for calculating the reduction amount of Cr(VI) is the following:

$$R = \frac{C_0 - C_e}{C_0} \times 100 \quad (1)$$

where R (%) represents the reduction rate, and C_0 and C_e are the initial and final concentrations of Cr(VI) in solution.

The changes of ORP, pH, and Cr-distribution in the system

The pH and ORP values of 50 mg/L BCr and Fe + BCr systems were measured with a pH meter every 10 h to observe changes. The distribution of Cr in BCr and Fe + BCr systems was studied as follows: The reaction system was centrifuged at 8000 rpm for 10 min to obtain supernatant and precipitate. The Cr(VI) and total Cr in the supernatant were measured as extracellular Cr(VI) and extracellular Cr(total). The precipitate was added with 1 M HCl and placed in a shaker at a rate of 150 r/min for 1 h. After centrifugation, the supernatant was taken to measure Cr(total), which is the Cr content adsorbed on the cell membrane (Gao et al. 2021). The remaining precipitate was added again with 1 M hydrochloric acid in an ice bath for 200 W ultrasonic treatment. The ultrasonic time was 3 s, the intermittent time was 7 s, lasted for 1 h. The supernatant was centrifuged to measure the total Cr to obtain the total Cr content of the cytoplasm. The distribution of Cr was calculated by the following equation:

$$Cr(III)_{extracellular} = Cr(total)_{extracellular} - Cr(VI)_{extracellular} \quad (2)$$

$$Cr(total) = Cr(III)_{extracellular} + Cr(VI)_{extracellular} + Cr_{cytoplasm} + Cr_{membrane} \quad (3)$$

Effects of NADH and cytochrome c on the reduction of cell components

To determine whether reduction process was enzyme-mediated and enzymes distribution, the cultures at later stage of logarithmic growth were divided into three parts and centrifuged at 8000 rpm for 10 min. One portion was filtered through a 0.22-μm filter and used as crude extracellular extract. The precipitate was washed three times with Tris–HCl buffer (pH = 8.5, 10 mmol/L), and then treated with a 200-W ultrasound in an ice bath for 3 s with an intermittent time of 7 s for 30 min. After ultrasonic treatment, the cytoplasmic components were obtained by centrifugation at 8000 rpm and 4℃, and the precipitate was the cell membrane component. The other two were washed three times by Tris–HCl buffer to become resting cells, and one of them was heated at 121 °C for 30 min to lose its biological activity. All samples were treated with Tris–HCl as medium and Cr stock solution to a concentration of 50 mg/L. All above were operated in an anaerobic glove box and cultured anaerobically at 40 °C for 10 h, and all reagents were free of dissolved oxygen. NADH was added to each sample of group 1 to reach 1 g/L, cytochrome c was added to each sample of group 2 to 1 g/L, and the control group was not treated.

Determination of relevant metabolites

To study the effect of Fe(III) on the growth and metabolism of strain RCr in the presence of Cr(VI). NAD^+ and NADH were analyzed using the WST assay kit (Beyotime Biotechnology) according to the instructions. Cytochrome c was determinated as follows: cells were collected by centrifugation at 8000 rpm for 5 min, then washed three times with 50 mM PBS buffer (pH 7.40), and then resuspended in 2 mL of the solution (1 mg/mL lysozyme, 20% (w/v) sucrose, 0.01 M Tris, pH 8.40) at 25 °C in an oscillating incubator at 141 r/min for 1 h. After centrifugation

at 8000 rpm for 10 min, the cells were resuspended in the Tris-Mg^{2+} solution (0.01 M $MgCl_2$, 0.01 M Tris, pH 8.40) and centrifuged at 8000 rpm for 5 min at 25 °C (Zheng et al. 2023b). The supernatant was collected and the concentration of cytochrome c was measured by adsorbance at 520 nm using an ultraviolet spectrophotometer (UV-5200, Metash instruments).

Characterization of reduction products

To analyze the changes of cell surface after reduction, scanning electron microscope (SEM, Hitachi SU8010) and energy dispersive spectrometer (EDS, OXFORD X-MAX 80) were used for observation, and sample preparation referred to previous studies (Mohamed et al. 2020). From the harvest to the dehydration step, the TEM sample was prepared the same as the SEM sample, and then the bacterial cells were embedded in the resin (Sun et al. 2023a). Ultrathin sections of embedded bacteria were obtained using an ultramicrotome (Leica UC7, Germany) and stained with uranyl acetate and lead citrate. Finally, the samples were observed using a JEM-1200EX at 80 kV.

XPS, XRD, and FTIR were used to characterize the reaction products, surface functional groups, and the valence states of related elements on the cell surface. The sample preparation referred to previous studies (Sun et al. 2020). Strain RCr grew with 100 mg/L Cr(VI) under anaerobic condition. After 72 h, cells were collected by centrifugation at 10,000 rpm for 10 min and washed twice with phosphate-buffered solution (PBS). The collected particles were freeze-dried in a vacuum freeze dryer (BIOCOOL) and then ground into powder using a mortar and a pestle. The powder was analyzed by FTIR (Thermo Nicolet 5700). The scanning spectral range was 500 ~ 4000 cm^{-1} (Yan et al. 2021). XPS was performed on a Thermo Fisher (Nexsa G2 X). XRD was performed on LabX XRD-6100 (Shimadzu).

Quantification of mechanisms of reduction

Three reaction systems were added in this experiment: Fe + BCr + P: ferric citrate + bacteria + chromate + phenanthroline; BCr + P: bacteria + chromate + phenanthroline; Fe + B + P: ferric citrate + bacteria + phenanthroline (phenanthroline 2.5 g/L).

The reaction of Fe + BCr and Fe + BCr + P began at the same time under the same conditions. When the Cr(VI) of the former reacted completely (reaction time according to 2.3), the Cr(VI) concentration of the latter was measured, and the amount of electrons transferred by bioreduction could be calculated. The amount of electrons transferred by chemical reduction could be obtained by subtracting the amount of electrons transferred by bioreduction from the total amount of electrons required for Cr(VI) reduction (Zheng et al. 2023a).

The concentration of Fe(II) in the complete reaction of Cr(VI) in Fe + BCr system was measured, and the reduction amount of Cr(VI) in BCr system at the same reaction time was used as a control. Thus, the number of electrons transferred by bioreduction of heavy metals could be calculated.

By comparing the Fe(III) reduction rate of Fe + B with or without P and the Cr(VI) reduction rate of BCr with or without P, transferring the number of electrons to a reduced metal was calculated to verify the feasibility of the experimental method.

Statistical analysis

All experiments were carried out in triplicate. The error bars represent standard deviations of triplicate measurement. IBM SPSS Statistics 26 was used for one-way analysis of variance, and Waller-Duncan was used for significant difference analysis; the lowercase letters in the figure were significant differences ($p < 0.05$).

Results and discussions

Screening and isolation of bacteria

After several rounds of separation and purification, the strain named RCr which had the highest reduction efficiency was applied for subsequent experiments. After 16S rRNA gene sequencing, the strain RCr was confirmed as *Bacillus cereus*, and a phylogenetic tree of it was constructed based on genetic similarities (Fig. S1). Its preservation number is GDMCC No: 63556.

Optimization of conditions

With the increase of the values of four more sensitive factors of Cr(VI) reduction, the response value increased first and then decreased. When the pH was 9.23, inoculation amount was 4.3%, temperature was 40.4 °C, carbon source (glucose) was 3.05 g/L, and the response value of maximum reduction rate could reach 97.06%, which was the best reduction condition (Fig. 1). Under this condition for 50 mg/L Cr(VI), the addition of different amounts of iron citrate can accelerate the reduction, and the promotion effect was the best at 1.5 g/L (Fig. S2). Therefore, all subsequent experiments were performed with pH 9, inoculation amount 4%, temperature 40 °C, carbon source (glucose) 3 g/L, and iron citrate 1.5 g/L, respectively, unless stated otherwise.

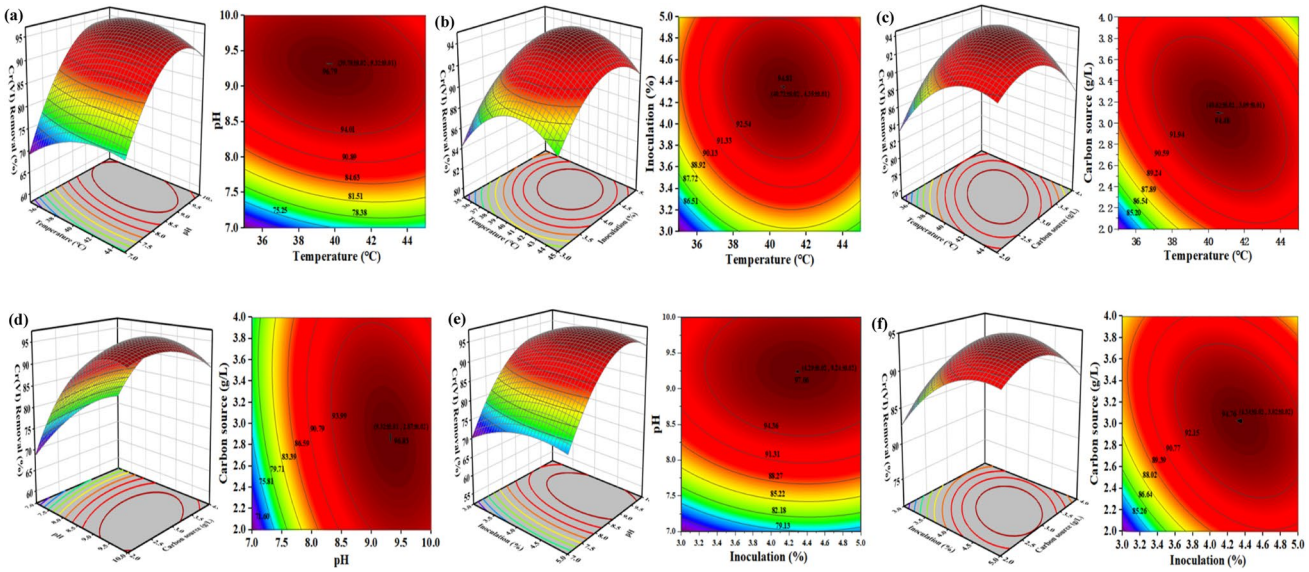

Fig. 1 Effect of bacterial reduction of Cr(VI) under different treatments (response surface methodology)

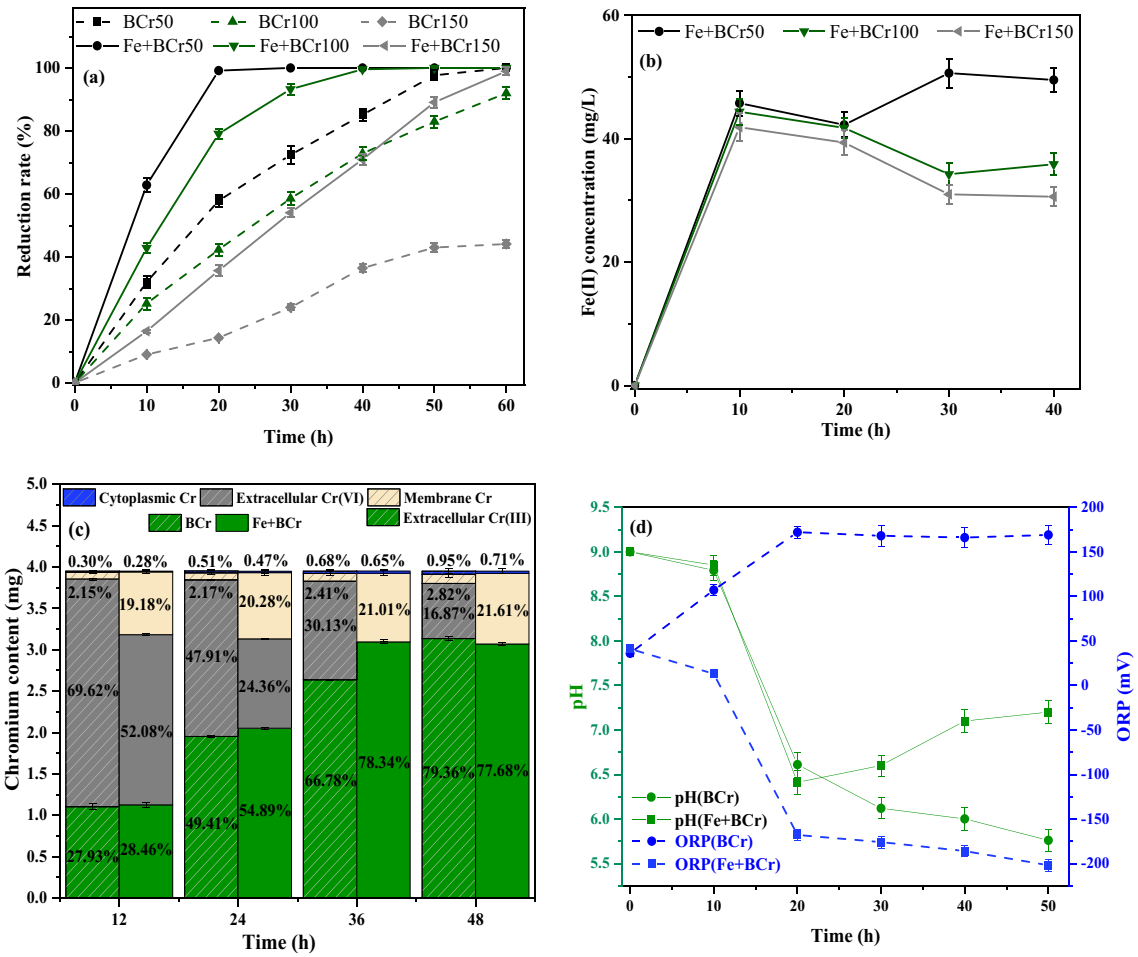

Fig. 2 (**a**) Effect of Fe(III) on the reduction of Cr(VI). (**b**) Fe(II) concentration under the optimal Fe(III) dosage. (**c**) Effect of Fe(III) on distribution of chromium. (**d**) Changes of pH and ORP of reduction systems

Effects of Fe(III) on reduction

In Fe + BCr system (Fig. 2a), under low, medium, high concentrations of 50, 100, and 150 mg/L, Cr(VI) reacts completely in approximately 20, 40, and 60 h, which is faster than BCr system. Fe(III) increased the average reduction rate from 120.43 to 220.61 mg/(L•h), which increased by approximately 83.19%, the amount of chromium reduction and bacterial biomass also increased significantly (Fig. S3), especially under high concentration of Cr(VI), the addition of Fe(III) reduced the stimulating effect of Cr on bacteria, enhanced the Cr resistance of bacteria, and promotes the reduction. Fe + BCr system produced approximately 40 mg/L Fe(II) (Fig. 2b). It means that the bioreduction rate of Fe(III) was always higher than that of Cr(VI). Therefore, the Fe(III)/Fe(II) shuttle speed is accelerated, and the proportion of indirect reduction is increased.

During the reduction of the BCr system, especially at 10 ~ 20 h when the cells are in logarithmic growth period (Fig. S3), pH decreased from 9 to approximately 6 (Fig. 2d), which may be due to the consumption of OH^- and the production of Cr(III) by cell metabolism. The decrease of pH made more CrO_4^{2-} convert into $HCrO_4^-$ and increased ORP (Jobby et al. 2018).

In Fe + BCr system, pH decreased to 6.5, then rise to 7.0. At 20 h, most of Cr(VI) was reduced and the cells have entered a stable period, when reproduction and growth were slowed down, and the decaying cells would also release OH^- (Dhal et al. 2013). Under acidic conditions, Cr(VI) reacted with Fe(II) more vigorously, which increased the consumption of H^+, so the pH began to rise. Due to accumulation of Fe(II) and slow consumption of OH^-, ORP continued to decline, forming a stronger reduction environment.

The Cr distribution of the two systems at 100 mg/L was compared. The Cr(VI) of the bacterial pickling solution of the two systems was below the detection limit, so the total Cr concentration of the pickling solution was tested in Fig. 2c. More Cr(III) was immobilized on the strain of the Fe + BCr system, probably because Cr(VI) reacted with Fe(II) adsorbed on the cell surface to produce more inorganic micro-precipitation. The reduction products were all mainly distributed in the suspension and mainly existed as organo-Cr(III) complexes (Huang et al. 2021). It is speculated that the main reduction process of Cr(VI) is that the chromate contacts the reductase successively to obtain electrons, and then complexes with the organic matter in the solution.

Cr in the cytoplasm increased gradually in both treatments, but was always less than 1%, presumably because CrO_4^{2-} entered the cell via the ionically similar SO_4^{2-}/PO_4^{2-} channel and exited from the cell after intracellular reduction, and the product accumulated less in the cell (Pushkar et al. 2021). The intracellular Cr content of Fe + BCr system is lower than that of BCr system, which may be because Fe(III) enabled bacteria to produce more teichoic acid (Lian et al. 2022), adsorbed a large amount of Fe(II), and reacted with approaching CrO_4^{2-}. At the same time, more inorganic micro-precipitation generated also played a blocking role for contact reduction (Li et al. 2020). The Fe + BCr system became acidic after 20 h,

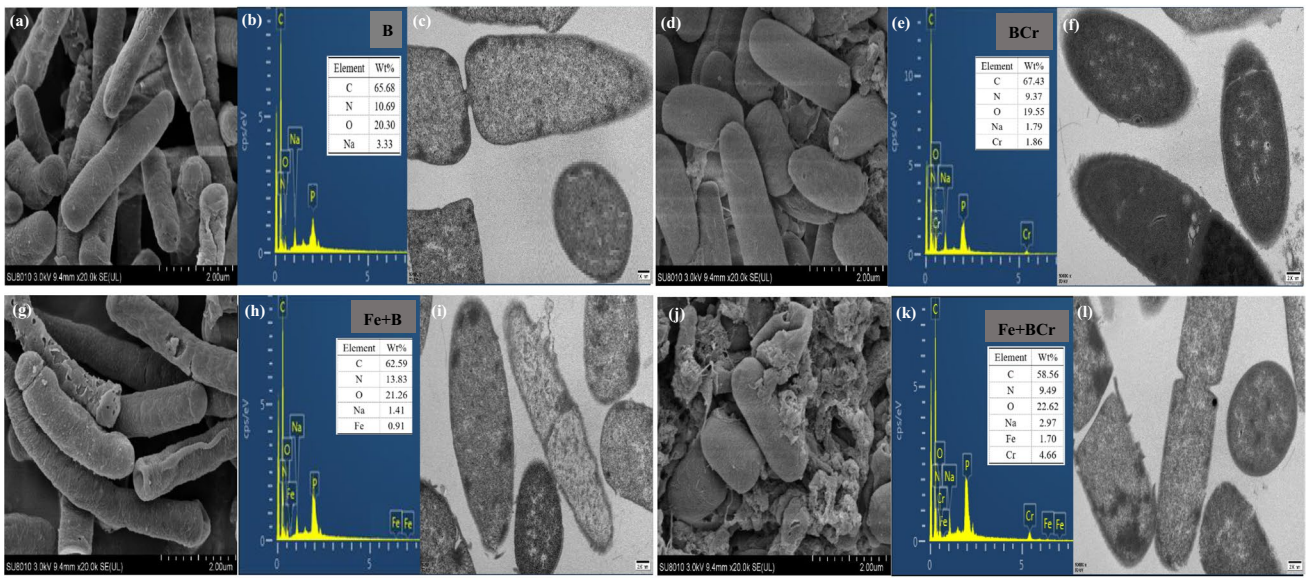

Fig. 3 Microscopic investigations of strain RCr under different treatments. (**a**, **d**, **g**, **j**) bacterial SEM images; (**b**, **e**, **h**, **k**) EDS spectra of bacteria; (**c**, **f**, **i**, **l**) TEM images of bacteria

which made the chemical reduction between active Fe(II) and $HCrO_4^-$ became faster.

Characterization of reduction products

Untreated strain RCr was rod-shaped, and the surface with flagella was full and smooth (Fig. 3). After treated with 100 mg/L Cr(VI) for 48 h, bacteria became smaller and began to gather, particles increased on the surface, cell membrane, and other structures were no longer clear; meanwhile, obvious precipitates appeared. Moreover, the EDS analysis confirmed the presence of Cr precipitates in the bacteria. After treatment with 1.5 g/L ferric citrate alone for 48 h, scaly precipitates appeared on the surface of bacteria, and a small number of precipitates appeared near the inner membrane of the cell. Besides, the EDS analysis also showed that iron precipitates existed in the bacteria. After treatment with 100 mg/L Cr(VI) and 1.5 g/L ferric citrate for 48 h, a large amount of particles appeared on the surface of the bacteria, and less precipitates appeared in the cells, which could be Cr and iron precipitates combined with EDS analysis.

Crystal mineral composition of cell surface was analyzed by XRD (Fig. 4a). The precipitate in B system was mainly salt. In BCr system, inorganic micro-precipitates such as hydroxyl Cr and Cr oxide appeared. In Fe + B system, the precipitate was mainly basic ferrous carbonate. And in Fe + BCr, more $FeCr_2O_4$ appeared in the system, which may be produced by the reaction of Fe(II) produced by bioreduction with $HCrO_4^-$. Fe(III) converts part of the reduction product into stable chemical raw material, which is an environmentally friendly and inexpensive preparation method, and has certain application potential.

FTIR was used to study the changes of functional groups on the surface of bacteria (Fig. 4b). Compared with B, the – OH/N–H adsorption peak intensity of BCr near 3414 cm^{-1} was weakened, indicating that they had complex reactions with Cr, and the intensity of P-O adsorption peak of B + Fe near 1050 cm^{-1} increased significantly, which may be due to the fact that iron ions promoted the pyruvate phosphate cleavage reaction of bacteria. C = C adsorption peak of B at 1618 cm^{-1} disappeared in the other three systems, indicating that iron ions and Cr(III) could be complexed with it. The strengths of – OH/N–H adsorption peak, $-CH_2$ adsorption peak near 2930 cm^{-1}, – COOH adsorption peak near 1545 cm^{-1}, C = O adsorption peak near 1451 cm^{-1}, and C-O adsorption peak near 1241 cm^{-1} of the system with Fe(III) were significantly weaker than that of the system without Fe(III), indicating that iron ions are more easily complexed than Cr(III). The intensity of P-O adsorption peak of B + Fe near 1050 cm^{-1} increased significantly, which may be due to the fact that iron ions promoted the pyruvate phosphate cleavage reaction of bacteria.

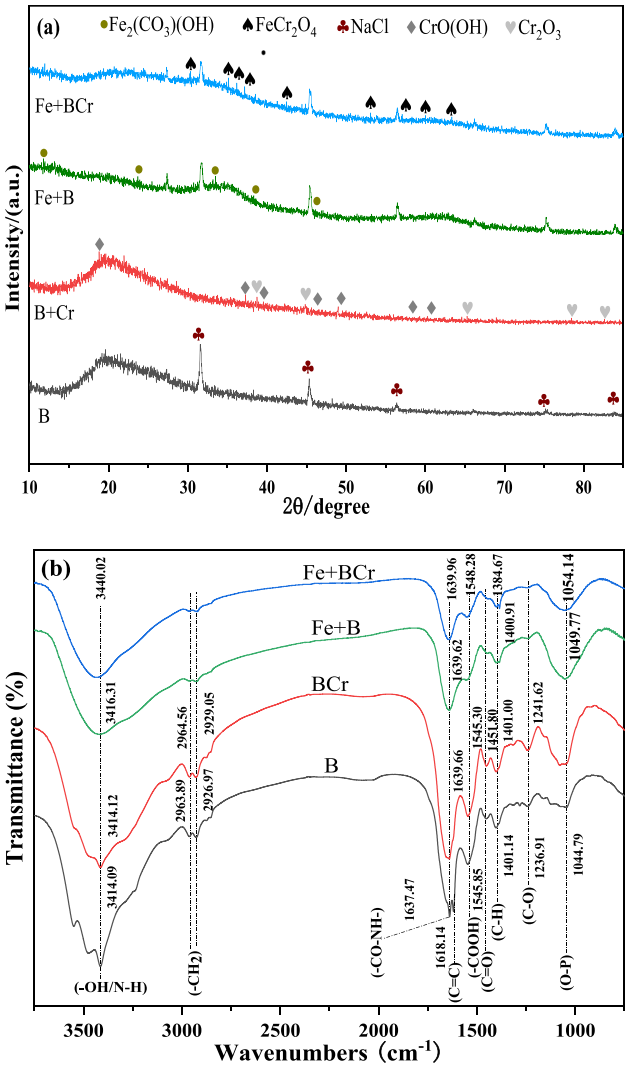

Fig. 4 XRD (**a**) and FTIR (**b**) of strain RCr under different treatments

XPS was used to analyze the elemental composition and electronic structure of the cell surface of the four systems (Fig. 5). In the C1s spectrum, after adding Fe(III), the C = O peak disappeared and completely complexed with iron ions. Compared with B, the proportion of C–C/C-H peaks in Fe + B decreased by 5.61%, and the C = O peak of BCr reacted less with Cr(III); the content decreased by 2.01%. In the O1s spectrum, compared with B, the proportion of C = O peak and C-O peak in BCr decreased by 6.25 and 3.11%; for Fe + B, the CO_3^{2-} peak disappeared; the proportion of C = O peak decreased by 26.42%; the complexation of iron ions was mainly carbonyl group (in line with infrared analysis); and the FeO peak (530.71) increased by 26.05%, the Fe_2O_3 peak (529.80) increased by 7.93%, while for Fe + BCr, the iron–oxygen peaks disappeared, which verified that the addition of Fe(III) promoted the production of more Fe–Cr

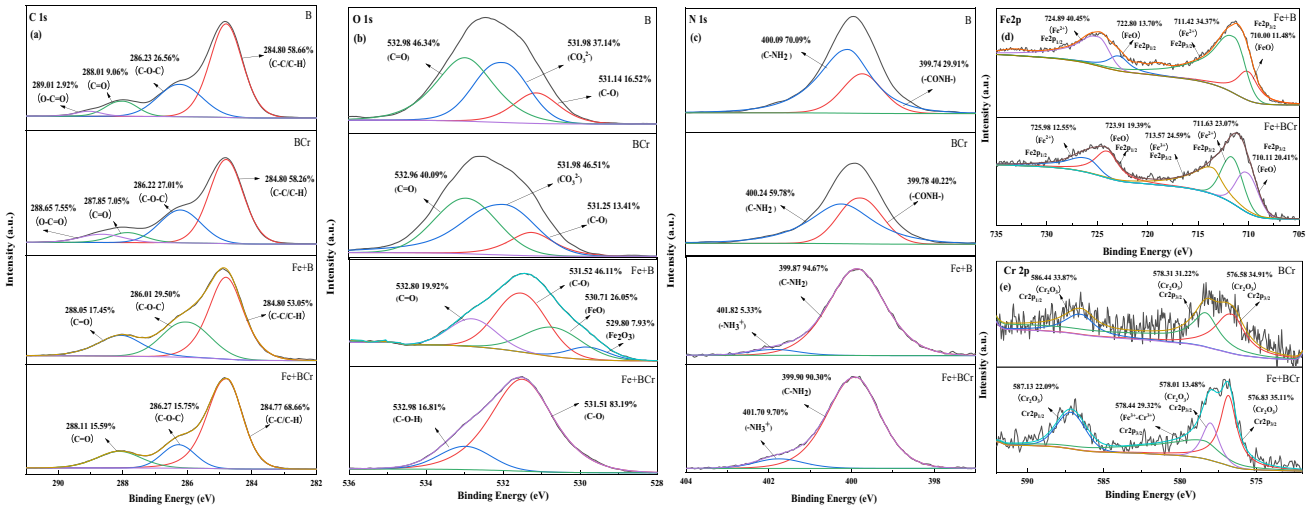

Fig. 5 XPS spectra of strain RCr under different treatments (**a**: C 1 s, **b**: O 1 s, **c**: N 1 s, **d**: Fe 2p, **e**: Cr 2p)

micro-precipitation and reduced the complexation of oxygen-containing functional groups.

In the N1s spectrum, C-NH_2 decreased by 10.31% in BCr compared with B, indicating that amino group reacted with Cr. After Fe(III) addition, the – CONH – peak disappeared, and all reacted with iron ions. At the same time, the proportion of C-NH_2 in Fe-containing systems exceeded 90%, and the C-NH_2 of Fe + BCr only decreased by 4.37% compared with Fe + B, indicating that the presence of iron ions weakened the complexation of Cr. In the Cr2p spectrum, both Fe + BCr and BCr produced more Cr_2O_3, Interestingly, 29.32% of Fe^{2+}-Cr^{3+} compounds were produced after Fe(III) was added.

In the Fe2p spectrum, compared with BF, the proportion of Fe(II) in Fe + BCr decreased from 74.82 to 35.62%. Among them, 24.59% of Fe(II) reacted with Cr(VI) to generate Fe(III), and 14.61% was converted into FeO. FeO is mainly a chemical state formed by complexation with oxygen-containing functional groups and chemical precipitation. The solubility of Fe(III) is very low, so the above iron ions are mainly Fe(II). This is consistent with the results of FTIR and XRD analysis.

Effects of Fe(III) on NADH and cytochrome c

Through the reduction experiment of each component of the cell (Fig. 6a), the reduction characteristics of the strain and its components could be clarified, and the main reduction mode of Cr(VI) under anaerobic conditions could be further determined. Comparing with resting cell, the bacteria sterilized at 121℃ had almost no reducing capacity, indicating that strain RCr was mainly related to the enzyme reduction. Additionally, after treatment with 1% SDS, the membrane was destroyed, and the membrane proteins were encapsulated, which became permeable cells, almost lost the reducing ability, probably because the reaction chain related to the cell membrane was interrupted, while the cell membrane was the main site of reduction (Sun et al. 2023b).

The reduction activity of extracellular secretions to Cr(VI) (5.0%) was slightly higher than that of membrane (3.8%) and cytoplasm (3.9%), indicating that the reductases produced by the cytoplasm were mainly secreted into extracellular as secretory enzymes. The reduction rate of resting cells was 18.2%, which was much higher than that of cytoplasm and cell membrane, much lower than that of the complete system (31.9%), indicating that there was a synergistic effect between cytoplasm, cell membrane, and extracellular fraction during the reduction process.

NADH and cytochrome c are important coenzymes in cells, which play an important role in electron generation and electron transfer (He et al. 2021), so the effects of NADH and cytochrome c on the reduction of each component of cell were analyzed. After adding NADH, the reduction rates of extracellular secretions and cytoplasm increased significantly from 5.0 and 3.9% to 31.1 and 18.1%, it was speculated that a large number of reduction-related enzymes in cytoplasm and secretions used NADH to reduce Cr(VI) inside and outside the cells, and the main product of organo-Cr(III) complexes were excreted from the cells (Gong et al. 2018). The increase in the reduction rate of membrane fraction was relatively small (3.8 to 5.9%), more metabolic enzymes in cells may be distributed on it (Kaila and Wikström 2021). After addition of cytochrome c, the reduction rate of cytoplasm fraction and extracellular secretions was improved slightly, from 3.9 to 5.0% and 5.0 to 9.1% respectively, while the membrane fraction was almost unchanged, possibly due to cytochrome was mainly distributed on the

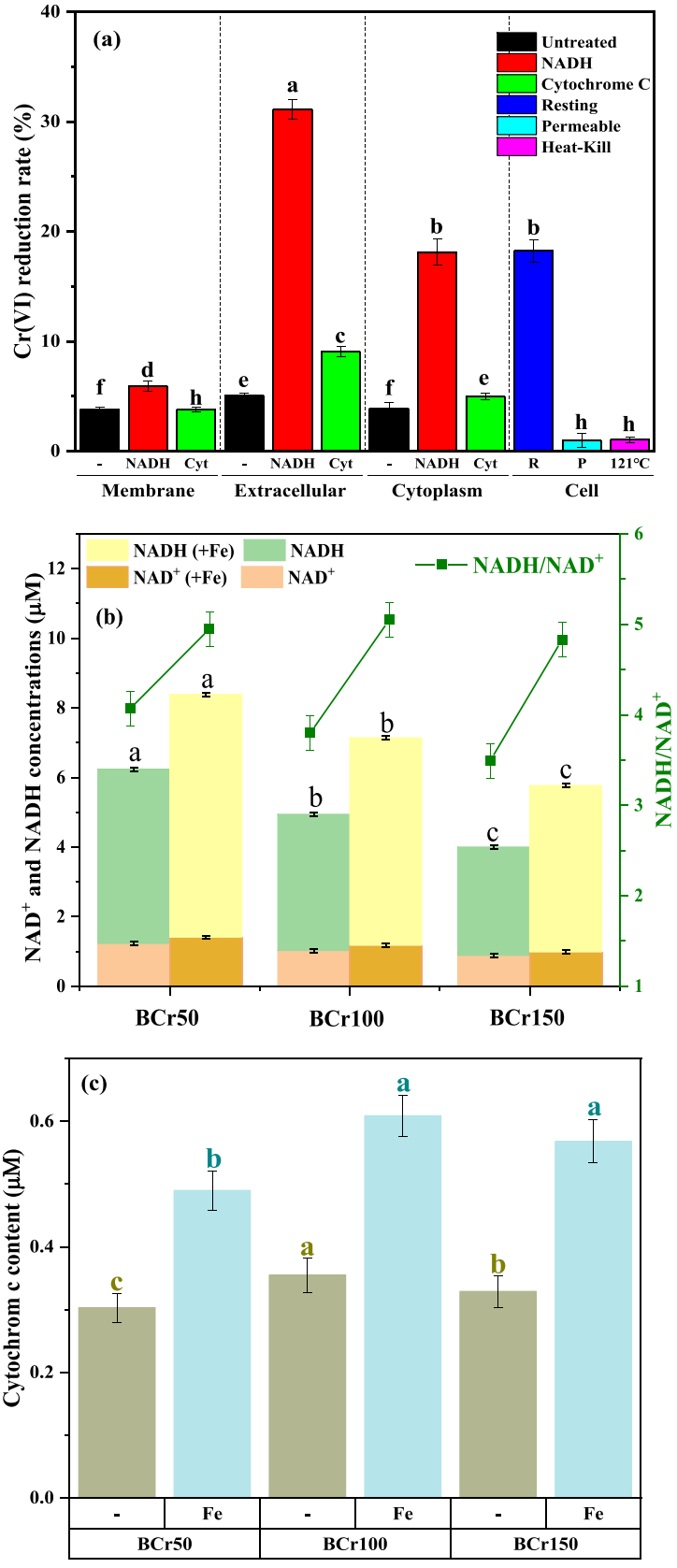

Fig. 6 (**a**) Effect of NADH and cytochrome c on Cr(VI) reduction of cell components. (**b**, **c**) Effect of Fe(III) on NADH and NAD^+, cytochrome c

cell membrane, which accelerated electron transfer between enzymes. In summary, NADH and cytochrome c were key factors in accelerating the reduction.

As shown in Fig. 6b, the addition of Fe(III) increased the capability of bacteria to produce electrons (NADH) by 48.51% on average, indicating that the intracellular metabolic reaction was enhanced. NADH/NAD^+ increased by 32% on average, indicating that reducing capacity of cells was greatly enhanced forming a strong reducing state. Cytochrome c is considered to be an important enzyme for electron transfer in cells (Han et al. 2016). It increased by 68.63% on average (Fig. 6c), which is beneficial to accelerate the electron transport rate. With the increase of Cr(VI) concentration, electron generation became more effective; nevertheless, the effect of electron transfer became weak.

Quantitative analysis

After 20, 40, and 60 h of reaction, feasibility study results of experimental methods showed that there was no significant difference in the effect of phenanthroline on the reduction of Cr(VI) and Fe(III) by strain RCr (Table. S1). Therefore, it is feasible to use phenanthroline to mask Fe(II). A small amount of phenanthroline have no inhibitory effect on the growth of bacteria and have no effect on metabolism (El-Attar et al. 2023). The antibacterial activity of phenanthroline metal chelates is not strong (Mahmoud et al. 2023).

Total electron quantity of dissimilating metals in Fe + BCr system was improved by 110% on average than that in BCr system (Fig. 7a). This suggested that more electrons on the dissimilatory metal reductases, and the electron transfer rate between enzymes became faster, quite possible because that the redox cycle of Fe(III)/Fe(II) as an electron shuttle was a rapid single electron transfer, which could accelerate the electron transfer between the bacteria and the terminal electron acceptor Cr(VI); meanwhile, iron ions in solution were easier to bind to negatively charged bacteria and secretory enzymes than chromate anions.

At medium and high concentrations, indirect reduction became the main mechanism of Cr(VI) reduction in Fe + BCr, the proportion was 68% and 77% (Fig. 7b). This may be because the system with Fe(III) was initially alkaline, which made the chemical reaction rate of Fe(II) and Cr(VI) was slow, and accumulated a large amount of Fe(II). With the progress of the reaction, pH decreased, made the biological reduction was inhibited, while the chemical reaction of Fe(II) and Cr(VI) was more rapid, so the concentration of Fe(II) decreased, but Fe(II) had not reacted completely; the rate of bioreduction of Fe(III) was always greater than Fe(II) reducing Cr(VI). Therefore, the Fe(III)/Fe(II) shuttle speed was accelerated, which increased the proportion of indirect reduction.

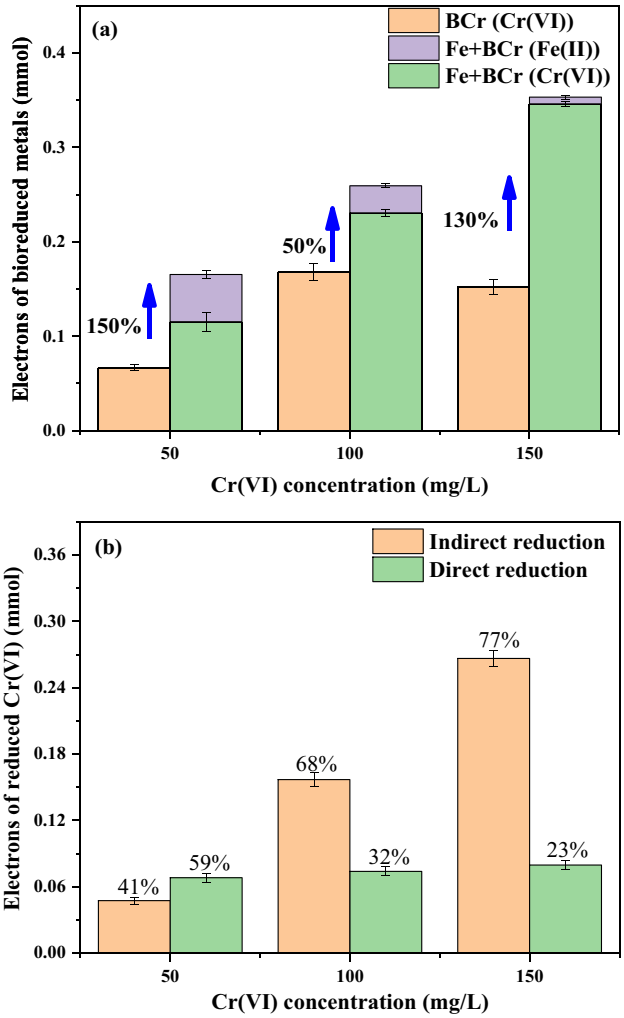

Fig. 7 (**a**) Total electrons of bioreducing metals in two systems. (**b**) Electrons transfer proportion in direct/indirect reduction of Cr(VI) in Fe + BCr system

Mechanisms of Fe(III) promoting bioreduction of Cr(VI)

Based on the above experimental analysis and characterization, mechanism of Fe(III) promoting the reduction of Cr(VI) by strain RCr was summarized (Fig. 8). First, Cr(VI) mainly relied on enzyme-mediated bioreduction. NADH was produced by metabolic reaction (Li et al. 2017). The intracellular and extracellular reductases used the electrons of NADH to reduce Cr(VI), and large amount of extracellular reductases were secreted to reduce Cr(VI) by using the electrons on their own surface or obtained on the cell surface (Bollella and Katz 2020). After adding Fe(III), the growth and reproduction of bacteria were promoted, the secretion of NADH and cytochrome c was increased, the generation and transfer of electrons were accelerated, and the amount

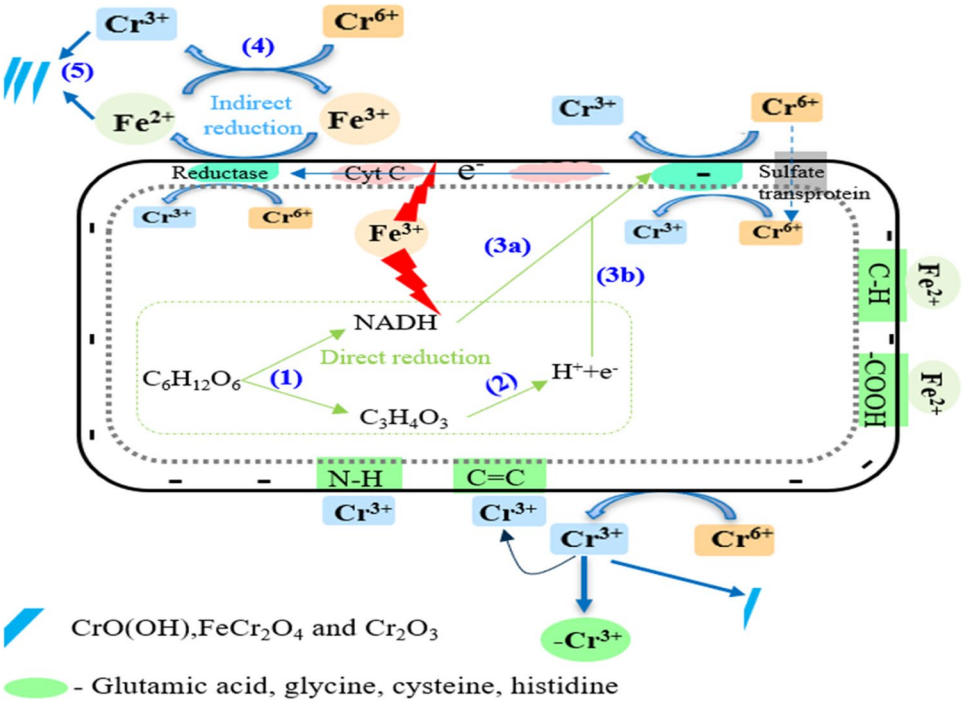

Fig. 8 Main electron transport pathways of Fe(III) promoting strain RCr for Cr(VI)-reduction

of electrons for biological reduction of Cr(VI) (direct reduction) and Fe(III) was increased.

On the other hand, Fe(III) was easier to contact with the negatively charged bacterial surface than chromate, and the Fe(III)/Fe(II) shuttle was faster. Especially after 20 h of reaction, the system had become acidic and the chemical reduction was strengthened, resulting in the indirect reduction mediated by Fe (III) was higher than the number of electrons transferred by direct biological reduction at medium and high concentrations, and the proportion of indirect reduction transfer electrons increased. Fe(III) promoted the electron production and transmission of bacteria to enhance the reduction of heavy metals, whereas whether bacteria would chemically reduce high-valent metals by bioreduction of Fe(II) produced by Fe(III) did not explore (He et al. 2021).

Furthermore, Fe(III) minerals need AQDS to enhance bacterial reduction rate, possibly because the reduction environment of minerals is alkaline and less iron ions are produced (Meng et al. 2022). However, after adding ferric sulfate, the production of Fe(III) and Fe(II) would not be conducive to the reduction of Cr(VI) until Cr(VI) was completely reduced by *Acidocella aromatica* strain PFBC in an extremely acidic environment with minimal oxygen, which may be because under extremely acidic conditions, bacterial growth is inhibited, metabolism is slow, and electron production is very low (Masaki et al. 2015).

Typical electron donor glucose was used as an example to analyze the pathway of Fe(III) promoting reduction: glucose entered the cell through the glycolysis pathway (EMP) to produce NADH, pyruvate, and energy (the main production mode in anaerobic environment), and these products were involved in the respiratory chain of the cell membrane. Part of CrO_4^{2-} could enter cells via SO_4^{2-} and PO_4^{2-} channels and gradually be reduced by intracellular NADH-dependent reductase (Banerjee et al. 2019). Pyruvic acid produced acetyl phosphate under the action of ferredoxin pyruvate oxidoreductase (FTIR found the vibration of the peak of phosphorus acyl group) and release CO_2 and H_2, H_2 produced electrons and hydrogen ions under the action of hydrogenase, electronegative hydrogenase contacted cytochromes, made it into a reduced cytochromes, various cytochromes connected to transfer electrons to the chromate reductases of cell membrane, and CrO_4^{2-} could contact terminal intracellular or extracellular chromate reductase to get electrons. This part of the biological reduction process was enhanced under the action of Fe(III). Strain RCr could simultaneously reduce Fe(III) and Cr(VI), forming Fe(III)/Fe(II)-mediated electron shuttle (indirect reduction) and bioreduct directly in contact with Cr(VI), especially in the logarithmic growth phase; the system become acidic and the indirect reaction become more intense. Finally, produced soluble organo-Cr(III) complexes precipitates such as CrO(OH), Cr_2O_3, Fe(II), and $FeCr_2O_4$ (Eqs. (1)-(5)) (Gong et al. 2017; Paavilainen et al. 1999; Huang et al. 2016, 2019; Pradhan et al. 2017).

(1) EMP (Embden -Meyerhof-Parnas) pathway:

$$C_6H_{12}O_6 + 2NAD^+ + 2ADP + 2Pi \xrightarrow{\substack{Metabolic \\ enzyme}} 2C_3H_4O_3 + 2NADH + 2H^+ + 2ATP + 2H_2O$$

(2) Pyruvate fermentation:

$$CH_3 - CO - COOH + H_3PO_4 \xrightarrow{\text{Pyruvate ferredoxin oxidore ductase}} CH_3 - CO - P + CO_2 + H_2$$

$$H_2 \xrightarrow{\text{Hydrogenase}} 2H^+ + 2e^-$$

(3) Bioreduction of Cr(VI):

$$13H^+ + 2CrO_4^{2-} + 3NADH \xrightarrow{\text{Cytochrome c, Cr reductase}} 2Cr^{3+} + 3NAD^+ + 8H_2O \quad (a)$$

$$8H^+ + CrO_4^{2-} + 3e^- \xrightarrow{\text{Hydrogenase, cytochrome c, Cr reductase}} Cr^{3+} + 4H_2O \quad (b)$$

(4) Fe(III)/Fe(II)-mediated electron shuttle reduction (Fe(II) was produced by bioreduction of Fe(III)):

$$3Fe^{2+} + HCrO_4^- + 7H^+ \rightarrow 3Fe^{3+} + Cr^{3+} + 4H_2O$$

$$6Fe^{2+} + 2CrO_4^- + 8H^+ \rightarrow 6Fe^{3+} + Cr_2O_4^{2-} + 4H_2O$$

(5) Main products:

$$Cr^{3+} + 3OH^- \rightarrow CrO(OH) + H_2O$$

$$2CrO(OH) \rightarrow Cr_2O_3 + H_2O$$

$$Fe^{2+} + Cr_2O_4^{2-} \rightarrow FeCr_2O_4$$

Conclusions

Under the optimal reduction conditions, Fe(III) could sustainably enhance the strain's bioreduction and resistance to Cr(VI). Moreover, Fe(III) was helpful for the precipitation, mainly $FeCr_2O_4$, CrO(OH), and Cr_2O_3 that were attached to the cell surface, while more than 80% of the reduction products were still organo-Cr(III) complexes. Cell membrane was the main site of Cr(VI) reduction, and Fe(II) and Cr(III) were mainly complexed with C-H, – COOH and N–H, and C = C respectively. Above all, Fe(III) increased the secretion of cytochrome c and NADH, promoted the generation and transmission of electrons, and increased the amount of electrons reducing heavy metals. To Cr(VI), indirect reduction accounted for 62% and direct reduction accounted for 38%. The ingenious combination of biochemistry to promote reduction is one of the promising methods, and Fe(III)/Fe(II) is a good shuttle.

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Author contribution Yishuo Wang: writing—original draft, visualization. Fei Huang: writing—review and editing. Jiaxin Liu: data curation. Haolin Li: methodology. Jinjing Bai: investigation. Peng Wang: resources. Xin Rao: conceptualization. Mingzhi Huang: writing—review and editing. Rongbo Xiao: writing—review and editing. Qianjun Liu: supervision. Xiao Zhou: methodology.

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Data availability Data will be made available on request.

Declarations

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Competing interests The authors declare no competing interests.

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