Immolization of As(V) in *Rhizopus oryzae* Investigated by Batch and XAFS Techniques

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**ABSTRACT:** Arsenic (As) contamination in aqueous solutions has become an increasing public concern due to the immense harm to human health. Herein, bioaccumulation of arsenate (As(V)) by *Rhizopus oryzae* in aqueous systems was investigated under different environmental conditions, such as different pH’s, ionic strengths, mycelia dosages, mycelia growths, and temperatures. The results showed that As(V) could be bioaccumulated efficiently by *R. oryzae*, and the maximum bioaccumulation capacity of As(V) in *R. oryzae* was 52.4 mg/g at T = 299 K, which was much higher than that for other biomaterials under similar conditions. *R. oryzae* generated a higher content of thiol compounds under As(V) stress to immobilize As(V) from aqueous solutions. X-ray absorption near-edge spectroscopy analysis indicated that As(V) was partly reduced to As(III) with increasing contact time, which increased As(V) bioaccumulation in mycelia. In addition, extended X-ray absorption fine structure analysis showed that the As–S complex played an important role in As(V) immobilization by mycelia. This study provided an in-depth investigation of intracellular As speciation and coordination in *R. oryzae* on the molecular scale, which was crucial to understand the interaction mechanisms of As(V) with fungi during environmental cleanup.

**INTRODUCTION**

Arsenic (As) is known to be one of the most powerful poisons in the world, which can enter drinking water through natural processes (i.e., volcanic emissions, biological activities, and weathering of minerals containing As) and anthropogenic processes (e.g., discharges from industrial wastes and burning of fossil fuels and use of arsenical pesticides). High levels of arsenic in drinking water have been detected in many countries, including Bangladesh, Vietnam, and China. Long-term exposure to As-containing wastewater can result in serious diseases, such as cancer (i.e., cancer of the lung, skin, kidney, nasal passages, liver, and prostate), and can negatively affect the gastrointestinal tract and cardiac, vascular, and central nervous systems. Hence, it is an urgent necessity to reduce As in aqueous solutions to levels below the World Health Organization limit of As in drinking water (10 μg/L).

In recent years, the removal of As from aqueous solutions using various materials, such as nanomaterials, resins, minerals, and microorganisms, has been extensively investigated. Bioremediation of As(V) by microorganisms has been a focus of research because it is environmentally friendly, produces only a few by-products, and is cost-effective. Microorganisms have evolved various mechanisms to deal with As toxicity, including reducing the number of intracellular As molecules and biotransformation of As species by oxidation, reduction, and methylation. It is reported that fungi living in As-contaminated sites have evolved different cellular and molecular systems to survive and have unique merits, such as high bioaccumulation and tolerance to As, high cell wall binding capacity, and high intracellular As uptake capacity. Whereas less information about fungal immobilization for As(V) bioremediation is available.

In this study, the As(V)-resistant fungus *Rhizopus oryzae* was isolated from As-contaminated sites and was evaluated for its...
bioaccumulation efficiency for the bioaccumulation of As(V) from wastewater by the batch technique. Subsequently, the resistance of the fungus to As(V) after different aging times was investigated by X-ray absorption spectrometry (XAFS), including X-ray absorption near-edge spectroscopy (XANES) and extended X-ray absorption fine structure (EXAFS) spectroscopy, to understand the interaction mechanism of As immobilized in R. oryzae.

**RESULTS AND DISCUSSION**

**Identification and Characterization.** The isolate exhibiting resistance to As(V) was subjected to molecular identification. Polymerase chain reaction (PCR) of the internal transcribed spacer (ITS) region yielded a PCR product of 743 bp and blasted to the NCBI-GenBank showing 99% homology to R. oryzae (accession number KM491890.1). Therefore, the fungus was identified as R. oryzae.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) micrographs of mycelia are shown in Figure 1. Before As(V) bioaccumulation, the mycelia were flat and had fewer branches and more folding (Figure 1a), whereas after As(V) bioaccumulation, the mycelia were cylindrical and exhibited more branches (Figure 1b). From the TEM images (Figure 1c,d), it can be seen that the fungal cell structure had obviously changed after As(V) bioaccumulation. The fungal intracellular inclusions significantly increased after As(V) treatment. The above results indicated that more fungal intracellular inclusions were induced to adapt to As(V) stress.

![Figure 1. SEM images of R. oryzae (a) and As(V)-loaded R. oryzae (b). TEM images of R. oryzae (c) and As(V)-loaded R. oryzae (d).](image)

![Figure 2. Potentiometric acid–base titration of R. oryzae (a). Mycelial growth under As(V) stress (b).](image)
The titration curve was plotted for a 0.01 mol/L NaNO₃ solution and displayed nearly identical buffering capacities across the pH range studied (Figure 2a). The surface of \textit{R. oryzae} was positively charged at pH < 6.5, whereas the surface of \textit{R. oryzae} was negatively charged at pH > 6.5. The point of zero change (pH\textsubscript{pzc}) of \textit{R. oryzae} was measured to be 6.5.

**Mycelia Growth under As(V) Stress.** The growth change of the mycelia with an increase in As(V) concentration was studied. Figure 2b shows a weak increase in the biomass of \textit{R. oryzae} with an increase in the As(V) concentration from 0 to 70 mg/L. This is because that the fungal metabolism by utilizing the culture environment created a need for the growth of its own energy.³⁷ For As(V) concentrations higher than 70 mg/L, the biomass of \textit{R. oryzae} decreased rapidly with increasing As(V) concentration. Fungal resistance to As(V) in the environment has a certain tolerance threshold, and As(V) concentrations higher than the threshold value may affect the growth of fungi.²⁷

**Role of pH and Ionic Strength in As(V) Bioaccumulation.** The effects of As(V) bioaccumulation on mycelia as a function of the pH of the medium are shown in Figure 3a. One can see that the bioaccumulation of As(V) by the mycelia decreased slightly with an increase in the pH from 2.0 and 6.0, and a significant decrease was observed at pH > 6.0. The pH values of the system greatly affected As(V) bioaccumulation by both the ionization status of functional groups on mycelia and As speciation. As shown in Figure 3b, As(V) was present in H₂AsO₄⁻ from pH's 3.0 to 6.0 and in HAsO₄²⁻ from pH's 8.0...
to 10.5.\textsuperscript{17} As shown in Figure 2a, a negative charge of the mycelial surface was observed at pH > 6.5. Therefore, the significant decrease in As(V) on the mycelia at pH > 6.5 was mainly attributed to the electrostatic repulsion between H\textsubscript{2}AsO\textsubscript{4}\textsuperscript{-}/HAsO\textsubscript{4}\textsuperscript{2-} and the negatively charged mycelial surface.

The effect of ionic strength on the bioaccumulation of As(V) in mycelia is also shown in Figure 4a. The removal of As(V) significantly decreased with increasing NaNO\textsubscript{3} concentrations (from 0.01 to 0.06 mol/L). The ionic strength-dependent adsorption indicated that ion exchange or outer-sphere surface complexation mainly contributed to As(V) bioaccumulation. Furthermore, partial aggregation of mycelia at higher ionic strengths also resulted in a decrease in the available sites on the mycelia.\textsuperscript{38}

**Effect of R. oryzae Dosage.** The effect of R. oryzae dosage on As(V) bioaccumulation and the extent of removal of As(V) are shown in Figure 4b. As(V) bioaccumulation increased with an increase in R. oryzae dosage in the early stage, and the bioaccumulation remained high at dosages >1.1 g/L. This trend could be explained by the fact that functional groups on the mycelia increased with an increase in R. oryzae dosage, whereas K\textsubscript{d} reduced gradually with an increase in the R. oryzae dosage. Partial aggregation of mycelia at a higher dosage resulted in a decrease in the effective surface area for As(V) bioaccumulation.\textsuperscript{39}

**Bioaccumulation Kinetics.** The kinetics of As(V) bioaccumulation was studied using pseudo-first-order (ln(Q – Q\textsubscript{e}) = ln Q – K\textsubscript{f} t) and pseudo-second-order (\(\frac{t}{Q\textsubscript{e}} = \frac{1}{KQ\textsubscript{e}^2} + \frac{t}{Q\textsubscript{e}}\)) equations, where Q\textsubscript{e} refers to the adsorbability of As(V) (mg/g) at time t (h) and K\textsubscript{f} (1/h) and K\textsubscript{s} (g/(mg h)) are the bioaccumulation rate constants.\textsuperscript{40} The major portion of As(V) was completely coordinated to the adsorbents within 12 h (Figure 4c). Therefore, all bioaccumulation experiments were performed for 24 h to ensure bioaccumulation equilibrium. Data points were fitted better with the pseudo-second-order kinetic model as compared to that with the pseudo-first-order kinetic model, and the kinetic parameters from both models are listed in Table 1. The results on the kinetics indicate that R. oryzae possesses a high bioaccumulation efficiency for As(V).

**Table 1. Parameters for the Bioaccumulation Kinetic Data using Different Models**

| constants        | pseudo-first-order | pseudo-second-order |
|------------------|--------------------|---------------------|
| Q (mg/g)         | 15.221             | 16.302              |
| K (1/h)          | 0.712              |                     |
| K\textsuperscript{s} (g/(mg h)) | 0.052              |                     |
| R\textsuperscript{2} | 0.983              | 0.998               |

**Bioaccumulation Isotherms.** Figure 5a shows the isotherms for As(V) bioaccumulation by R. oryzae at pH 5.5. The As(V) bioaccumulation capacity of R. oryzae increased rapidly with an increase in the As(V) dosage at the initial phase, and it later arrived at the platform. It was also observed that the isotherms showed an increase in bioaccumulation as the temperature increased, and the maximum bioaccumulation occurred at a temperature of 319 K, which indicated the endothermic nature of As(V) bioaccumulation. The bioaccumulation data were fitted to Langmuir (Q = b \times Q\textsubscript{max} \times C_e/(1 + b \times C_e)) and Freundlich (Q = K\textsubscript{f} \times C_e\textsuperscript{n}) models (Q and Q\textsubscript{max} are the amount of As(V) adsorbed on the mycelia and the maximum adsorption amount of As(V) accumulated on the mycelia (mg/g) at complete monolayer coverage, respectively; b (L/mg) is the Langmuir constant related to the bioaccumulation heat; K\textsubscript{f} (mg\textsuperscript{1-n}/L/g) represents the bioaccumulation capacity when the equilibrium concentration of As(V) is 1, and n represents the degree of dependence of bioaccumulation at equilibrium concentration). It was found that the bioaccumulation of As(V) on R. oryzae correlated well with the Langmuir equation as compared to that with the Freundlich equation (Table 2 and Figure 5a). The C\textsubscript{e}max values of As(V) on the mycelia was 52.4 mg/g at 299 K and was higher than those of many corresponding biomaterials reported in the literature (Table 3).\textsuperscript{41–46}

**Intracellular Thiol Content.** The intracellular thiol content is shown in Figure 5b. The level of intracellular thiol increased in response to As(V) exposure. The maximum increases in the thiol content were 143% (at 200 mg/L) and 201% (at 400 mg/L) after 24 h of exposure, whereas the increases were 87% (at 200 mg/L) and 132% (at 400 mg/L) after 72 h of exposure. The increase in the intracellular thiol content of R. oryzae may indicate the role of thiols in the detoxification or tolerance of As(V). These results are in accordance with the thiol groups, which may form complex with As(V) as a part of tolerance mechanism.\textsuperscript{34} Canovas et al. also showed that soluble thiol species played a key role in the response of Aspergillus sp. P37 to arsenic, and thiol–As complexes were formed and contributed in the vacuoles of Aspergillus sp. P37.\textsuperscript{47}

**Fourier Transform Infrared (FTIR) Analysis.** The FTIR spectra of R. oryzae and As(V)-loaded R. oryzae are displayed in Figure 6a. The characteristic bands of As(V)-loaded R. oryzae shifted in comparison to those for R. oryzae, such as the bands corresponding to the –OH stretching vibration (from 3461 to 3597 cm\textsuperscript{-1}), N–H stretching and C=O stretching vibrations of the amide group (from 1664 to 1636 cm\textsuperscript{-1}), and C–N stretching vibrations (from 1074 to 1119 cm\textsuperscript{-1}). Besides, the weak peak at 2551 cm\textsuperscript{-1} represented the disappearance of the thiol groups after As(V) bioaccumulation in the mycelia.\textsuperscript{48} Hence, hydroxyl, amino, and thiol groups are possibly involved in As(V) bioaccumulation in the mycelia.\textsuperscript{49–51}

**XAFS Spectral Analysis.** The intracellular As of fungal mycelia was investigated by the XANES and EXAFS techniques. The XANES spectra of As in R. oryzae are presented in Figure 6b and demonstrate differences between 11 868 and 11 873 eV. The absorption edge energies of As(III) and As(V) were 11 870.9 and 11 874.4 eV, respectively.\textsuperscript{24,52} The results showed that As speciation in fungal mycelia transformed with an increase in aging time. After 4 h of culture at pH 5.5, only As(V) was detected. Both As(V) and As(III) were found in the mycelia of R. oryzae after 4 days of culture, and the predominant valence state of As in the mycelia was As(V). After 8 days of culture, both As(V) and As(III) were detected, and the As(III) content was higher than that of As(V) within the mycelia. These results indicated that R. oryzae had a strong ability to reduce As(V) to As(III) and immobilized As(III), which thereby decreased the arsenic toxicity.\textsuperscript{53}

The best fits of the EXAFS spectra of As in R. oryzae are shown in Figure 7. The fitted structural parameters (coordination number (CN), R, and σ\textsuperscript{2}) are listed in Table 4.
The ^3\text{-}weighted \(\chi(k)\) spectra (Figure 7a) and radial structure functions (RSFs) (Figure 7b) of the samples were all different, which suggested that As in the mycelia of \textit{R. oryzae} samples had different coordination structures. At a cultivation period of 4 h and a of pH 5.5, the peak at \(\sim1.67 \pm 0.02\) Å, with CN varying from 3.6 to 4, was indicative of the first shell of As\(^{\text{-}O}\), which was in good agreement with that for arsenate.\(^{18,54}\) After 4 days of culture, the first coordination shell surrounding the As atom still contained oxygens, and the As\(^{\text{-}O}\) distances changed from 1.69 to 1.76 Å, which was similar to that for As(III). Moreover, the second shell distinctly showed that As was coordinated with sulfur atoms.\(^{55}\) Especially after 8 days of culture, the CN of As\(^{\text{-}S}\) increased from 1.4 to 1.7 and the CN of As\(^{\text{-}O}\) decreased, indicating that the amount of As coordinated to sulfur increased as the aging time increased.\(^{56}\) Furthermore, the As speciation in the mycelia from EXAFS analysis was consistent with the XANES results. XAFS data provided information on the average As coordination in the mycelia illuminated by synchrotron light, and it was difficult to acquire exact information on a pure compound from the mixture. At best, we can suggest that As was present as a mixture of coordinated compounds As(III)\(^{\text{-}O}\) and As(III)\(^{\text{-}S}\). As(III) had a strong affinity for thiols; thus, the thiol group was probably responsible for the coordination with As(III). Former researches have shown that As stimulated the production of thiol compounds,\(^{55,57}\) and the As atom was bound to S atoms from different cysteine (Cys) residues, with R values varying from 2.2 to 2.25 Å.\(^{57,59}\) \textit{R. oryzae} was isolated from As-contaminated soils and used to bioremediate As(V) under different environmental conditions.

### Table 2. Parameters for the Langmuir and Freundlich Isotherm Models

| \(T\) (K) | \(Q_{\text{max}}\) (mg/g) | \(b\) (L/mg) | \(R^2\) | \(K_F\) (mg\(^2\)-L\(^{-1}\)/g) | \(n\) | \(R^2\) |
|------|----------------|--------|------|----------------|----|------|
| 299  | 52.4           | 0.851  | 0.994| 5.91           | 0.469 | 0.959 |
| 309  | 65.9           | 0.092  | 0.997| 9.03           | 0.521 | 0.978 |
| 319  | 70.2           | 0.204  | 0.992| 16.58         | 0.421 | 0.945 |

### Table 3. Comparison of the Maximum Bioaccumulation Capacities of As(V) on Various Biosorbents

| biosorbents            | experimental conditions | \(Q_{\text{max}}\) (mg/g) | refs |
|------------------------|-------------------------|----------------|------|
| \textit{Inonotus hispidus} | pH = 6.0, \(T = 293\) K    | 51.9          | 41   |
| mycan/HDTMA            | pH = 3.0, \(T = 293\) K    | 57.85         | 42   |
| \textit{Lessonia nigrescens} | pH = 2.5, \(T = 293\) K | 45.2          | 43   |
|                        | pH = 4.5, \(T = 293\) K    | 33.3          |      |
|                        | pH = 6.5, \(T = 293\) K    | 28.2          |      |
| iron oxide-coated sponge | pH \(= 7.2, T = 303\) K | 4.5           | 44   |
| tea fungal biomass      | pH = 7.2, \(T = 303\) K    | 4.95          | 45   |
| sulfate-reducing bacteria | pH = 6.5, \(T = 298\) K | 1.76          | 46   |
| \textit{R. oryzae}     | pH = 5.5, \(T = 299\) K    | 52.4          |      |

\textit{R. oryzae} was isolated from As-contaminated soils and used to bioremediate As(V) under different environmental conditions.
ditions. *R. oryzae* showed a high capacity to accumulate As(V), and the equilibrium bioaccumulation data can be satisfactorily simulated by the Langmuir model. The results from XAFS analysis indicated that most of the intracellular As(V) was reduced to As(III) and complexed with thiols after 8 days of culture, which contributed to understanding the interaction mechanisms of As in *R. oryzae* under As(V) stress. After As(V) is bioreduced to As(III) on *R. oryzae*, it can be immobilized on *R. oryzae* and can thereby decrease As(III) pollution. These findings showed that *R. oryzae* could be an efficient and promising bioremediation material for As(V) pollution.

**Experimental Section**

**Isolation and Identification of As(V)-Resistant Fungus.** A soil sample was collected from As-contaminated sites in Dengjiaotang, Chenzhou City, and Hunan Province, China. The soil sample (1.0 g) was suspended in 100 mL of sterile distilled water. Serial dilutions were conducted to obtain a solution of 10⁻⁵ dilution for fungal isolation. Then, a 200 μL suspension was plated onto a sterile potato dextrose agar (PDA) plate containing 500 mg/g As(V). The plate was incubated at 26 °C for 4 days of culture. All colonies that appeared on each plate were marked, the colony-forming units were calculated, and for 4 days of culture. All colonies that appeared on each plate containing 500 mg/g As(V). The plate was incubated at 26 °C. Finally, the solid phase was separated from the liquid phase by centrifugation at 9000 rpm for 20 min. An atomic absorption spectrophotometer (Shimazu ICPE-9000) was used to measure the As(V) concentration in the supernatant. The results from XAFS analysis indicated that most of the intracellular As(V) was reduced to As(III) and complexed with thiols after 8 days of culture, which contributed to understanding the interaction mechanisms of As in *R. oryzae* under As(V) stress. After As(V) is bioreduced to As(III) on *R. oryzae*, it can be immobilized on *R. oryzae* and can thereby decrease As(III) pollution. These findings showed that *R. oryzae* could be an efficient and promising bioremediation material for As(V) pollution.

**Characterization of Mycelia.** Morphological analysis of *R. oryzae* was performed by SEM (FEI-JSM 6320F) and TEM (JEM-2010, Japan). For SEM analysis, fungal mycelia of *R. oryzae* and As(V)-adsorbed *R. oryzae* were fixed with 5% glutaraldehyde at 4 °C for 4 h; washed with 0.1 mol/L phosphate buffer; and then dehydrated sequentially in 10, 30, 70, 90, and 100% ethanol; they were finally critical-point-dried and gold-coated with stubs for analysis. For TEM analysis, the samples were prefixed with 5% glutaraldehyde, infiltrated with resin, and then sectioned to a 70 nm thickness with an ultramicrotome (Leica EM UC7, Austria) and collected on copper grids for analysis. The functional groups of the mycelia were analyzed by an automatic titrator system (DLS8; Mettler Toledo, Germany) and an infrared spectrophotometer (Perkin-Elmer 100).

**Bioaccumulation Procedures.** Bioaccumulation of As(V) by mycelia was studied under ambient conditions. The different concentrations of mycelia suspensions, As(V) and NaNO₃ solution were added into Erlenmeyer flasks. Thereafter, the pH was adjusted to the desired value with negligible amounts of HNO₃ or NaOH solution. After bioaccumulation equilibrium was reached, the solid phase was separated from the liquid phase by centrifugation at 9000 rpm for 20 min. An atomic absorption spectrophotometer (Shimazu ICPE-9000) was used to measure the As(V) concentration in the supernatant. The bioaccumulation percentage and As(V) accumulation capacity (Q; mg/g) are described in eqs 1 and 2.

\[
\text{Bioaccumulation\% (}) = \left( \frac{C_0 - C_e}{C_0} \right) \times 100\% / \text{V/m}
\]

Where \( C_0 \) and \( C_e \) (mg/L) are the initial and equilibrium concentrations, respectively, and \( m \) and \( V \) are the mass of the mycelia and volume of the suspension, respectively. All tests were conducted in triplicate.

**Assay of Intracellular Thiol Content.** The concentration of intracellular thiol groups was determined using a UV–vis spectrophotometer (Shimadzu, Japan) at 412 nm. Briefly, 0.2 g of fresh mycelia was ground in a 2 mL centrifuge tube in liquid nitrogen, extracted with 1.0 mL of 5% sulfoalicylic acid, and then centrifuged at 12 000 rpm for 15 min at 4 °C. Finally, 200 μL of the supernatant was mixed with 600 μL of 0.2 mol/L H₂O₂, 500 μL of 100 mmol/L EDTA, and 500 μL of 10 mmol/L CuSO₄, and then incubated at 37 °C for 1 h. The absorbance was measured at 412 nm.
Tris—HCl (pH 8.0) and 40 μL of 10 mmol/L S,S′-dithiobis(2-nitrobenzoic acid).

X-ray Absorption Spectroscopy. Briefly, the PDA liquid medium in 250 mL flask bottles containing 500 mg/L As(V) and 2.0 g/L mycelia at 26 °C for different aging times of culture, and fungal mycelia were washed with sterile Milli-Q water and phosphate-buffered saline to remove the residual As(V). Then, XAFS of the fungal samples was conducted at the Shanghai Synchrotron Radiation Facility, with an electron storage ring energy of 3.5 GeV and a maximum storage beam current of 210 mA. A double-crystal Si(111) monochromator was used. A multielement pixel high-purity Ge solid-state detector was used to record the fluorescence signal. Sodium arsenite and sodium arsenate were used as references for XAFS evaluation. As K edge EXAFS spectra were extracted from the subtraction of pre-edge, background, and post-edge, the correction of energy. The k^2-weighted EXAFS data and Fourier-transformed RSFs were analyzed using the Athena and Artemis interfaces of IFFEFIT 7.0 software.\(^{1,2}\) The CN, interatomic distance (\(R\)), and Debye–Waller factor (\(\sigma^2\)) were allowed to be adjustable parameters.

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**Notes**
The authors declare no competing financial interest.

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