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RESEARCH ARTICLE

Hexavalent chromium reduction and bioremediation potential of *Fusarium proliferatum* S4 isolated from chromium-contaminated soil

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Abstract

Microbial remediation, utilizing reduction of Cr(VI) to Cr(III), is considered a promising method for lowering toxic environmental chromium levels. In this study, a Cr(VI)-resistant fungal strain, *Fusarium proliferatum* S4 (*F. proliferatum*), was isolated from seriously chromium-polluted soil at Haibei Chemical Plant, China. This strain for treatment chromium-containing solution resulted in 100.00%, 93%, and 74% removal at initial concentrations of 10, 30, and 50 mg L^{-1} Cr(VI), respectively, after 12 days of treatment in a batch mode. Contributions of different cell fractions to Cr(VI) removal were explored. The Cr(VI) removal capacity of various cell components from strong to weak was as follows: cytoplasm, cell secretions, and cell debris. Observations obtained by scanning electron microscopy and transmission electron microscopy with energy dispersive X-ray spectroscopy revealed that not only the cell surfaces but also the intracellular contents were involved Cr through adsorption, reduction, or accumulation. Fourier transform infrared spectra indicated that a large number of functional groups (amino, carbonyl, carboxyl, and phosphate groups) participated in chromium binding on the cell surface. X-ray photoelectron spectroscopy confirmed the presence of Cr on the cell surface only as Cr(III). The results have important implications for an in-depth understanding of microbial chromate reduction by *F. proliferatum*. This study provides an insight into the microbial Cr(VI) bioreduction efficiency, and mechanisms in the chromium-contaminated environment.

Keywords *Fusarium proliferatum* · Chromium · Bioremediation · Reduction mechanism

Introduction

Chromium (Cr) is an important industrial pollutant, which is extensively used in leather tanning, stainless-steel production, plating chrome, and electroplating (Kantar et al. 2008). Although chromium can exist in different oxidation states (+2 to +6), Cr(III) and Cr(VI) are the most dominant stable states in natural systems (Dhal et al. 2013; Rai et al. 1987). Cr(III) mainly exists in the forms of $Cr(OH)^{2+}$, $Cr(OH_3)$, $Cr(OH)_4^-$, and $Cr(OH)_5^{2-}$, which is much less toxic than Cr(VI) (Lu et al. 2017). Cr(VI) can have extremely negative effects on all forms of living organisms at elevated concentrations due to its strong oxidation and high carcinogenic properties, which has become the second most common metal hazard to human beings and ecosystems (Bennett et al. 2013; Lai et al. 2016). At present, Cr(VI) has been listed as a priority pollutant in different regulatory agencies worldwide including the United States Environmental Protection Agency. Therefore, it remains a major global concern to control Cr(VI) pollution in the environment.

Nowadays, the transformation of Cr(VI) to Cr(III) has been considered to be a feasible, economical, and promising way to substantially lower the toxicity of chromium pollution. There are many physio-chemical techniques for the Cr(VI) removal from various environmental compartments, such as ion exchange, absorption, membrane separation, reverse osmosis, electrochemical treatment, and chemical reduction (Anupam et al. 2015, Miretzky and Cirelli 2010, Sarkar et al. 2010). Nevertheless, these physicochemical techniques have caused secondary pollution along with high cost, high energy requirement, and technical complexity. Therefore, biological reduction has been thought to be an

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effective technique to remediate chromium containment. The study on microbial remediation of chromium pollution in the environment was first reported in the 1970s (Xiao et al. 2012). With the development of researches, microorganisms including bacteria, parts of fungi, and yeasts have been found to decrease Cr(VI) concentrations and transform into Cr(III) for lowering toxic environmental chromium levels (Table S1). *Paecilomyces lilacinus* (Xu et al. 2017), *Aspergillus niger* (Xu et al. 2020a), *Pseudomonas* (Yasir et al. 2021), *Enterobacter* (Mubashar et al. 2020), *Saccharomyces cerevisiae* (Jankovic et al. 2020), and *Escherichia* (Rohand et al. 2021) have shown to possess Cr(VI) removal capacities. However, the processes and mechanisms of Cr(VI) removal by microorganisms still need further study.

In this study, an efficient Cr(VI) reducing fungi was isolated from the chromium-polluted soil at Haibei Chemical Plant, China. A variety of analytical techniques including morphological, biochemical, and physiological strategies were used to discuss the potential of this strain for bioremediation of chromium-contaminated soil. In addition, Cr(VI) reduction and bioremediation mechanisms by this strain also were explored in this research.

Materials and methods

Sample collection and media

Soil samples were collected from the Haibei Chemical Plant (Qinghai province, China) and stored at 4 °C in a refrigerator. Profile soil samples from different depths (typically 0–4 m with 1 m intervals) were taken after removal of litter within an area of 0.5 km^2. All samples were sealed in polyvinyl chloride bags, homogenized, and transported to the laboratory for follow-up bioassay experiments. The culture medium used in isolating Cr(VI)-resistant microorganisms contained: 1 g beef extract, 3 g tryptone, 0.5 g yeast extract, 3 g glucose, 2 g NaCl, 0.5 g $NaNO_3$, 5×10^{-3} g $MgSO_4{\cdot}7H_2O$, and 0.1 g NH_4Cl in 1 L deionized water. $K_2Cr_2O_7$ was used as the source of Cr(VI), and the concentrations were set as 0, 10, 30, and 50 mg L^{-1}. All chemicals used in domestication and batch experiments were analytically reagent grade.

Isolation and identification of Cr(VI)-tolerant fungus

The Cr-contaminated soil (0.1 g) was added to a 100 mL aqueous medium (without Cr) and then shaken in an LRH-150 incubator (Shanghai Qixin Scientific Instrument Co., Ltd., Shanghai, China) at 150 rpm and 30 ± 0.5 °C for 2 days. Then, 1 mL of the culture was inoculated into fresh medium with 200 mg L^{-1} Cr(VI) and cultured for an additional 2 days. Then, the strain was gradually cultured by increasing the Cr(VI) concentration in the medium. After five cycles, the mixture suspension (0.1 mL) was spread evenly on solid medium containing 500 mg L^{-1} Cr(VI). According to the high tolerance and removal of Cr(VI), a fungal strain named S4 was selected through continuous isolation and screening. The colonies of strain S4 were flat with a faint yellow color on the surface of the solid medium (Fig. S1a), and the spores were scattered with diameters of 1–5 μm (Fig. S1b–e).

The identification of strain S4 was done by the Allwegene Tech., Beijing, China. The ITS sequences were compared with the National Coalition Building Institute (NCBI) online database, and Blast analysis was performed to identify the species of strain S4 (Fig. S2). The results showed that strain S4 belonged to *Fusarium proliferatum* (*F. proliferatum*).

Separation of cell components

In order to explore the Cr(VI) removal ability of different fractions of *F. proliferatum* as well as whether it was enzyme-mediated, three cell fractions comprising cell secretions, cytoplasm, and cell debris were separated in this experiment. The detailed steps for separating each fraction were as follows (Desai et al. 2008; Kang et al. 2017; Xu et al. 2020a): (i) mycelium pellets were collected via centrifugation (10,000 rpm, 10 min) after incubation in the liquid medium at 30 ± 0.5 °C for 2 days. The supernatant obtained after centrifugation was considered cell secretions. (ii) Mycelium pellets were washed twice and resuspended in 50 mL potassium phosphate buffer solution (0.1 mM, pH 7.0), homogenized in a shaker (120 rpm, 30 min), and placed in an ice bath. (iii) Cell suspensions in a buffer solution were disrupted using an ultrasonic cell breaker for 30 min (2-s pulses, 4-s intervals). (iv) Suspensions after cell fragmentation were centrifuged again, and the supernatant was filtered to obtain the cytoplasm using a 0.22-μm filter membrane. The precipitates obtained by centrifugation were used as cell debris. In addition, experiments using boiled cell secretions, cytoplasm, and cell debris were set up to investigate whether the Cr(VI) reduction process was mediated by enzymes.

Removal of Cr(VI) by fungal biomass

To investigate the time-dependent Cr removal by the isolate, *F. proliferatum* was treated with 0, 10, 30, and 50 mg L^{-1} Cr(VI) (three replicates each) for 2, 4, 6, 8, 10, and 12 days. All experiments were carried out in Erlenmeyer flasks containing 1 mL inoculum and 249 mL culture medium containing 0, 10, 30, and 50 mg L^{-1} Cr(VI). The flasks were incubated at 30 ± 0.5 °C and shaken in incubator at 150 rpm. In addition, the sterilized medium with an initial concentration of 50 mg L^{-1} Cr(VI) was used as a control group.

After each incubation period, the extracted mycelium pellets and 1 mL supernatant were used to measure the fungal biomass and residual Cr concentration in the culture medium, respectively. Mycelium pellets were extracted by filtering through filter paper followed by repeated rinsing with deionized water. Subsequently, the mycelium pellets were oven-dried (Shanghai Shinbae industrial Corporation, Shanghai, China) at 45 °C to constant weight, and the results indicated the fungal biomass. The residual Cr(VI) and total Cr concentrations in the supernatants were determined after filtration with 0.22-μm cellulose acetate filter membranes. The concentration of Cr(VI) was analyzed colorimetrically by the 1,5-diphenylcarbazide method (DPC) by UV–vis spectrophotometry (Evolution 220 Thermo) at 540 nm (APHA 1998). The total Cr was then determined by inductively coupled plasma optical emission spectroscopy (ICP–OES; Spectro Blue SOP). The Cr(III) concentration was then obtained by the difference between total Cr and Cr(VI) concentrations. The removal rate ($R\%$) of Cr(VI) by *F. proliferatum* was calculated using the following formula:

$$R\% = \frac{C_i - C_f}{C_i} \times 100$$

where $R\%$ is the removal rate of Cr(VI), C_i (mg L^{-1}) is the initial concentration of Cr(VI) in solution, and C_f (mg L^{-1}) is the final concentration of Cr(VI) in solution.

Characterization methods of the fungal cells

Scanning electron microscopy (SEM) was performed to observe the surface morphological characteristics of *F. proliferatum* with and without Cr(VI) treatment. *F. proliferatum* was cultivated in a medium with 0 and 50 mg L^{-1} Cr(VI) at 30 °C and 150 rpm in a shaking incubator. After cultivation for 12 days, fungal pellets were collected, washed three times (5 min each time) in 0.1 M phosphate buffer (pH = 7), and fixed on the surface of conductive adhesive tape. Due to the microbial samples being non-conductive, a small amount of gold powder was sprayed before SEM observation to increase the electrical conductivity. All samples were stored in a refrigerator at 4 °C for 24 h. A FEI Quanta 200 FEG SEM with energy-dispersive spectroscopy (SEM/EDS) was employed for observation and analysis. The EDS provided a means for component identification of extracellular Cr-containing precipitates formed by *F. proliferatum.* The SEM and EDS were operated at 15 kV and 120 Pa.

Transmission electron microscopy (TEM) was used to identify the structure of fungal cells and to determine intracellular Cr distributions. The fungal strain was cultivated in the medium with 0 and 50 mg L^{-1} at 30 °C (150 rpm) for 12 days. Subsequently, the fungal pellets were collected, washed three times (5 min each time) in 0.1 M phosphate buffer (pH = 7), and fixed in 2.5% glutaraldehyde at temperature of 4 °C for 24 h. The sample was fixed in 1% osmic acid at 1 °C for 1 h and washed three times (7 min each time) in 0.1 M phosphate buffer (pH = 7). Then, the cells were dehydrated and embedded with acetone and resin. After the embedding operation was completed, the cells were sectioned to a thickness of 90 nm by an ultrathin microtome and fixed on a copper network containing a micro-sand carbon film. A Tecnai F30 microscope operating at a high voltage of 300 kV was used to observe the cell morphology and to assess the elemental mapping.

Fourier transform infrared (FT-IR) spectral analysis was performed to investigate the changes of functional groups on the cell wall of *F. proliferatum* under Cr(VI) treatment. Mycelia were obtained from the culture solution of *F. proliferatum* containing 0, 10, 30, and 50 mg L^{-1} Cr(VI) at 30 °C (150 rpm) after 12 days of incubation, respectively. After the mycelia were filtered, freeze-dried, and ground, the prepared samples were analyzed using an infrared absorption spectrometer (Nicolet is50, Thermo Fisher Scientific, USA) with a scanning range of 40–4000 cm^{-1} at a resolution of 4 cm^{-1}.

X-ray photoelectron spectroscopy (XPS, AXIS Supra) analysis was used to determine the valence states of chromium attached to the cell surface of *F. proliferatum*. *F. proliferatum* was incubated in the medium with 50 mg L^{-1} Cr(VI) at 30 °C (150 rpm) for 12 days. The cells were then obtained by centrifugation, washed with sterilized deionized water three times, and freeze-dried in a vacuum freeze-drier for 48 h. The obtained dried samples were fully ground, adhered to an iron tape, and pressed into tablets before being tested by XPS. Spectra of Cr 2p1/2 and Cr 2p3/2 were referenced to a C 1 s photopeak at 284.6 eV.

Results and discussion

Effect of initial Cr(VI) concentration on Cr(VI) removal efficiency

F. proliferatum could tolerate up to 500 mg L^{-1} Cr(VI), and the fungi had a high Cr(VI) removal rate in aqueous medium containing less than 50 mg L^{-1} Cr(VI). Several experiments were performed at different initial Cr(VI) concentrations to explore the effect of this parameter on Cr(VI) removal. The Cr(VI) removal efficiencies obtained for the different initial concentrations are shown in Fig. 1a. There was clearly Cr(VI) removal by *F. proliferatum* at various initial Cr(VI) concentrations ranging from 10 to 50 mg L^{-1}. To observe the effects of abiotic factors (e.g., medium composition or volatilization) on Cr(VI) removal, two types of sterile control groups were set up in parallel. The removal of Cr(VI) in the two control groups was less than 5%, indicating that the Cr(VI) removal in the experiment groups was caused

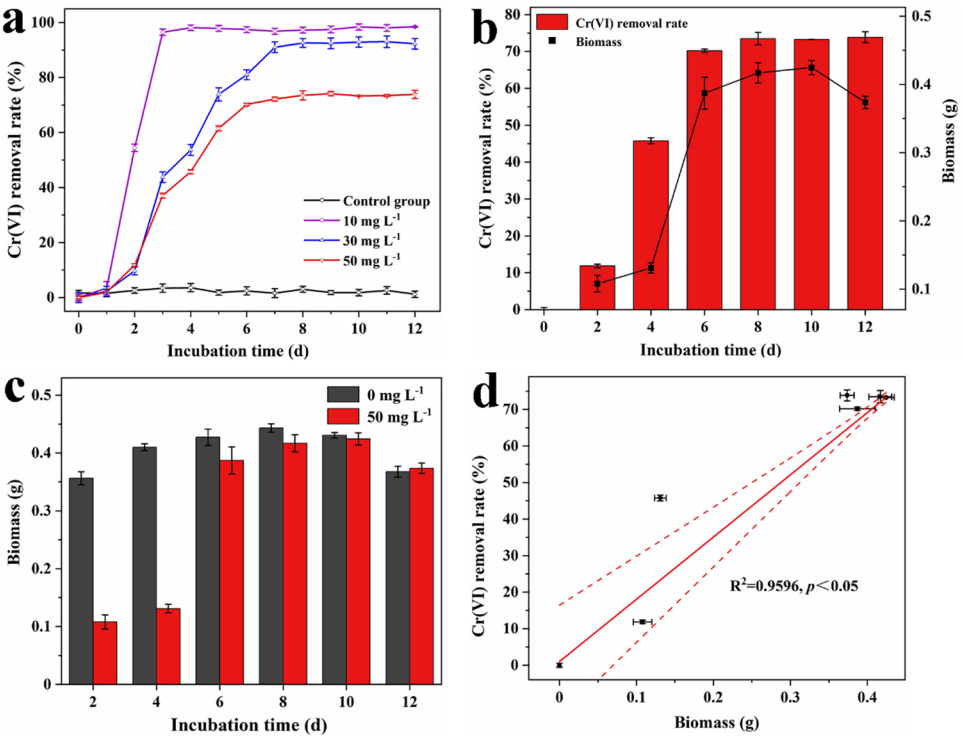

Fig. 1 **a** Cr(VI) removal rate by *F. proliferatum* at different initial Cr(VI) concentrations (10, 30, and 50 mg L^{-1}), the control group represented 50 mg L^{-1} Cr(VI) medium without *F. proliferatum*. **b** Variation characteristics of fungal growth and removal rate under 50 mg L^{-1} Cr(VI). **c** The growth of *F. proliferatum* with and without 50 mg $L.^{-1}$ Cr(VI). **d** The correlation between biomass and Cr(VI) removal rate (%), the red dotted lines represent 95% confidence intervals

by *F. proliferatum*, and abiotic factors had little effect on the removal of Cr(VI). Media with different Cr(VI) concentrations showed significant Cr(VI) removal efficiency during the first 6 days, and then, the Cr(VI) concentration in the medium gradually stabilized. Compared with previous research (Shi et al. 2018; Xu et al. 2020a), hexavalent chromium in the media could be removed more quickly by the methods employed in this study. The Cr(VI) removal rate by strain S4 basically reached the peak (100%, 93%, and 74%) after 8 days under 10, 30, and 50 mg L^{-1} initial Cr(VI) concentrations, respectively. From the results, the maximum removal rate gradually decreased as the initial Cr(VI) concentration increased. Therefore, a longer interaction would be required to reach peak removal with increasing initial Cr(VI) concentration. Similar trends were reported for *Pseudomonas aeruginosa* CCTCC AB93066 and *Stenotrophomonas acidaminiphila* 4–1 (Kang et al. 2017; Li et al. 2021). However, if the initial Cr(VI) concentration was above 100 mg L^{-1}, the final Cr(VI) concentration was decreased by only about 20% (not shown). As predicted, if the initial Cr(VI) concentration continued to increase, the growth and activity of the fungi would eventually be inhibited, and thus, the Cr(VI) reduction efficiency would be extremely low. The high Cr(VI) tolerance of the isolate indicated that this fungus may have significant bioremediation potential for in situ use in environments with high level of chromium pollution.

In order to determine whether the Cr(VI) removal was caused by bioreduction or bioaccumulation, the supernatant of the culture solution was extracted for the measurement of total Cr after 12 days. The remaining Cr(III) concentration represented the bioreduction of Cr(VI). The values of cell accumulation and adsorption of Cr were expressed by the difference between the initial Cr(VI) concentration and the final Cr concentration (including Cr(III) and Cr(VI); see details in the "Removal of Cr(VI) by fungal biomass" section) in the supernatant. As shown in Fig. S3, the reduction rates of extracellular Cr(VI) were 98.34%, 89.73%, and 58.24% at initial Cr(VI) concentrations of 10, 30, and 50 mg L^{-1}, respectively. Meanwhile, with the increase of initial Cr(VI) concentration, the bioaccumulation rate of Cr increased from 0.14 to 15.63%. Previous researchers have reported that a variety of microorganisms could reduce Cr(VI) to soluble Cr(III) by secreting substances that remain in the medium (Li et al. 2016; Xu et al. 2009). In addition, a small portion of Cr (III) could bind to the fungal surface via combining with functional groups and negatively charged ligands by electrostatic attraction.

Effect of biomass on Cr(VI) removal efficiency

In this study, the effect of Cr(VI) on fungal growth was evaluated by comparing the growth process of *F. proliferatum* in the presence and absence of 50 mg L^{-1} Cr(VI) (Fig. 1c). The biomass of *F. proliferatum* cultivated in the Cr(VI)-free medium reached the logarithmic phase faster than that of the same fungal strain in the presence of Cr(VI). It was clear that the growth of strain S4 was inhibited during the early growth phase due to the addition of Cr(VI) in the medium. However,

after the 8th day, the biomass in each experimental group tended to be constant. The difference between the maximum biomass of the two experimental groups (with and without 50 mg L^{-1} Cr(VI)) was not significant. We speculated that strain S4 grew slowly in the initial stage of culture due to the high concentration of Cr(VI). Subsequently, the strain gradually adapted to the cultivation environment containing 50 mg L^{-1} Cr(VI), then grew rapidly, and finally reached maximum biomass similar to that of the Cr(VI)-free culture. These results indicated that *F. proliferatum* could adapt to less than 50 mg L^{-1} Cr(VI) concentration in a short time and that this concentration would not have a negative impact on the growth of the fungi.

The relationship between fungal growth and Cr(VI) removal rate was also investigated in this study (Fig. 1b). After 2 days of adaptive cultivation, strain S4 inoculated in the medium with 50 mg L^{-1} Cr(VI) showed logarithmic growth, accompanied by the rapid decrease of Cr(VI) concentration. From the 2nd to 8th day, the upward trend of Cr(VI) removal rate was correlated with fungal biomass, indicating that biomass was one of the crucial factors affecting the Cr(VI) removal efficiency. Previous studies have also shown a close relationship between heavy metal removal and microbial biomass (Bhattacharya and Gupta 2013, Dogan et al. 2011; Xu et al. 2020b). Furthermore, there was a strong positive correlation between the biomass of the strain and removal rate of chromium ($R^2 = 0.9596$) (Fig. 1d), indicating that the removal amount of chromium was largely controlled by the fungal biomass.

pH is essential for the growth of microorganisms. Fig. S4 shows that the pH varied among different systems. In the culture medium containing the fungal strain and 50 mg L^{-1} Cr(VI), the pH did not vary significantly in the early growth stages of the fungus. With the growth and metabolism of the fungus, the pH of the culture solution gradually decreased. In addition, the pH in the culture medium containing Cr(VI) decreased more than that in the culture medium without Cr(VI); this might have been caused by the presence of Cr(VI) in the aqueous solution that stimulated the strains to produce more metabolites. The protonation of the microbial cell surface caused by low acidity conditions not only helps to bind negatively charged chromate but also provides abundant protons for the reduction of Cr(VI) (Silva et al. 2012). For *F. proliferatum*, we speculated that the strain S4 had secreted a large number of metabolites (e.g., organic acids and organic nutrients that could reduce Cr(VI) and combine with Cr(III) to form soluble organic complexes during the fungal growth process).

Contribution of different cell fractions to Cr(VI) removal.

In this study, we examined the Cr(VI) removal ability of three cell fractions (cell secretions, cytoplasm, and cell debris) as well as whether the reduction capacities were enzyme-mediated. The Cr(VI) removal rates with an initial concentration of 50 mg L^{-1} by different cell fractions and enzyme-inactivated samples (the boiled cell fractions) after 6 h of interaction are shown in Fig. S5. The total cell fractions reduced the Cr(VI) concentration in the aqueous solution by approximately 27.15% and the contributions of the three cell fractions to the Cr(VI) removal were as follows: cytoplasm (17.26%), followed by cell secretions (5.29%), and cell debris (4.6%). Nevertheless, in boiled samples, the Cr(VI) concentration was decreased slightly only in the cell secretion fraction, and neither cytoplasm nor cell debris exhibited Cr(VI) removal capacity. This suggested that the reduction of toxic and carcinogenic Cr(VI) to less toxic Cr(III) could be catalyzed by chromate reductase.

Many researchers have found significant differences in the Cr(VI) removal capacity of different cell components (Acevedo-Aguilar et al. 2006; Chang et al. 2016; Shi et al. 2018). Batool et al. (2012) reported that cell secretion and cell debris of *Ochrobactrum intermedium* isolated from tannery wastewater could remove Cr(VI) via being reduced by chromate reductase in the cytoplasm. In contrast, the main Cr(VI) reduction process occurred in the cell debris (11.40%) rather than the cytoplasm (2.40%) in *Acinetobacter haemolyticus* (Ahmad et al. 2013). In this experiment, the Cr(VI) removal rate by cytoplasm and cell debris of *F. proliferatum* reached 17.26% and 5.39%, whereas the corresponding boiled samples exhibited none, thereby suggesting that chromate reductase in the cytoplasm and cell debris played an important role in the Cr(VI) reduction. The removal rate of Cr(VI) by cell secretions reached 4.61%, indicating that cell secretions, including proteins, organic acids, and various polysaccharides, could protect cells from the toxic effects of the external environment and partially remove Cr(VI) outside cells. In addition, boiled cell secretions (enzyme inactivation) could still remove 2.26% of Cr(VI), indicating that in addition to chromate reductase, there were other non-enzymatic organic substances that could participate in the removal of Cr(VI). Some studies have reported that organic acids (e.g., malic acid and oxalic acid) could reduce Cr(VI) (Ding et al. 2019; Shi et al. 2018). Similarly, we speculated that the Cr(VI) removal was via the action of chromate reductase and non-enzymatic organic acids. Therefore, the combined effects of different cell fractions finally reduced Cr(VI) concentration in the medium. The reduction of Cr(VI) by *F. proliferatum* largely depended on the intracellular chromate reductase that could reduce Cr(VI) to less toxic Cr(III) inside the cell. In addition to the Cr(VI) reduction in cells, extracellular metabolites, including chromate reductase and non-enzymatic organic substances, also participated in the removal process of Cr(VI).

Effect of Cr(VI) on fungal surface morphology and functional groups

SEM–EDS analysis

To gain insight into the effect of Cr(VI) on cells of *F. proliferatum* under growth conditions, SEM was used to observe the morphological changes of fungal mycelia with different concentrations of Cr(VI) (0, 10, 30, and 50 mg L^{-1}).

Figure 2 shows the SEM image of cells grown in the presence and absence of Cr(VI). SEM analysis showed that, under the condition of Cr(VI)-free culture, the hyphae of strain S4 were distinctly clear and plump with a smooth homogeneous surface (Fig. 2a). In contrast, as the concentration of Cr(VI) increased, the surface of hyphae became rougher as well as being curlier and more wrinkled (Fig. 2b–d). Contrary to expectations, the hyphae were not deformed seriously under the highest concentration of Cr(VI). These results implied that *F. proliferatum* could tolerate relatively high concentrations of Cr(VI), with no major impact on mycelial morphology. Meanwhile, a small number of amorphous precipitates were present on the hyphal surface under the Cr(VI) concentration of 50 mg L^{-1}, and a Cr peak was found in the EDS image (Fig. 2e). Nevertheless, the Cr peak was not found for the hyphal surface in the Cr(VI)-free and lower Cr(VI) concentration (10 and 30 mg L^{-1}) cultures, confirming that the appearance of chromium on the mycelium surface was completely caused by the addition of chromium in the external environment. With lower Cr(VI) concentrations, the lack of precipitation on the mycelium surface may have been due to the poor accumulation ability of strain S4 to Cr(VI) under low concentrations, and most of the Cr(VI) existed in the solution in the form of reduced state. Similar observations were made by Xu et al. (2020a) during the removal of Cr(VI) by *Aspergillus niger*. We preliminarily speculated that the formation of chromium-containing precipitates might be caused by reducing of Cr(VI) to Cr(III)-containing substances with low solubility.

FT-IR analysis

To obtain the functional groups of cells involved in the Cr adsorption process, the FT-IR spectra of cells under the conditions of 0, 10, 30, and 50 mg L^{-1} Cr(VI) were examined. The corresponding FT-IR spectra are shown in Fig. 3.

For Cr(VI)-free cells, the FT-IR spectra showed the following features: a maximum peak of 3250.5 cm^{-1} represented the overlapping stretching vibrations of –NH groups (Chen et al. 2012; Pei et al. 2009); the absorption peak at 2924.8 cm^{-1} was assigned to the C–H stretching vibrations of alkyl chains; the peak at 1034.0 cm^{-1} was attributed to the phosphate group;

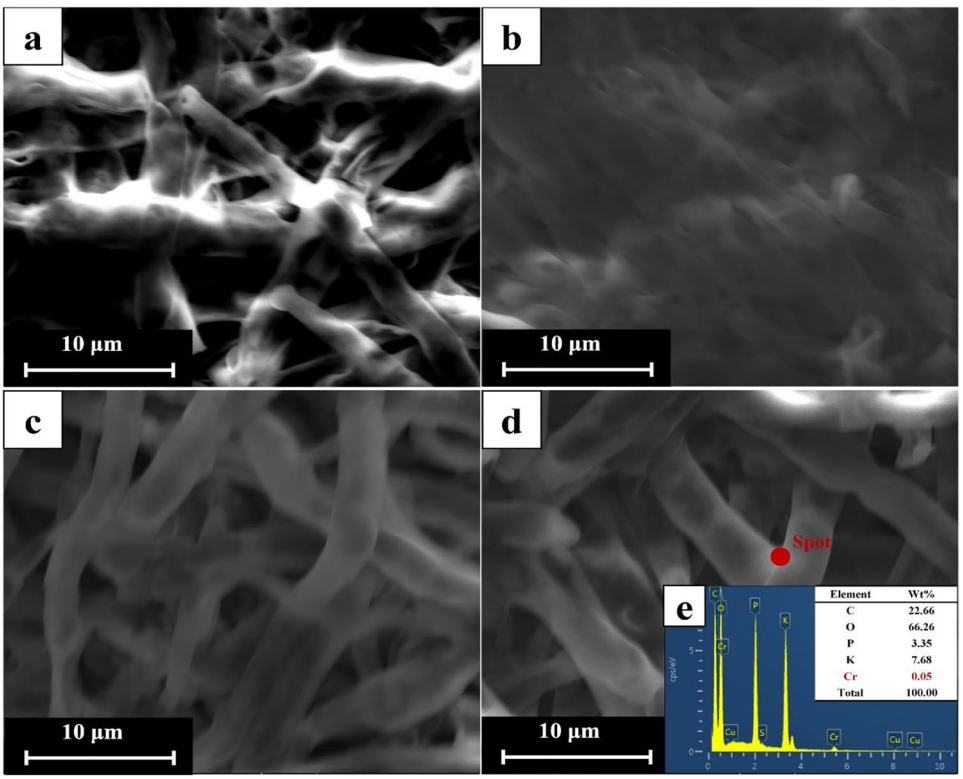

Fig. 2 Scanning electron microscopy (SEM) images of *F. proliferatum* incubated **a** without Cr(VI) and in **b** 10 mg L^{-1}, **c** 30 mg L^{-1}, **d** 50 mg $L.^{-1}$, **e** EDS of the hyphal surface. The samples were collected on day 12

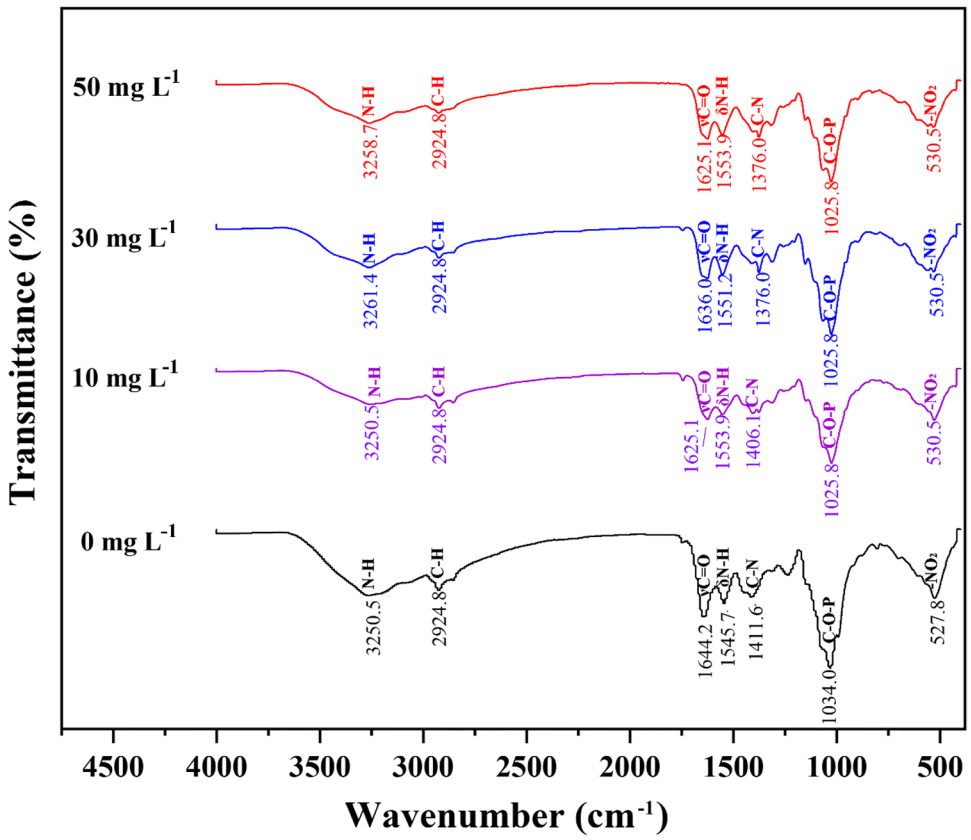

Fig. 3 Fourier transform infrared (FT-IR) Spectral analysis of *F. proliferatum* incubated without Cr(VI) and containing 10 mg L^{-1}, 30 mg L^{-1}, 50 mg $L.^{-1}$ Cr(VI). The samples were collected on day 12

the absorbances at 1644.2, 1545.7, and 1411.6 cm^{-1} were related to the amide I, amide II, and amide III, respectively; the peak at located about 527.8 cm^{-1} was reported as a nitro-compound. After Cr(VI) treatment, the chromium-exposed biomass exhibited changes in proteins (N–H stretching, 3250.5 cm^{-1} shifting to 3250.5, 3261.4, and 3258.7 cm^{-1}), amide I ($\nu C=O$ stretching vibration, 1644.2 cm^{-1} shifting to 1625.1, 1636.0, and 1625.1 cm^{-1}), amide II (δN–H bending vibration, 1545.7 cm^{-1} shifting to 1553.9, 1551.2, and 1553.9 cm^{-1}), amide III (C–N absorption band, 1411.6 cm^{-1} shifting to 1406.1, 1376.0, and 1376.0 cm^{-1}), and phosphate moieties (C–O–P antisymmetric stretching, 1034.0 cm^{-1} shifting to 1025.8, 1025.8, and 1025.8 cm^{-1}). The altered vibrational bands indicated interactions between Cr and amino, carbonyl, carboxyl, and phosphate groups, leading to the adsorption of Cr on the cell surface of strain S4. This finding was consistent with those of previous studies. Kang et al. (2017) revealed that amino and carbonyl groups present on the cell surface of *Pseudomonas aeruginosa* were responsible for Cr adsorption. The changes involving phosphate and sulfonate groups on cell surfaces due to cell wall interactions with chromium were also reported by Ertugay and Bayhan (2008) for *Agaricus bisporus*.

XPS analysis

The XPS spectra of *F. proliferatum* cultured with 50 mg L^{-1} Cr(VI) were obtained to determine the oxidation states of chromium adsorbed and accumulated on the cell. As shown in Fig. S6, two distinct peaks derived from the Cr 2p 3/2 and Cr 2p 1/2 core levels appeared at 577.04 and 586.75 eV, respectively, for the Cr(VI)-loaded cells of strain S4. By comparing the peaks of Cr 2p corresponding to Cr(III) and Cr(VI), the binding energies of Cr 2p 3/2 and Cr 2p 1/2 were found to be inconsistent with those of Cr(VI) compounds (e.g., $K_2Cr_2O_7$, K_2CrO_4) but were similar to those of Cr(III) compounds. The spectrum in Fig. S6 confirmed the presence of Cr on the cell surface as only Cr(III). These results suggested that the Cr adsorbed on the cell surface was in the Cr(III) state, possibly as CrOOH or $Cr(OH)_3{\cdot}0.4\ H_2O$ (Kang et al. 2017; Pan et al. 2014; Sarkar et al. 2013). The form of Cr(III) on the cell surface may be derived from spontaneous Cr(VI) reduction by electron donor groups due to the relatively high redox potential of Cr(VI) (Chang et al. 2019; Park et al. 2005; Silva et al. 2012). We speculated that the adsorbed Cr(VI) on the cell surface acting as a terminal electron acceptor was completely converted to the Cr(III) state. Furthermore, on entering the cells,

Cr(VI) would be reduced to Cr(III) that could combine with proteins or nucleic acids to form aggregations inside the cells (Pei et al. 2009; Zheng et al. 2015).

Intracellular and extracellular distribution characteristics of Cr

In order to elucidate detailed intracellular and extracellular distribution characteristics of chromium in the cells of *F. proliferatum*, TEM observations after Cr(VI) treatments were performed, as evinced by EDS analysis. In terms of cell morphology, the cells of *F. proliferatum* had a regular shape with complete organelles under Cr-free culture conditions. However, after contact with 50 mg L^{-1} Cr(VI) for 12 days, the cells became slightly deformed, with nuclei and vacuoles displaying creases. In addition, in most of the cells, thin electron-dense granules were detected in the cytoplasmic region and cell wall after the interaction with 50 mg L^{-1} Cr(VI) (Fig. 4b), whereas similar particles were not observed in the Cr(VI)-free cells (Fig. 4a).

Meanwhile, STEM–EDS measurement was conducted to identify the elemental composition of particles via the emergence of carbon, oxygen, and chromium (Fig. 4c). The existence of chromium inside the cells confirmed that Cr could enter the cell and that it was accumulated by *F. proliferatum* from the extracellular environment. Therefore, we assumed that these black precipitates in cells were formed by biomineralization of Cr. Kumar and Dwivedi (2019) also reported that *Aspergillus flavus* CR500 isolated from electroplating wastewater could form Cr_2O_3 nanoparticles on the cell walls. Opaque particles appeared in the cell cytoplasm of *P. aeruginosa* treated with 10 mg L^{-1} of Cr(VI) (Kang et al. 2017). It was unfortunate that precipitates formed inside cells were sparse and amorphous, resulting in precipitate types not being measured.

Mechanism of Cr(VI) removal by F. proliferatum

In the present study, in order to clarify the mechanisms of Cr(VI) reduction and Cr(III) immobilization, a variety of analytical techniques were used to explore the Cr binding site and elemental distribution in *F. proliferatum* cells. SEM and TEM revealed that Cr(VI) was removed not only on the cell surfaces but also inside the cell by adsorption, reduction, and accumulation. The conformational changes in functional groups and Cr valence owing to metal adsorption or reduction were confirmed by FT-IR and XPS analyses. The mass balance analysis showed that Cr tended to be preserved in solution in the form of Cr(III), with a lesser content of chromium in the fungal inner portions. In view of the above results, we speculated the following possible mechanism in which *F. proliferatum* reduced Cr(VI) (Fig. 5): in the presence of *F. proliferatum*, some portion of Cr(VI) was reduced to Cr(III) through extracellular secretions, including reductase and non-enzymatic organic substances, as well as by intracellular chromate reductase. The reduction of Cr(VI) in the cytoplasm showed that Cr(VI) could enter microbial cells via uptake systems and be transformed into Cr(III) via chromate reductase. It was regrettable that species of reductase were not ascertained. Previous studies have shown that soluble reductase and membrane-associated enzymes including ChrR, YieF, Tkw3, lipolyl dehydrogenase, and glutathione reductase could catalyze the reduction of Cr(VI) to Cr(III) in microorganisms (Donati et al. 2003, Opperman and van Heerden 2008, Qamar et al. 2011). Therefore, a possible reduction pattern of chromium was speculated to be present in *F. proliferatum*. Another amount of Cr(VI) was coordinated with certain functional groups (e.g., amino, carbonyl, carboxyl, and phosphate groups) on the cell surfaces and immobilized in the form of Cr(III) by reductase. In addition, after 12 days of culture, we found that although the

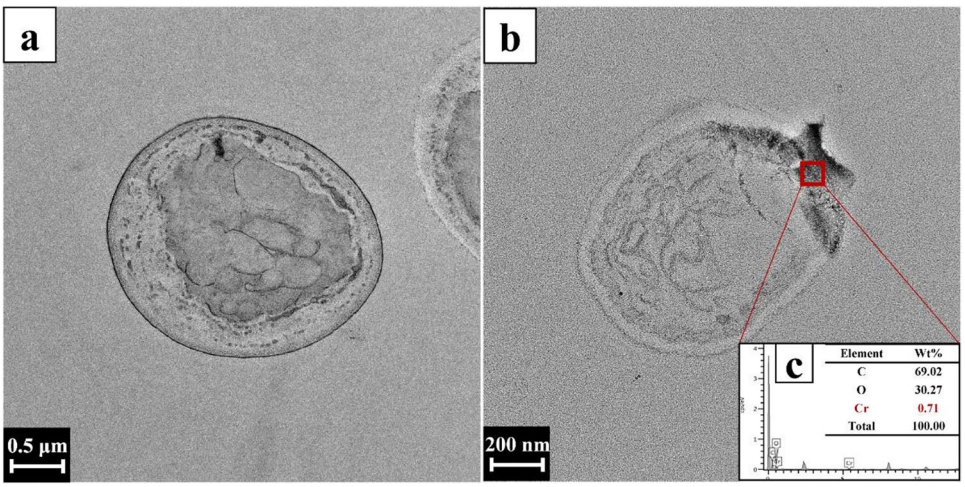

Fig. 4 Transmission electron microscopy (TEM) images of *F. proliferatum* incubated **a** without Cr(VI) and **b** with 50 mg L^{-1} Cr(VI), **c** TEM-EDS results for cell after 50 mg $L.^{-1}$ Cr(VI) treatment. The samples were collected on day 12

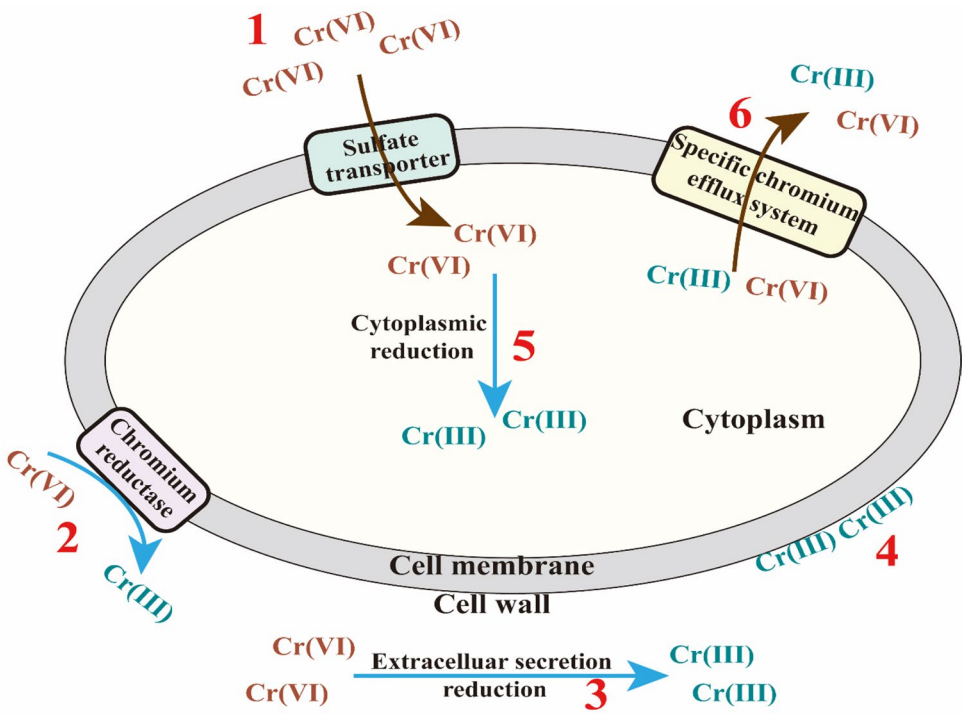

Fig. 5 Possible chromium removal mechanisms by *F. proliferatum*. The brown arrows indicate the chromium migration process; the blue arrows indicate the reduction process of Cr(VI). (1) Cr(VI) migrates into the cell; (2) Cr(VI) is reduced by chromium reductase on the cell surface; (3) Cr(VI) is reduced by extracellular secretion; (4) Chromium is adsorbed on the cell surface; (5) Cr(VI) is reduced by reductase in cell; (6) Intracellular chromium is expelled by the specific efflux system

concentration of Cr(VI) was significantly reduced, the total Cr concentration in the medium was only decreased by less than 15%, implying that the reduced Cr(III) in the cytoplasm was transported outside the cell by specific efflux systems. Nevertheless, the way in which trivalent chromium was expelled from cells was unclear and thus will require required further study in the future.

Conclusions

The Cr(VI)-tolerant *F. proliferatum* isolated from Cr-contaminated soil from a chemical plant exhibited high Cr(VI) removal efficiency from aqueous solution, reaching up to 100%, 93%, and 74%, when grown under optimum conditions and on exposure to 10, 30, and 50 mg L^{-1} Cr(VI). This fungal strain could not only effectively reduce Cr(VI) to less toxic Cr(III) but could also absorb/accumulate Cr in solution, with the process proceeding gradually. The Cr(VI) reduction capacity increased almost linearly with increasing biomass dosage, suggesting that the removal amount of Cr(VI) was largely controlled by the fungal biomass. Meanwhile, we found that a small fraction of Cr(III) was immobilized on the cell surface, confirming that amino, carbonyl, carboxyl, and phosphate groups contributed most to the absorption of Cr(III). Both cell secretions and cell-associated fractions (cytoplasm and cell debris) were involved in the Cr(VI) reduction process, and this was primarily carried out through enzyme action. These findings indicated that *F. proliferatum* is a potential candidate for cost-effective in situ bioremediation of Cr(VI)-contaminated sites.

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Declarations

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