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Arsenic (As) Removal Using *Talaromyces* sp. KM-31 Isolated from As-Contaminated Mine Soil

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Abstract: Bioremediation is an environmentally-benign and cost-effective approach to removing arsenic from contaminated areas. A fungal strain hyper-tolerant to arsenic was isolated from soil from a mine site and used for the removal of arsenic. The isolated fungus was identified as *Talaromyces* sp., and its growth rate, arsenic tolerance, and removal rates were investigated for As(III) and As(V). Arsenic tolerance tests revealed that the fungus was highly resistant to arsenic, tolerating concentrations up to 1000 mg/L. Robust mycelial growth was observed in potato dextrose broth containing either As(III) or As(V), and there was no difference in growth between that in arsenic-free medium and medium amended with up to 300 mg/L of either arsenic species. The isolate showed relatively low growth rates at As(V) concentrations >500 mg/L, and almost no growth at As(III) concentrations >300 mg/L. Both arsenic species were effectively removed from aqueous medium (>70%) in tests of the biosorption of arsenic onto mycelial biomass. Surface modification of the biomass with Fe(III) (hydr)oxides significantly enhanced arsenic removal efficiency. The findings indicate that this soil fungal strain has promise for use in bioremediation strategies to remove arsenic from highly contaminated aqueous systems.

Keywords: arsenic removal; *Talaromyces* sp. KM-31; biosorption; Fe(III) hydroxide; Fe(III) oxide

1. Introduction

Arsenic (As) is a major pollutant in the environment, derived from the weathering of rocks and volcanic activity, and from anthropogenic inputs including industrial activities, mining operations, and agriculture [1,2]. In the natural environment, arsenic is present in a variety of inorganic forms such as As(V) and As(III), and organic forms such as monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and trimethylarsenic oxide (TMA). Microbes can convert arsenic from one form to another via oxidation/reduction and methylation/demethylation processes [3]. As(V) occurs predominantly under oxidizing conditions, whereas As(III) is mainly found under reducing conditions [4]. In particular, As-contaminated soil is of concern, as human exposure to arsenic can occur through both the food chain and the water supply [2,5]. Various technologies to remediate arsenic-polluted soil and water have been developed, including stabilization and solidification, but the application of these methods has been limited for economic reasons and because of disposal problems [6,7].

Microbial biogeochemical transformation of inorganic elements is a result of assimilatory processes in which an element is incorporated into biomass, and/or dissimilatory processes in which transformation results in energy generation and/or detoxification [8]. Fungi are able to tolerate and/or detoxify metals using several mechanisms, including redox transformation, biosorption, extracellular

and intracellular precipitation, and active uptake. As a result, the responses of microorganisms to toxic metals can dramatically alter metal abundance and speciation [9]. Biosorption is considered to be a promising method for the removal of metal ions from aqueous solutions. Algae, fungi, and bacteria have been used as biomass-derived biosorbents for several metals [10–12]. Biosorption of toxic metals and metalloids by fungal biomass in both living and non-living forms provides a cost-effective and suitable technology for remediating soil and water contaminated by toxic metals [13–15].

Many fungal species have been found to be capable of arsenic removal, and the accumulation of arsenic by microorganisms has been recognized as a potential remediation method because it is environmentally-friendly and cost-effective [7]. Fungal strains capable of arsenic accumulation or biosorption include *Phaeolus schweinitzii* [16], *Fusarium oxysporum* [17], *Sinorhizobium meliloti* [18], *Neosartorya fischeri* [15], *Aspergillus candidus* [19], *Trichoderma* sp., *Neocosmospora* sp., *Rhizopus* sp. [20], and *Paecilomyces* sp. [21]. Given the variety of mechanisms of heavy metal resistance and the reported removal of arsenic by fungi, it is likely that arsenic-tolerant fungi with substantial arsenic removal ability will be present at As-contaminated sites. Therefore, it is important to expand investigations to screen native fungi present at As-contaminated sites for their potential for arsenic remediation.

The surface charge on fungal mycelium is typically negative over pH ranges of 3.0~10.0 [20,22]. Although few fungal species are able to remove or volatilize anionic metal(loid)s, including As(V), their negative surface charge is unfavorable for adsorptive removal of anionic pollutants. Therefore, we hypothesized that surface modification with a cationic agent could enhance arsenic removal ability. The synthesis and utilization of iron oxide materials having specific properties and functions have been widely investigated because of their high surface area to volume ratios [23]. In past studies [24], iron oxide materials have been widely used to remove a range of metalloids and heavy metals, including arsenic, lead, mercury, cadmium, and copper. Moreover, iron oxides have low toxicity, chemical inertness, and biocompatibility, and hence have enormous potential in biotechnology applications [25]. Surface-coating media and preparation methods play an important role in determining surface chemical properties, size distribution, morphology, and magnetic properties; therefore fungi- or protein-mediated biological methods for iron oxide nanomaterials as emerging methods necessitate wide development [26]. Nonetheless, iron oxide-based biotechnologies for heavy metal adsorption are at a relatively early stage in terms of wide application.

In the present study, microorganism in soil from an arsenic-contaminated mine was isolated and screened for arsenic removal ability, yielding in a single culturable fungal isolate. The objectives of this study were: (1) To explore the tolerance and growth of the arsenic-tolerant fungal isolate in the presence of As(III) and As(V); (2) to assess the arsenic removal and transformation activity of the arsenic-tolerant isolate; and (3) to test the removal of arsenic from an aqueous system using mycelial pellets and Fe(III) (hydr)oxides-coated dry fungal biomass.

2. Materials and Methods

2.1. Chemicals

Sodium arsenite ($NaAsO_2$) and sodium arsenate dibasic heptahydrate ($Na_2HAsO_4{\cdot}7H_2O$) were obtained from Merck (Darmstadt, Germany). For quantification, an authentic analytical arsenic standard (1000 mg/L) was obtained from Junsei Chemicals (Kyoto, Japan). Potato dextrose broth and agar media were obtained from Fluka (Darmstadt, Germany). All chemicals used were of analytical grade or the highest purity.

2.2. Fungal Isolation and Molecular Identification

An arsenic-resistant filamentous fungus was isolated by selective enrichment of soil suspension (100-fold dilution) with arsenic-polluted soil (5000 mg/kg of total arsenic) collected at a former arsenic mine site located near the city of Cheongyang, Republic of Korea. The isolate was cultured in mineral medium containing 0.25% KH_2PO_4, 0.20% $MgSO_4$, 0.50% $(NH_4)_2SO_4$, 0.50% NaCl, and 0.25% glucose

supplemented with 500 mg/L $NaAsO_2$. The pH of the medium was maintained at 5.3 using 100 mM citrate phosphate buffer. The cultures were incubated at 28 °C for 7 days. Molecular identification of the arsenic-tolerant isolate was achieved by sequencing the internal transcribed spacers ITS1 and ITS2 of the test fungus. The ITS regions were PCR-amplified using the universal primers ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′. Extraction of genomic DNA was performed by using the Power Soil DNA extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA) and the extracts thus obtained were pooled prior to PCR amplification. PCR cycling conditions were as follows: One initial cycle of denaturation at 94 °C for 3 min, followed by 34 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), and at 72 °C for 45 s (elongation), and an extension for 5 min at 72 °C. To identify the isolate, the PCR-amplified ITS DNA was sequenced, and the sequence was used in a BLAST search of the NCBI database. Pairwise nucleotide sequence comparisons, DNA alignments using CLUSTALW, and phylogenetic relationships to the most closely related fungal strains were analyzed using the MEGA 5.0 software package. The obtained sequence was deposited in GenBank (accession No. MK955349).

2.3. Arsenic Tolerance Assay

A fungal isolate was tested for tolerance to arsenic in potato dextrose agar (PDA) medium containing various concentrations (50, 100, 200, 300, 500, 800, and 1000 mg/L) of As(III) or As(V) as sodium arsenite and sodium arsenate, respectively. Mycelial discs (5 mm) from pure 7-day-old cultures grown on PDA were inoculated onto Petri dishes (9 cm) containing As(III) or As(V), and incubated at 30 °C. Mycelial growth was monitored by measuring the radial growth of the mycelial colony. For the determination of dry cell weight, the PDA plate was mixed with 100 mL of distilled water and boiled to dissolve the agar. The agar solution containing the fungal biomass was filtered through dry No. 5 Whatman filter paper, and the filter paper with the retained fungal cells was then dried in an oven at 60 °C for 24 h until a constant dry weight was attained. Arsenic tolerance was calculated by comparing the radial growth of each fungus in control medium containing no arsenic. A tolerance index was used, defined as the change in radius of the fungus in the presence of arsenic divided by its change in radius in the same period in the control. The fungal isolate that grew was regarded as tolerant to arsenic and was used in further experiments.

2.4. Arsenic Biosorption Experiments

An arsenic biosorption experiment was conducted using pre-grown mycelium of *Talaromyces* sp. KM-31. A culture of the fungus that had been grown in potato dextrose broth for 7 days was filtered onto a glass fiber filter disc (47 mm) and washed with deionized water. A known quantity of mycelial biomass (60 mg/mL of dry weight) was transferred into Erlenmeyer flasks containing deionized water and various concentrations of As(III) or As(V) (1~25 mg/L). Arsenic biosorption/removal was monitored at different time intervals over a period of 7 days. The uptake of arsenic by live and inactivated *Talaromyces* sp. KM-31 mycelium was analyzed using the Langmuir adsorption isotherm model, and biosorption efficiencies were compared [27]. Inactivated mycelium was obtained from fungal cultures grown in PDB that were dried in an oven at 80 °C for 24 h after harvesting.

2.5. Arsenic Removal by Modified Fungal Biomass

To assess whether arsenic removal is enhanced by coating with Fe(III) (hydr)oxides, the surface of the fungal biomass was modified by addition of a Fe(III) hydroxide or nano-sized Fe(III) oxide coating. For surface coating, wet mycelium was initially suspended in 1 M $FeCl_3{\cdot}6H_2O$ Fe(III) chloride solution, then 3 M NaOH or $NaBH_4$ was added and the mixture stirred well to form particles of Fe(III) hydroxide or nFe(III)-oxide, respectively. The mycelium coated with Fe(III) (hydr)oxides was then separated and dried overnight in a vacuum oven at 90 °C. The dried mycelium was ground to a fine powder for use in arsenic removal experiments.

2.6. Analytical Methods

To determine the arsenic content, dried mine soil or treated fungal material was placed in a Teflon vessel and extracted in a microwave oven using concentrated nitric acid (HNO_3), following EPA Method 3051A [28]. Following cooling, each sample was filtered and the volume was adjusted using ultra-pure water. Arsenic content was determined using inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Elemental, Franklin, MA, USA). As speciation was conducted by using a solid phase extraction resin (Supelclean^{TM} LC-SAX SPE tube, Sigma-Aldrich, Seoul, Korea) that can remove anionic As(V) (As_{total} − As(III) = As(V)). All samples were analyzed in triplicate to check precision. The analytical precision among replicates (n = 3) was within 5%. Final results were reported as mean values of the triplicate measurements, and were expressed as mg/kg dry weight. To determine the residual concentration of arsenic in the medium, samples of the growth medium in the flasks after incubation were centrifuged at 16,000 rpm for 20 min and the arsenic concentrations of the resulting cell-free supernatant was determined using ICP-MS.

3. Results and Discussion

3.1. Isolation and Identification of anAarsenic-Tolerant Fungal Strain

Only one type of fungus was cultured on both PDA and PDB from As-contaminated mine soil. The ITS DNA sequence of the purified fungal isolate showed maximum identity with the sequence of *Talaromyces helicus*, and phylogenetic analysis also showed that the isolate was closely related to *T. helices* (GenBank accession No. FJ430764). The purpose of this investigation was to obtain soil fungi from highly arsenic-contaminated mine soils for assessment of their potential for use in bioremediation of arsenic-contaminated aqueous systems. A wide range of fungi from all major taxonomic groups have been observed in metal-polluted habitats [29], and various fungi have been isolated from soil polluted with heavy metals in various parts of the world, including *Aspergillus*, *Rhizopus*, *Penicillium*, *Fusarium*, *Chaetomium*, *Geomyces*, and *Paecilomyces* spp. [12,14,29,30]. *Talaromyces* sp. are known to be tolerant of harsh conditions, making them potentially useful in extreme environments, including acidic and metal(loid)-contaminated mine drainage and soils [31,32]. Past studies have shown metal ion adsorption by *Talaromyces* sp. Romero et al. used *T. helicus* to degrade biphenyl treated with high levels of copper (Cu), and showed the detoxification abilities of this species and its adaptation to heavy metals and biacrylic compounds [33]. In addition, *T. emersonii* has been shown to have uranium (U) biosorption ability [34]. Using metal-resistant fungal strains in bioremediation strategies could increase the detoxification and biosorption of pollutants in contaminated areas.

3.2. Growth and Arsenic Tolerance of Talaromyces sp. KM-31

The isolated *Talaromyces* sp. KM-31 strain grew well on PDA medium augmented with up to 200 mg/L As(III) or 300 mg/L As(V) (Figure 1).

The isolate showed relatively low growth rates at As(V) concentrations >500 mg/L and almost no growth at As(III) concentrations >300 mg/L, indicating that, although the fungal strain was tolerant to arsenic, high concentrations inhibited growth. The isolate was more tolerant of As(V) than As(III) because the latter has a higher toxicity. After 15 days of incubation, the tolerance index values over a range of As(III) and As(V) concentrations were 1.0 or near that value, indicating that growth was not affected on solid PDA medium (Table 1 and Table S1).

In PDB media, the isolate grew well up to 200 mg/L As(III) or 300 mg/L As(V). The tolerance index results show that growth was completely inhibited at concentrations >300 mg/L As(III), while As(V) did not completely inhibit growth even at concentrations >500 mg/L. However, 30−65% inhibition of mycelial growth occurred at concentrations >300 mg/L, indicating that the isolate was highly tolerant of As(V) in the complex organic medium. For cultures 15 days old, the rate of removal of As(V) was 3- to 5-fold higher than that for As(III). The variation in tolerance of this strain to As(III) or As(V) may be exhibited by redox conditions because of the presence of one or more types of tolerance strategy or

resistance mechanisms [35,36]. For different species of metal(loid), or among different fungal strains, differing biological mechanisms are involved in fungal survival [8,10,13,37]. In-vitro evaluation of tolerance of *Aspergillus* and *Rhizopus* over a wide range of arsenic concentrations [38] revealed that As-resistant isolates tolerated arsenate concentrations up to 1000 mg/L, and maintained their tolerance index in a range of 0.35 to 0.98 (Table 1). The radial growth patterns of *Talaromyces* sp. KM-31 in the present study appear to reflect tolerance development in the fungus (Table 1 and Figure 1); this could be used as a basis for selecting tolerant strains in metal(loid) removal assays. Intracellular accumulation of arsenic in vacuoles may play a major role in detoxification of arsenic in tolerant fungi [39], although other explanations for arsenic tolerance include detoxification by arsenic biomethylation and radial growth stimulation in the presence of different concentrations of arsenic in PDA media [40].

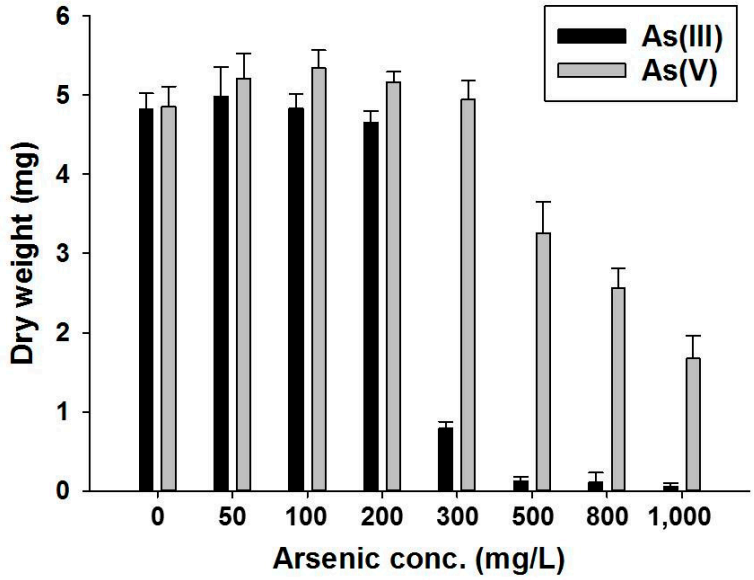

Figure 1. Changes in biomass of *Talaromyces* sp. KM-31 after 15 days of cultivation on PDA medium augmented with arsenic. The As(III) and As(V) concentrations ranged from 0 to 1000 mg/L.

Table 1. Arsenic tolerance index for *Talaromyces* sp. KM-31 at varying concentrations of As(III) or As(V) in PDB and PDA media after 15 days of cultivation, and arsenic removal rates in PDB medium.

Arsenic Tolerance Index in Solid and Liquid Media					Arsenic Removal (%)	
As (mg/L)	PDA		PDB		PDB	
	As(III)	As(V)	As(III)	As(V)	As(III)	As(V)
0	1.0	1.0	1.0	1.0	0	0
50	0.98	0.96	0.98	0.98	5.24	26.2
100	0.96	0.97	0.98	0.98	4.41	17.25
200	0.96	0.96	0.97	0.97	2.8	7.53
300	0.98	0.97	0.13	0.96	0.41	1.24
500	0.96	0.97	0.008	0.67	0	0.78
800	0.96	0.97	0.008	0.5	0	0.52
1000	0.96	0.98	0.004	0.35	0	0.32

3.3. Arsenic Removal by Live Mycelial Pellets

Arsenic removal by live mycelium of *Talaromyces* sp. KM-31 was assessed in an aqueous system (Figure 2). Effective removal of relatively low concentrations of arsenic (1.0~5.0 mg/L) by live biomass was observed, but as the concentration increased, the extent of removal decreased. The live mycelium preferentially removed As(V) compared with As(III), and showed no reduction or oxidation of either

arsenic form. This is consistent with the data on *Talaromyces* sp. KM-31 growth patterns (Figure 1 and Table 1).

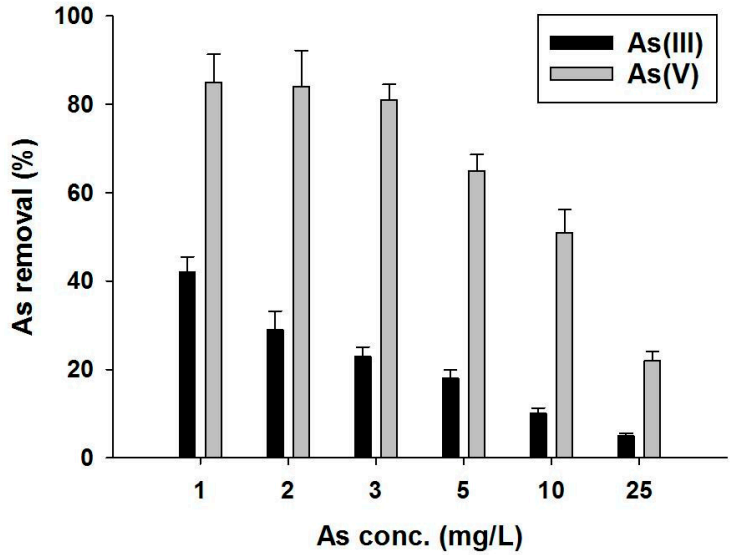

Figure 2. Percent change in live mycelial biomass of *Talaromyces* sp. KM-31 at various initial arsenic concentrations (1.0~25.0 mg/L) in an arsenic removal assay. The experimental conditions were biomass 60 g/L, contact time 72 h, pH 6.0, temperature 30 °C, and shaking at 160 rpm.

The removal of As(V) was analyzed using adsorption isotherms (Figure 3), which showed that the experimental data fitted well with the Langmuir isotherm model. The isotherm studies revealed that the adsorption capacity increased as the concentration of As(V) increased. According to the Langmuir isotherm, the maximum arsenic absorption capacity was 0.005 mg/g for live mycelium and 0.0008 mg/g for inactivated mycelium, and the isotherm constants (K_L) were 0.8948 L/mg and 1.027 L/mg for live and inactivated mycelium, respectively (Figure 3). The mycelial sorption of arsenic was directly estimated by digesting the mycelium after it had been exposed to arsenic, and then comparing the arsenic concentration in solution with that estimated based on removal efficiency. A mass balance difference was observed between the removed and residual arsenic contents, suggesting that the live mycelium may have volatilized the absorbed arsenic, in agreement with previous reports [41].

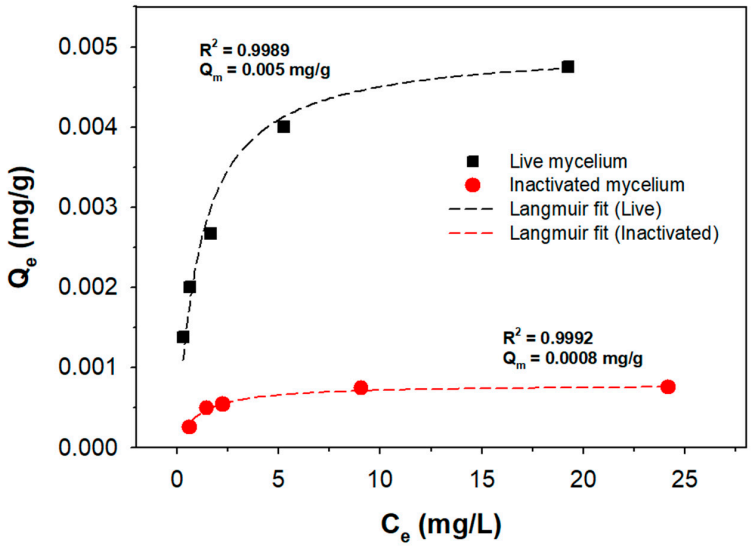

Figure 3. Isotherm for the removal of arsenic. The Langmuir isotherm is shown. The experimental conditions were biomass 60 g/L, contact time 72 h, pH 6.0, temperature 30 °C, and shaking at 160 rpm.

The inactivated mycelial biomass showed poor removal ability compared with the live mycelium, indicating that active arsenic biosorption mechanisms are involved [14]. The biosorption mechanisms may differ among fungal species depending on redox chemistry and biomass specificity [42,43]. Previous studies have reported that metal tolerant strains of fungi, including *Rhizopus nigricans* and *Aspergillus* spp., could accumulate cadmium (Cd) and chromium (Cr) from aqueous solution [38], although the biosorption levels varied among different fungal strains [44–46]. Similarly, in this study, we showed that the live mycelium of *Talaromyces* sp. KM-31 accumulated arsenic, but with greater tolerance of As(V). Biosorption of toxic metal(loid)s involves ionic species on the fungal cell surface, including extracellular polysaccharides, proteins, and chitins [14,38]. Most fungal species have chitin and chitosan as integral parts of their cell wall structure, and hence have potential for effective biosorption of metal(loid)s [47]. The metal biosorption by *Rhizopus arrhizus* was predominantly associated with cell wall chitin and chitosan [44], but deacetylated amino groups of glucosamine may also act as binding sites for metals [20,48]. Melanins and phenolic polymers also have oxygen-containing moieties, including carboxyl, phenolic, alcoholic hydroxyl, carbonyl, and methoxy groups, that could potentially act as metal-binding sites [29]. It has been proposed that arsenic-tolerant fungi isolated from contaminated soil reduce arsenic uptake by enhanced production of melanin, which may bind the toxic element [49]. Arsenic binding by fungi seems mainly to involve passive biosorption to the cell wall, which promotes arsenic uptake through intracellular accumulation [20,48].

3.4. Arsenic Removal by Modified Biomass of Talaromyces sp. KM-31

The potential to enhance metal(loid) accumulation or sorption by *Talaromyces* sp. KM-31 was assessed by coating the surface of the mycelium with Fe(III) (hydr)oxides (Table 2).

Table 2. Arsenic removal (%) by untreated biomass, Fe(III) hydroxide-coated biomass, and Fe(III) oxide-coated biomass.

Treatment [a]	Arsenic Removal (mean ± SD [b], %)	
	As(III)	As(V)
Untreated biomass	4 ± 0.32	13 ± 0.97
Fe(III) hydroxide-coated biomass	69 ± 2.79	79 ± 3.65
nFe(III) oxide-coated biomass	91 ± 5.25	96 ± 4.18

[a] Sorbent, 1 g/L; sorbate, 5 mg/L; pH 6.0; temp, 30 °C; contact time, 24 h. [b] The mean and standard deviation of three replicate determinations.

We evaluated the arsenic removal rate by biomass of *Talaromyces* sp. KM-31 coated with Fe(III) (hydr)oxides. X-ray diffraction analysis showed that the two modes of coating used in the present work formed different Fe(III) oxide species (Figure 4). From diffraction peaks, the primary particle size of Fe(III) hydroxide and Fe(III) oxide coated on fungi was determined to be approximately 24 and 17 nm, respectively, which confirmed the formation of nano-sized Fe(III) (hydr)oxide on fungi. Arsenic removal data revealed that modification of the biomass enhanced removal of both arsenic species relative to uncoated biomass (Table 2). In the case of As(III), live biomass of *Talaromyces* sp. KM-31 coated with Fe(III) hydroxide removed 69.0% of the arsenic, while biomass coated with nano-sized Fe(III) oxide removed 91.0%, whereas untreated mycelium removed only 4.0%. In the case of As(V), the removal rates were higher than those for As(III), which is consistent with the growth and tolerance results. The untreated biomass removed 13.0% of the As(V), while the removal rates for treated biomass were 79.0% with the Fe(III) hydroxide coating and 96.0% with the Fe(III) oxide coating. Thus surface modification resulted in better affinity, and hence more effective removal of arsenic. This is arisen from an intrinsic property of Fe(III) oxide species favorable for arsenic adsorption (see Table S2). In past studies, the addition of coatings has been shown to be effective in arsenic removal. For example, sand coated with iron oxide showed greater arsenic removal than sand alone [50], orange juice industrial residue loaded with Fe(III) [51], and *Penicillium purpurogenum* [52]. In another study,

chemical modification of *Penicillium chrysogenum* biomass increased arsenate removal [53]. Similarly, in the present study, we found that Fe(III) (hydr)oxide modification of *Talaromyces* sp. KM-31 greatly enhanced its arsenic removal capacity. Fungal cells are inherently fragile, limiting freely-suspended biomass as a good sorbent for metal(loid) removal from aqueous solution. This fragility problem can be reduced by immobilization of fungal biomass in a suitable porous matrix [12]. Use of Fe(III) (hydr)oxides as an immobilization matrix has advantages, including large surface area, absence of toxicity, and ease of handling.

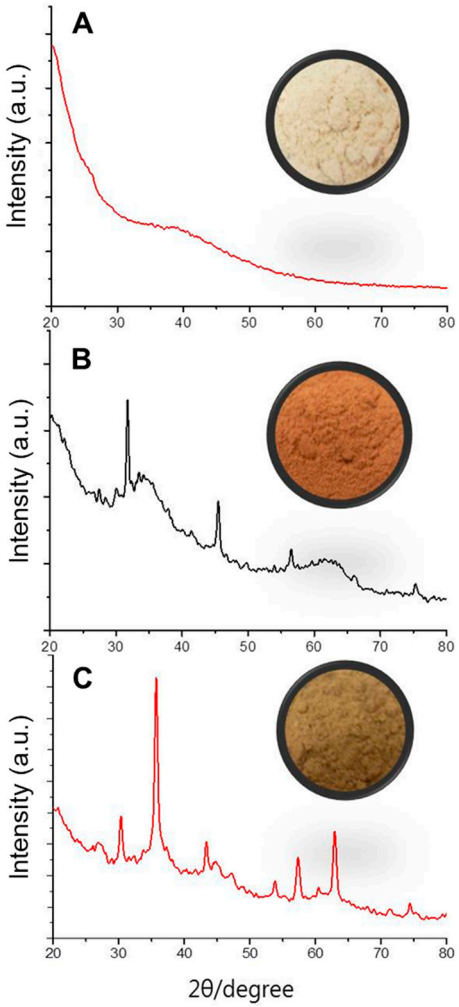

Figure 4. X-ray diffractograms and images (insets) for untreated *Talaromyces* sp. KM-31 and Fe(III) hydr(oxide)-coated biomass. (**A**) untreated biomass; (**B**) Fe(III) hydroxide-coated biomass; and (**C**) nFe(III) oxide-coated biomass.

4. Conclusions

Talaromyces sp. KM-31 isolated from highly As-contaminated soil from a mine site showed hyper-tolerance to arsenic, and was able to grow in the presence of arsenic concentrations up to 300 mg/L in a complex organic medium. Although the isolate tolerated arsenic and grew well in its presence, its arsenic removal ability was poor at high arsenic concentrations. At low arsenic concentrations, live mycelium was more effective at removing As(V) than inactivated mycelium. Powder prepared from mycelium of *Talaromyces* sp. KM-31 that had been coated with Fe(III) (hydr)oxide showed several-fold enhanced removal of both As(III) and As(V), suggesting that the modified biomass could be used for water purification. The present findings indicate that this Fe(III)-modified fungal strain has promise

for use as an efficient biosorbent in bioremediation-based approaches to the removal of arsenic from aqueous systems.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-163X/9/10/568/s1: Table S1: Growth of *Talaromyces* sp. KM-31 in PDA medium amended with As(III) or As(V).; Table S2: Arsenic removal (%) by Fe(III) oxides as a non-fungal reference.

Author Contributions: I.H.N. and J.H.K. designed the study; I.H.N., K.M., and J.R. performed the experiment and analyzed the data; J.R. and J.H.K. provided critical feedback and helped shape the research; I.H.N. wrote the final version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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