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Effects of alfalfa root exudates on the microbial remediation of nitrate-heavy metal complex pollution in groundwater

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ABSTRACT: The effects of root exudate addition on Fe(II) oxidation, nitrate degradation, and heavy metal removal by *Clostridium* sp. strain PXL2 were studied. The root exudate addition could increase the average oxidation rate of Fe(II) from 0.52 to 0.68 mM Fe(II)/d. The removal rate of nitrate, arsenic, and mercury could be increased from 54% to 95%, 84% to 99%, and 60% to 86%, respectively. The complexation of Fe(III) with the root exudates was analyzed by quenching titration. The results showed that there was a complexation of root exudates with Fe(III). This complexation resulted in increased precipitation of iron oxides and further promoted the removal of heavy metals.

KEYWORDS: root exudates, heavy metals, groundwater, ferrous oxidizing denitrifying bacteria

INTRODUCTION

Groundwater is an important water resource used as the main source of water for cities, industry, and agriculture. However, with increasing industrial and agricultural activities, groundwater pollution is expanding from point to surface, from shallow to deep, and from urban to rural areas. Thus, the problem of groundwater pollution has become increasingly prominent [1]. Nitrate-heavy metal complex pollution is a typical type of groundwater pollution. Researchers have conducted numerous studies on this issue [2].

Anaerobic ferrous oxidizing denitrifying bacteria can use nitrate as an electron acceptor for Fe(II) oxidation in anaerobic environments. The metabolism of this microorganism provides a method for alleviating nitrate-heavy metal complex pollution in groundwater [3, 4]. Many factors affect the remediation of compound pollution by anaerobic ferrous oxidizing denitrifying bacteria [5], which are related not only to differences in species, but also to the use of organic carbon sources.

Root exudates refer to various organic and inorganic substances released by plant roots in the rhizosphere. Root exudates include cell exfoliates and lysates, high-molecular-weight gels, and low-molecular-weight organic compounds [6]. Researches have shown that root exudates are of great significance for the removal of pollutants because they change the solubility and mobility of metal ions in the environment [7]. The removal rate of Cr(VI) can be increased when maize root exudates are used as a carbon source [8]. Montiel-Rozas et al [9] found that root exudates can regulate the bioavailability of heavy metals. Low-molecular-weight organic acids play an important role in heavy metal fixation. Habibul et al [10] found that root exudates can provide a carbon source for microorganisms in plant-microbial fuel cells. Bioelectrochemical reduction and removal of Cr(VI) have been achieved using biofuel cells. With the leaching of rainwater and the continuous erosion of groundwater, root exudates are gradually released into the groundwater becoming an important part of the groundwater ecosystem. The relationship between root exudates and the migration and transformation of heavy metals in the groundwater environment has become a concern.

In this study, a typical *Clostridium* sp. strain PXL2, with ferrous oxide denitrification ability, was isolated from the sediments of natural water bodies in Xinjiang. The *Clostridium* sp. strain PXL2 could effectively and simultaneously remove As(III) and nitrate from water owing to its Fe(II) oxidation and denitrification activities [11]. The common plant

alfalfa was selected as the studied object to analyze the role of root exudates in the microbial degradation of pollutants. This study lays a foundation for the application of oxidative denitrifying bacteria in the field of complex groundwater pollution treatment.

MATERIALS AND METHODS

Simulated experiment

The microorganisms used in the experiment originated from an enriched solution of *Clostridium* sp. strain PXL2, which was isolated and purified in our laboratory. The alfalfa used in the experiment was collected from farmland in the suburbs of Urumqi, Xinjiang, and healthy plants were selected as subjects. The roots of the selected plants were cleaned with tap water, rinsed with deionized water several times, placed in a clean beaker, cultivated in a plant incubator with deionized water for 48 h, and then removed. The culture solution was filtered through a 0.45 μm filter and stored at a low temperature in the dark until later use.

The bacterial solution in the stationary growth phase was inoculated into a culture medium consisting of 0.28 g ammonium chloride, 0.25 g dipotassium hydrogen phosphate, 0.10 g magnesium sulfate, 0.01 g calcium chloride, 0.10 g yeast extract, 0.50–2.00 g potassium nitrate, 0.20–1.00 g ferrous chloride tetrahydrate, and 1000 ml deionized water. The inoculation amount was 5% (v/v). The pH of the medium was 6.8–7.0. All the chemicals were analytically pure (China National Pharmaceutical Group Chemical Reagent Co., Ltd.).

The culture was inoculated at a cell density of about 6×10^5 cells per milliliter to the above-mentioned liquid medium containing various concentrations of Fe(II) and NO_3^-. Four levels of root exudates addition were designed. Less than 3 ml of samples were collected every 24 h to determine the concentrations of nitrate, nitrite, and Fe(II). All experiments were carried out in the G250 anaerobic workstation (Don Whitley Scientific, West Yorkshire Shipley, England) and triplicated.

Arsenic (As) and mercury (Hg) were added to the medium to simulate heavy metal pollution. Sodium arsenite and high mercury chloride were selected to simulate arsenic and mercury pollutions, respectively. The sodium arsenite and high mercury chloride were of premium grade purity (China National Pharmaceutical Group Chemical Reagent Co., Ltd.). The initial concentration of arsenic was 36.0 μM, and the initial concentration of mercury was 0.5 μM. Approximately, 5–50% (v/v) of root exudates were added to the medium. The supernatant was collected as samples to detect dissolved As and Hg in the water.

Analytical methods

The molecular weight characteristics of the root exudates were analyzed by high-performance liquid chromatography (Superdex TM 20010/300GL, General Electric Company, United States). The exclusion column was washed with a buffer solution (9.0 mM sodium chloride and 0.9 mM disodium phosphonate), which was also used as the carrier fluid. The washing rate and the carrier current rate were 0.8 ml/min and 0.5 ml/min, respectively. 14.4–97.4KDa complex proteins were used as internal standards.

The fluorescence characteristics of the root exudates were measured using a molecular fluorescence spectrophotometer (F-7000, Hitachi, Japan). The parameters were set as follows:- bandpass: excitation (Ex) = 5 nm, emission (Em) = 10 nm; response time: 0.5 s; and scanning speed: 1200 nm/min. The Ex wavelength range was 200–450 nm (at intervals of 5 nm), and the Em wavelength range was 200–550 nm (at intervals of 2 nm). The measured results were deduced from the experimental blank.

A spectrophotometer (DU 800 UV/Vis Spectrophotometer, Beckman Coulter, United States) was used to measure the concentrations of nitrate, nitrite, and Fe(II). The nitrate concentration was determined at 220 and 275 nm [12]. The nitrite concentration was monitored at 540 nm by the formation of purple azo compounds [13]. The Fe(II) concentration was measured using phenanthroline at 510 nm [14]. For the modified phenanthroline assay, the samples were mixed with 40 mM sulfamic acid (pH of approximately 1.8) instead of 25% (v/v) hydrochloric acid, as sulfamic acid could react rapidly with nitrite, while preventing Fe(II) oxidation by nitrite at an acidic pH [15]. The As and Hg concentrations were measured using an AFS-8X atomic fluorescence spectrophotometer (Jitian Instruments Co., Ltd., Beijing, China).

RESULTS AND DISCUSSION

Characteristic analysis of root exudates

The molecular weight distribution of the root exudates was analyzed using liquid chromatography. The peak time was based on the size exclusion signal. The peak times of the protein standards were 20.14, 30.77, 43.53, 51.66, 60.17, and 70.03 min

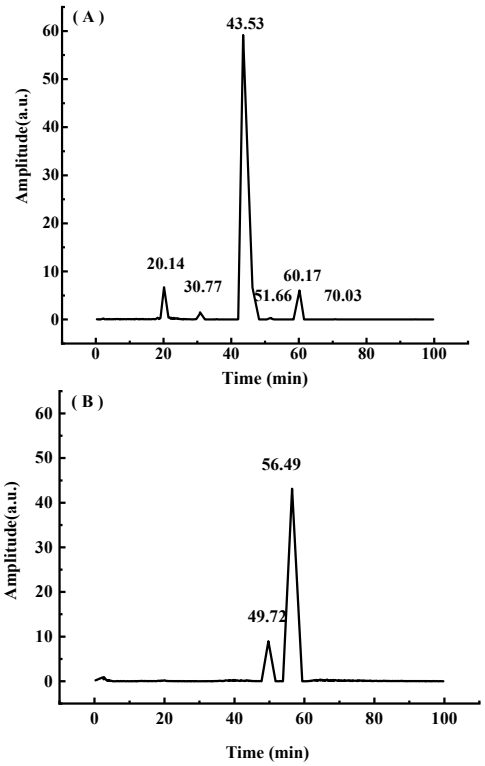

Fig. 1 Liquid chromatograph of protein standards and root exudates: (A) protein standards and (B) root exudates.

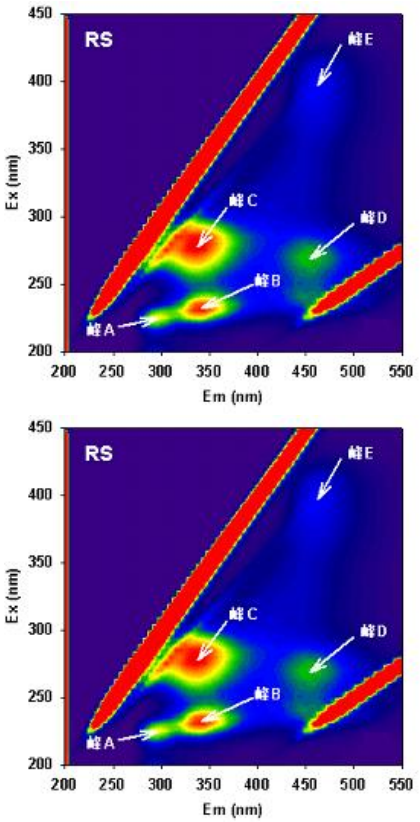

Fig. 2 Excitation-emission matrix (EEM) spectra of root exudates: (A) before interaction with Fe(III) and (B) after interaction with Fe(III).

(Fig. 1A), and the molecular weights corresponding to the peak times were 97.4, 66.2, 43.0, 31.0, 20.1, and 14.4 kDa, respectively. The peak times of the root exudates were 49.72 and 56.49 min (Fig. 1B). The molecular weight distribution of the root exudates was calculated based on the molecular weight of the control protein standard. The main components of the root exudates were substances with molecular weights of 20–31 kDa.

The three-dimensional fluorescence spectra of the root exudates are shown in Fig. 2A. Five distinct fluorescence peaks were detected in the fluorescence spectra. Peaks A and B are the fluorescence peaks of aromatic proteins. According to the positions of the fluorescence peaks, these proteins were derived from tyrosine [16–18]. Peak C is the fluorescence peak derived from tryptophan. Peaks D and E are the fluorescence peaks of humic acids.

Effects of added root exudates on ferrous oxide and nitrate degradation

The change of the Fe(II) concentration after inoculating *Clostridium* sp. strain PXL2 in the culture medium with different amounts of root exudates is shown in Fig. 3A. The addition of root exudates promoted the oxidation of Fe(II). Without root exudates, the average oxidation rate was 0.52 mM Fe(II)/d. The addition of 5% and 20% root exudates promoted ferrous oxidation during the first 3 days of the reaction. The concentration of Fe(II) was the same as that without added root exudates by the fifth day. When 50% root exudates were added, the average oxidation rate increased to 0.68 mM Fe(II)/d, which was 30% higher than that without root exudates. Low-molecular-weight organic acids in the root exudates can be used as a carbon source for microorganisms, thereby promoting the growth and reproduction of microorganisms [19] and accelerating their oxidation of Fe(II). However, some organic acids in the root secretion can be complexed with the Fe(III) formed by the biological oxidation of Fe(II), hence further promoting the conversion of Fe(II) to Fe(III).

With the continuous oxidation of Fe(II), the addition of different concentrations of root exu-

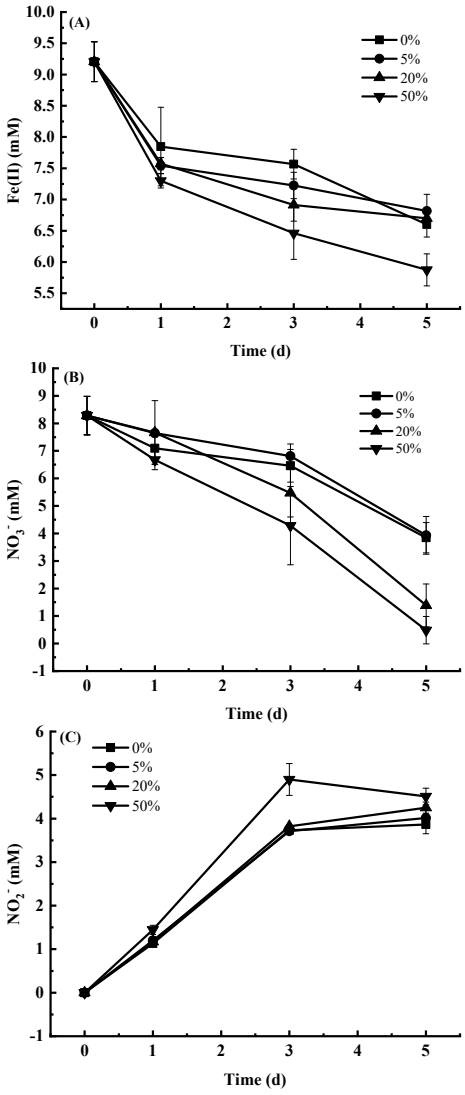

Fig. 3 Time profiles of the Fe(II), nitrate (NO_3^-), and nitrite (NO_2^-) concentrations in the culture of *Clostridium* sp. strain PXL2 with the stimulation of root exudates.

dates promoted the degradation of nitrate (Fig. 3B). When 5% root exudates were added, the promotion of nitrate degradation was observed on the first day of culture, and the degradation rate gradually coincided with that of the group without root exudates. This might have occurred because the active ingredients in root exudates that promote nitrate degradation were exhausted. On the fifth day of culture, the nitrate concentration decreased, and approximately 54% of the nitrate was removed. When 20% root exudates were added, the nitrate removal promotion effect was clearer. Approximately 83%–95% of the nitrate was removed after 5 days

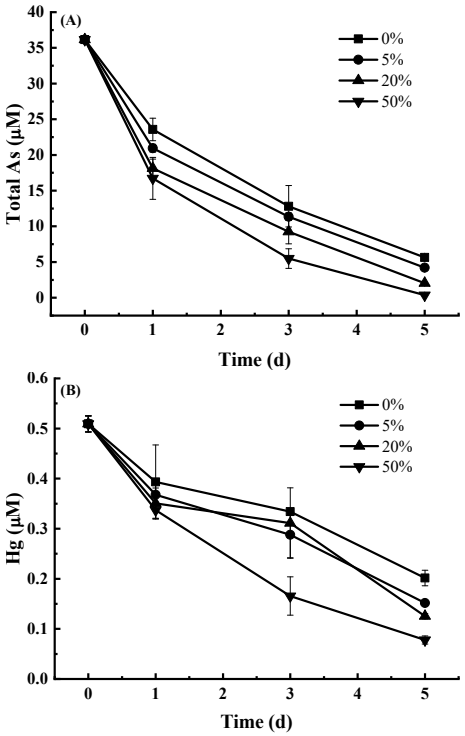

Fig. 4 Time profiles of As and Hg concentrations in the culture of *Clostridium* sp. strain PXL2 with the stimulation of root exudates.

of culture.

As nitrate was continuously degraded, nitrite gradually accumulated (Fig. 3C). The accumulated concentration of the culture group with 50% root exudates was slightly higher than that of the other culture groups, with the highest concentration reaching 4.8 mM. As the experiment progressed, the concentration of nitrite gradually stabilized or began to decrease, thereby indicating that nitrite is an intermediate product of nitrate degradation and that nitrite is further used for degradation.

Effects of root exudates on heavy metal removal

The effects of root exudate addition on heavy metal removal are shown in Fig. 4. When no root exudates were added, the removal rate of As was 84% after 5 days of inoculation. When 50% root exudates were added, the As removal rate increased to 99% (Fig. 4A). Similarly, root exudate addition was beneficial to Hg removal (Fig. 4B). After 5 days of inoculation with 50% root exudates, the Hg concentration decreased to 0.07 μM, and the mercury removal rate increased from 60% to 86%.

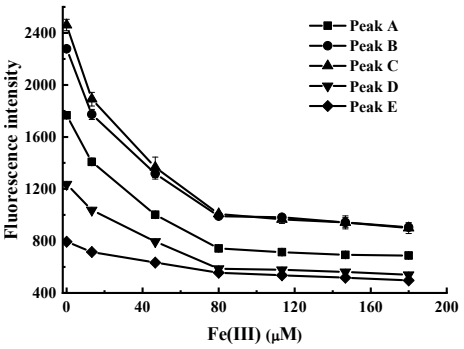

Fig. 5 Fluorescence intensity of root exudates with the increasing concentrations of Fe(III).

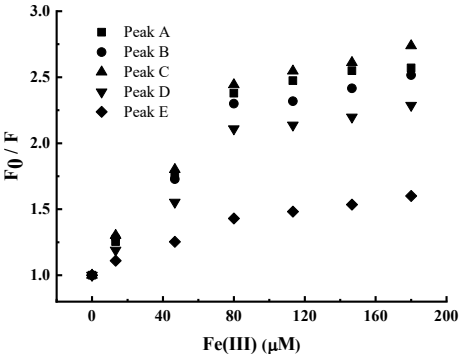

Fig. 6 Plots of F_0/F versus [Fe(III)] for root exudates titrated with Fe(III).

Analysis of the complexing ability between root exudates and Fe(III)

The complexing ability of the root exudates and Fe(III) was analyzed by quenching titration (Fig. 2B). In the reaction system, 180 μM Fe(III) fully reacted with the root exudates. The intensity of the fluorescence peaks in the three-dimensional fluorescence spectrum of the root exudates significantly reduced (compared with that in Fig. 2A). Thus, Fe(III) can interact with the fluorescent components in root exudates and effectively quench their fluorescence.

Table 1 The conditional stability constants ($\log K_a$), complexation constants ($\log K_b$), and complexation sites (n) of root exudates fluorophore-Fe(III) systems.

Fluorescence	$\log K_a$	$\log K_b$	n	R^2
Peak A	4.4144	3.0244	0.7317	0.9732
Peak B	4.5230	2.7218	0.6626	0.9776
Peak C	4.5154	2.9081	0.6956	0.9797
Peak D	4.3028	3.0347	0.7611	0.9750
Peak E	4.3157	0.2893	0.6633	0.9932

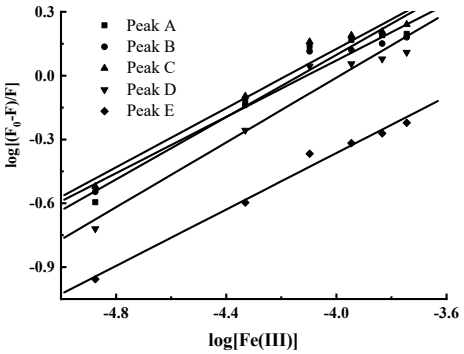

Fig. 7 Modified Stern-Volmer plots of root exudate fluorescence titrated with Fe(III).

The decrease in fluorescence intensity with the increase in Fe(III) concentration in the solution is shown in Fig. 5. The Stern-Volmer equation was used to fit the fluorescence quenching data, as follows Eq. (1):

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q] \qquad (1)$$

where F_0 is the initial fluorescence intensity, F is the fluorescence intensity after the addition of a quencher, K_q is the quenching rate constant, K_{sv} is the quenching constant, and τ_0 is the average lifetime of the fluorescence emitted by the fluorophore in the absence of a quencher. For biological macromolecules, τ_0 is generally 8–10 s [20]. $[Q]$ is the concentration of the quencher.

The curve of the fluorescence intensity of root exudates corresponding to different Fe(III) concentrations in the solution is shown in Fig. 6. The fluorescence intensity of root exudates exhibited a non-linear change with an increase in the Fe(III) concentration. This feature is significantly different from the typical Stern-Volmer linear curve. This means that the fluorescence quenching of root exudates by Fe(III) involves both dynamic and static quenching [20].

The Stern-Volmer correction equation (2) was used to analyze the fluorescence quenching data. These data can aid the understanding of the fluorescence quenching mechanism.

$$\frac{F_0}{F_0 - F} = \frac{1}{fK_a(Q)} + \frac{1}{f} \qquad (2)$$

where f is the percentage of fluorescence emitted by the fluorophore that complexes with the quencher and K_a is the effective quenching constant.

Lakowicz [20] proposed that the initial fluorescence (F_0) contains two parts that are quenchable (F_0a) and non-quenchable (F_{0b}), as follows:

$$F_0 = F_{0a} + F_{0b} \tag{3}$$

$$f = \frac{F_{0a}}{F_{0a} + F_{0b}} \tag{4}$$

In the presence of a quencher, only F_{0a} will change. The obtained fluorescence intensity can be used to fit the Stern-Volmer correction equation to calculate the fluorescence quenching parameters. By substituting the fluorescence quenching data of Fe(III) by root secretions into Eq. (2) and fitting the data, the obtained function had a good linear correlation ($R^2 > 0.996$; $p < 0.0001$). The conditional stability constants ($\log K_a$) are given in Table 1. The conditional stability constants of peaks A, B, and C were slightly higher than those of peaks D and E, thereby indicating that the stabilities of the complexes of Fe(III) with tyrosine and tryptophan are strong, whereas those of the complexes with phytic acid are less stable.

When the equilibrium sites of small molecules and large molecules are combined, the change in the fluorescence intensity of the fluorophores can be substituted into Eq. (5) to calculate the complexation constant (K_b) and complexation sites (n) of the fluorophores and the quencher molecules [21]:

$$\log \frac{F_0 - F}{F} = \log K_b + n \log[Q] \tag{5}$$

A larger K_b value indicates stronger complexation ability between the fluorophore and the quencher, and a larger value of n, a greater the number of quencher molecules that the fluorescent group can complex. The $\log K_b$ and n calculated according to Eq. (5) are given in Table 1. The complexation constant of peak E was significantly lower than those of the other four peaks, thereby indicating that the complexation between humic acids and Fe(III) represented by peak E was significantly weaker than those with root exudates derived from tryptophan.

The function fitting curve is shown in Fig. 7, where $\log[(F_0 - F)/F]$ is the dependent variable, and $\log$[Fe(III)] is the independent variable. The curve showed a good linear correlation ($R^2 > 0.973$; $p < 0.001$).

Because of their ubiquitous nature, significant metabolic capacities, and related adaptation potentials, microorganisms play a key role in the functioning and transformation of biogeochemical cycles. Processes such as denitrification and nitrogen fixation are specifically related to the activities of microorganisms [22]. The metabolism of microorganisms involved in plant root exudates plays an active role in the geochemical cycle of pollutants.

The involvement of root exudates affects the removal of heavy metals by iron oxides; for example, sunflower root secretions can promote the adsorption of hydrated iron oxide on Cd^{2+}. Studies have also shown that organic acids can help improve the adsorption of cadmium by iron oxides (goethite) [23, 24]. For example, the presence of different concentrations of citric acid can promote the effect of goethite on Cd^{2+} [23]. This may occur because Cd^{2+} can precipitate with organic acids, such as citric acid and oxalic acid.

Other studies have found that organic acids inhibit the adsorption of heavy metals by iron oxides (goethite) [25]. This may occur because organic ligands compete with heavy metal ions for adsorption sites, or because the presence of organic ligands inhibits the hydrolysis of heavy metal ions; thereby reducing the probability of heavy metal adsorption [26].

Some studies have indicated that the presence of organic acids affects the adsorption performance of iron oxide. The inhibition or promotion of adsorption depends on the concentration of organic acids. For example, in an acidic environment (pH = 5), a lower concentration of organic acids can promote the adsorption of heavy metals by goethite, whereas a higher concentration of organic acids can inhibit the adsorption reaction [27]. Whether the addition of root exudates promotes or inhibits the removal of heavy metals in the groundwater environment is greatly affected by the type of plant, the type of iron oxide mineral, the type and concentration of heavy metal ions, and the environmental conditions of the interaction.

Heavy metal solubility and bioavailability can be enhanced by microorganisms, thereby making microbial remediation an effective means of heavy metal removal from the environment [28–30]. Previous studies have shown that soluble organic matter affects the mobility of arsenic in different ways. Regarding the soil environment, dissolved organic matter from root exudates affects the distribution and morphological classification of arsenic in the rhizosphere. For example, in the rhizosphere, the root exudates of the arsenic-enriched plant yarrow activate insoluble iron arsenate, aluminum arsenate, and fixed arsenic, which exist in copper arsenic tailing-contaminated soil [31]. This process causes

the distribution of arsenic in the rhizosphere to be deficient [32]. Microbial communities such as *Pseudomonas* sp. LK9 affect the secretion of low-molecular-weight organic acids that can be combined with heavy metals [33, 34]. Humic acid, fulvic acid, and the soil mineral surface are strongly bound [35], therefore the binding sites of arsenic are blocked [36, 37]. When root exudates are continuously transferred and released into the groundwater environment, they also affect the migration and transformation of arsenic in the groundwater environment.

CONCLUSION

In this study, alfalfa root exudates were extracted. The basic properties of the root exudates were characterized. The three-dimensional fluorescence spectroscopy (excitation-emission matrix) results showed that the exudates contained aromatic proteins derived from tyrosine, substances derived from tryptophan, and substances derived from humic acids. The addition of root exudates promotes the oxidation of Fe(II), the degradation of nitrate, and the removal of heavy metals by *Clostridium* sp. strain PXL2. The complexation between Fe(III) and the root exudates was analyzed by quenching titration. The results showed that the fluorescence quenching of root exudates includes both dynamic and static quenching. The addition of root exudates is beneficial to promote the oxidation of ferrous iron, the degradation of nitrate, and the removal of As and Hg by the *Clostridium* sp. strain PXL2.

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