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Bioscorodite Production from As(III) and Fe(II) Salts under Oxidizing and Acidic Conditions of *Trichoderma atroviride* Culture

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Abstract: Arsenic (As) contamination of groundwater is widespread and significantly affects drinking water, posing a threat to public health due to its classification as a human carcinogen. Arsenic (As) can be removed from contaminated water using sustainable technologies (e.g., biotechnological processes). The process of removing Arsenic from water through reactions with iron under acidic and oxidizing conditions in a fungal broth has been proposed alongside the production of bioscorodite ($FeAsO_4{\cdot}2H_2O$) crystals by *Trichoderma atroviride* culture. This ascomycete was selected based on tests with three other fungi (*Aspergillus niger*, and the basidiomycetes, *Postia placenta*, and *Phanerochaete chrysosporium*) because it decreased the pH to 2.2, raised the redox potential (Eh) to 207 mV, and was the quickest to produce 0.39 µg/L of H_2O_2 in a modified Wunder medium. The Eh was further increased to 324.80 mV under improved fungal culture conditions, selected using a 2^{3-1} fractional factorial design (FFD). The fungal broth was then used for bioscorodite production by adding Fe(III)/As(III) salts and scorodite seeds at 92 °C for 21 h. Scorodite seeds and bioscorodite were characterized by X-ray diffraction (XRD) and scanning electron microscopy (SEM). Arsenic was determined in solution by atomic absorption spectrophotometry (AAS), and a 73% reduction in the initial As concentration (0.30 g/100 mL) was observed after bioscorodite production. Bioscorodite production under appropriate fungal culture conditions could be an option for sustainable As removal from water. The production of H_2O_2 by the fungus resulted in the oxidation of As(III) into As(V) and acidification of the culture broth, which created the necessary conditions for the production of bioscorodite without the need for chemical acids or oxidants. This approach is environmentally friendly and cost effective, making it a promising alternative for the treatment of arsenic-contaminated water.

Keywords: bioscorodite; *T. atroviride*; arsenic; iron; mycoremediation

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1. Introduction

Arsenic (As) is a metalloid that is widely distributed in the environment due to natural processes and anthropogenic activities. It is classified as a human carcinogen [1] and constitutes a threat to public health. In natural waters, As is generally found as a dissolved species, having formed arsenic species. Its predominant oxidation states are the As(III) and As(V), and less frequently As(0) and As (-III). As(V) is more commonly found, as arsenic acid (H_3AsO_4) is the major species found in water, whereas under reducing conditions, As(III) is found [2]. The redox potential (Eh) and pH fundamentally control the oxidation state and mobility of As. Among the various chemical forms, As(V) exists as arsenic acid (H_3AsO_4) and its corresponding dissociation products (H_2AsO_{4-}, $HAsO_4^{2-}$, and AsO_4^{3-}),

while As(III) occurs as arsenious acid (H_3AsO_3) and its derivatives (H_4AsO^{3+}, H_2AsO^{3-}, $HAsO_3{}^{2-}$, and $AsO_3{}^{3-}$), depending on the pH [3].

Organic arsenic compounds are considered less harmful because they are less easily absorbed by the body and have a lower toxicity than inorganic arsenic compounds. However, it is still important to monitor and control levels of both types of arsenic compounds in drinking water and food to ensure public health and safety.

High As concentrations (in μg/L) in water have been detected worldwide [4], in Thailand (5000), India (3200), Vietnam (3050), Bangladesh (2500), Argentina (9900) and Mongolia (2400). Arsenic contamination in drinking water is a major public health concern worldwide. According to the World Health Organization (WHO), an estimated 140 million people in at least 70 countries have been drinking water containing arsenic at levels above the WHO provisional guideline value of 10 μg/L [4]. In Mexico, various regions, such as Valle de Zimapan, Hidalgo, have reported arsenic concentrations exceeding the permissible limits for drinking water (14 to 1000 μg/L) [5]. This highlights the urgent need for effective and sustainable arsenic removal technologies to mitigate the risk of arsenic exposure and associated health problems.

As contamination is associated with three main environments: areas of geothermal activity, alluvial aquifers, and areas of mining activity [6]. However, there are few areas where the specific processes involved in the release of As into groundwater have been identified [7].

The technologies that have been applied in recent years for the removal of As from drinking water are as follows: (1) coagulation, flocculation, precipitation, and filtration; (2) adsorption and ion exchange; (3) processes based on membranes; and (4) biological treatments. Amongst these options, adsorption is the process that has received the most attention [8,9]. In Latin America, the main treatment methods used are chemical precipitation/filtration and reverse osmosis [10]. In biological processes, some microorganisms, such as bacteria or fungi, have been used to remove As in its reduced form of arsenite or its oxidized form of arsenate using various mechanisms, including biomass sorption, reduction-oxidation reactions (redox), and biomethylation processes [11].

It is therefore crucial to consider the oxidation state of the As agent and its mobility in water, among other factors, to control the Eh and pH in the broth culture of a biological process [12]. Scorodite ($FeAsO_4{\cdot}2H_2O$) is an attractive medium for As removal due to its high stability, high As fixation capacity, good crystallization state, and low water content in slag [13]. Recent studies have focused on the production of bio-sourced scorodite (bioscorodite) using extremophile bacteria [14], where the oxidation mechanisms that control the saturation levels necessary to induce crystal formation require high temperatures. The metal removal mechanism using *A. niger*, has been studied previously, but the exact mechanisms involved are dependent on the fungal species used [15]. There are options which involve using other fungi for As removal, with several species shown to have the ability to remove As from water [15,16].

In this study, the previously isolated ascomycete fungus *T. atroviride* [17] was selected and grown under appropriate culture conditions to exploit the acidic and oxidizing properties of the fungal broth culture for As(III) oxidation into As(V). The crystallization conditions were set to ensure a more stable and less toxic ($Fe^{3+}AsO_4{\cdot}2\ H_2O$) mineral.

In this study, it was hypothesized that the ascomycete *T. atroviride*, which has been reported to be an As remover, reduces the pH and increases the redox potential under selected culture conditions, facilitating bioscorodite formation and therefore As removal.

The main objective of this research was to select a fungus and its culture conditions to decrease the pH and increase the redox potential, in order to create the acidic and oxidizing conditions necessary for the production of bioscorodite. The study attempted to solve a practical problem that has relevance worldwide, providing an innovative solution to As contamination using a form of mycoremediation that has not been previously reported. The successful implementation of this technique has the potential to provide an economical and environmentally sustainable alternative to current As disposal practices. Low temperatures

are used for growing the fungus, which produce organic acids and can help to reduce the pH, eliminating the need to add expensive inorganic acids to the system. Moreover, the oxidizing conditions can promote the conversion of As(III) to As(V), which can facilitate its removal from the water.

2. Materials and Methods

2.1. Fungal Cultivation

The ascomycete, *A. niger*, and two basidiomycetes, *P. placenta*, and *P. chrysosporium* CDBB-h-298, were obtained from the Cinvestav Culture Collection, and *T. atroviride* was isolated from a natural wetland that has been impacted by municipal water discharges on the central coast of Veracruz, Mexico [17]. Stock cultures were maintained on potato dextrose agar (PDA) at 4 °C. Ascomycete fungi inoculum preparation and bioscorodite production were performed in a modified Wunder mineral medium [18] that consisted of dextrose, 10 g; polypeptone, 1 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.875 g; K_2HPO_4, 0.125 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; NaCl, 0.1 g; $MnSO_4 \cdot H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; and distilled water, 1 L. Basidiomycete fungi inoculum preparation was performed in a Sivakumar medium [19] that contained dextrose, 20 g; yeast extract, 2.5 g; KH_2PO_4, 1 g; $(NH_4)_2SO_4$, 0.05 g; $MgSO_4$, 0.5 g; $CaCl_2$, 0.1 g; $FeSO_4$, 0.01 g; $MnSO_4$, 0.001 g; $ZnSO_4$, 0.001 g; $CuSO_4$, 0.002 g; and distilled water, 1 L.

2.2. Evaluation of Fungi for Acidic and Oxidizing Culture Conditions

Biomass production, sugar consumption, pH, Eh, and hydrogen peroxide (H_2O_2) production were measured over 14 days of cultivation in a modified Wunder medium. The fungal biomass was determined by filtering the broth culture in a vacuum through a Whatman filter paper No. 40 at a constant weight. The fungal biomass was kept at 105 °C until the dry weight was stable. The pH and Eh of all samples were determined using a portable logging multiparameter meter (HI9829, Hanna Instruments, Woonsocket, RI, USA). A spectrophotometer (model 1800, Shimadzu, Kyoto, Japan) was used for spectrophotometric measurements. Reduced sugars were estimated by the 3,5-dinitro salicylic acid method [20]. The H_2O_2 concentration in the broth culture was determined using the iodine/iodate method [21]. The determination of the glucose oxidase enzyme activity in the liquid medium was carried out by adding the following reagents to test tubes: 50 µL of 1 mg/mL glucose (0.0055 M) and 950 µL of the fungus culture medium for samples and blanks. Then, 1 mL at 0.2 M of the chemical compound used for enzyme determination, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)-phosphate buffer (pH = 5.5, with an ABTS concentration of 0.41 mM), and 1 mL of peroxidase 5 U/mL were added. The mixture was incubated for 10 min at 37 °C, and the absorbance was read at 410 nm [22]. The H_2O_2 concentration was calculated from a calibration curve of H_2O_2 at 29−32%, with concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L, according to the equation $y = 0.3469x + 0.0037$ ($R^2 = 0.99$), where y = H_2O_2 concentration (mg/L) and x = absorbance in the filtered broth culture.

2.3. Effect of As(III) and As(V) on the Culture Parameters of Fungi

The effect of the As(III) and As(V) salts on the culture parameters were determined under the culture conditions obtained at the times when the highest Eh and lowest pH values of each fungus were reached, i.e., after four days for *A. niger* and *T. atroviride*, eight days for *P. chrysosporium*, and twelve days for *P. placenta*. The parameters analyzed were sugar consumption, biomass production, pH, Eh, and glucose oxidase enzyme activity. Samples were digested with nitric acid at 65−70% dilution and then filtered through a Whatman filter paper No. 40. A calibration curve was obtained to determine the As concentration of broth cultures by graphite furnace atomic absorption spectroscopy (AAS) (Avanta AAS, GBC Scientific Equipment Ltd., Dandenong, VIC, Australia).

2.4. Selection of Fungal Cultivation Conditions for Acidic and Oxidizing Parameters Using an Experiment with a 2^{3-1} Fractional Factorial Design (FFD)

The variables considered in the 2^{3-1} FFD [23] were: sources of carbon (C: lactose and dextrose), iron (Fe: $FeCl_3$ and $FeSO_4$), and phosphorous (P: KH_2PO_4 and K_2HPO_4). The independent variables included: fungal biomass production, pH, Eh, and H_2O_2 production, which were measured on the sixth day of culture. The matrix of the 2^{3-1} FFD used to select the culture conditions is presented in Table 1.

Table 1. Matrix for the 2^{3-1} fractional factorial experimental design for evaluation of the fungal culture conditions.

	Variable		
T	C	Fe	P
T1	Lactose (−1)	$FeCl_3$ (−1)	KH_2PO_4 (+1)
T2	Dextrose (+1)	$FeCl_3$ (−1)	K_2HPO_4 (−1)
T3	Lactose (−1)	$FeSO_4$ (+1)	K_2HPO_4 (−1)
T4	Dextrose (+1)	$FeSO_4$ (+1)	KH_2PO_4 (+1)

Note(s): T = Treatments; C = Carbon; Fe = Ferrous; P = Phosphorous. Coded variables: (−1) = Low level; (+1) = High level.

2.5. Scorodite Seed and Bioscorodite Production

Scorodite seed was prepared as follows. Flat-bottomed 250 mL flasks were used as As reaction vessels. First, 0.24 g of Fe(III) was mixed with iron sulfate ($Fe_2(SO_4)_3 \cdot nH_2O$) in 100 mL of distilled water with an acid pH (1.3 was achieved with H_2SO_4), then 0.28 g of As(V) was added as sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$). Later, concentrated H_2SO_4 was added to clarify the solution, and gypsum ($CaSO_4 \cdot 2H_2O$) (0.56 g) used as a seed. The flask was placed on a heating plate with constant shaking (125 rpm). Then it was fitted to a condenser and cooled by circulating cold water (4 °C) to avoid water loss by evaporation. The system was kept at 92 °C for 21 h, then the solution was filtered through a Whatman filter paper No. 40 to obtain the scorodite, which was dried at room temperature for further characterization. All chemicals used were of analytical grade.

The bioscorodite production was similar to the preparation process for scorodite seed, except that Fe(II) and As(III) salts were added to a 100 mL filtered fungal broth (T3 with lactose, $FeSO_4$, K_2HPO_4) selected from the 2^{3-1} FFD, where acidic (pH = 2.20) and oxidizing conditions, (Eh of 324.80 mV) were attained.

2.6. Scorodite Characterization

The structural characteristics of the scorodite and bioscorodite crystals were determined by X-ray diffraction (XRD) at a 2-theta range of 10−80° in an X-ray diffractometer (D2 Phaser, Bruker Daltonics, Bremen, Germany). A thermionic emission scanning electron microscopy (SEM) system (Vega3) with a LaB6 filament (Tescan, Brno, Czech Republic) was used to determine the morphology of the samples. A spectrophotometer (model 6300, Shimadzu) was used to measure the residual As.

2.7. Statistical Analysis

The mean ± the standard deviation was used to express the results. A comparison of means was achieved using the least significant difference (LSD) test with a significance level of $p < 0.05$. In the resulting figures, different letters have different statistical significances, and the same letters have the same statistical significance. Statistical Analysis System (SAS), version 9.0, was used to analyze the data, and each experiment was run three times.

3. Results and Discussion

3.1. Determination of Fungal Culture Parameters

The fungal growth parameters for *T. atroviride*, *A. niger*, *P. placenta*, and *P. chrysosporium* were determined over 14 days and are presented in Figure 1a–d. High growth rates for *T. atroviride* and *A. niger* (2.4 and 0.78 g/L/day, respectively) were achieved (Figure 1a), along with significant sugar consumption (3.90 and 5.72 g/L/day, respectively) (Figure 1b).

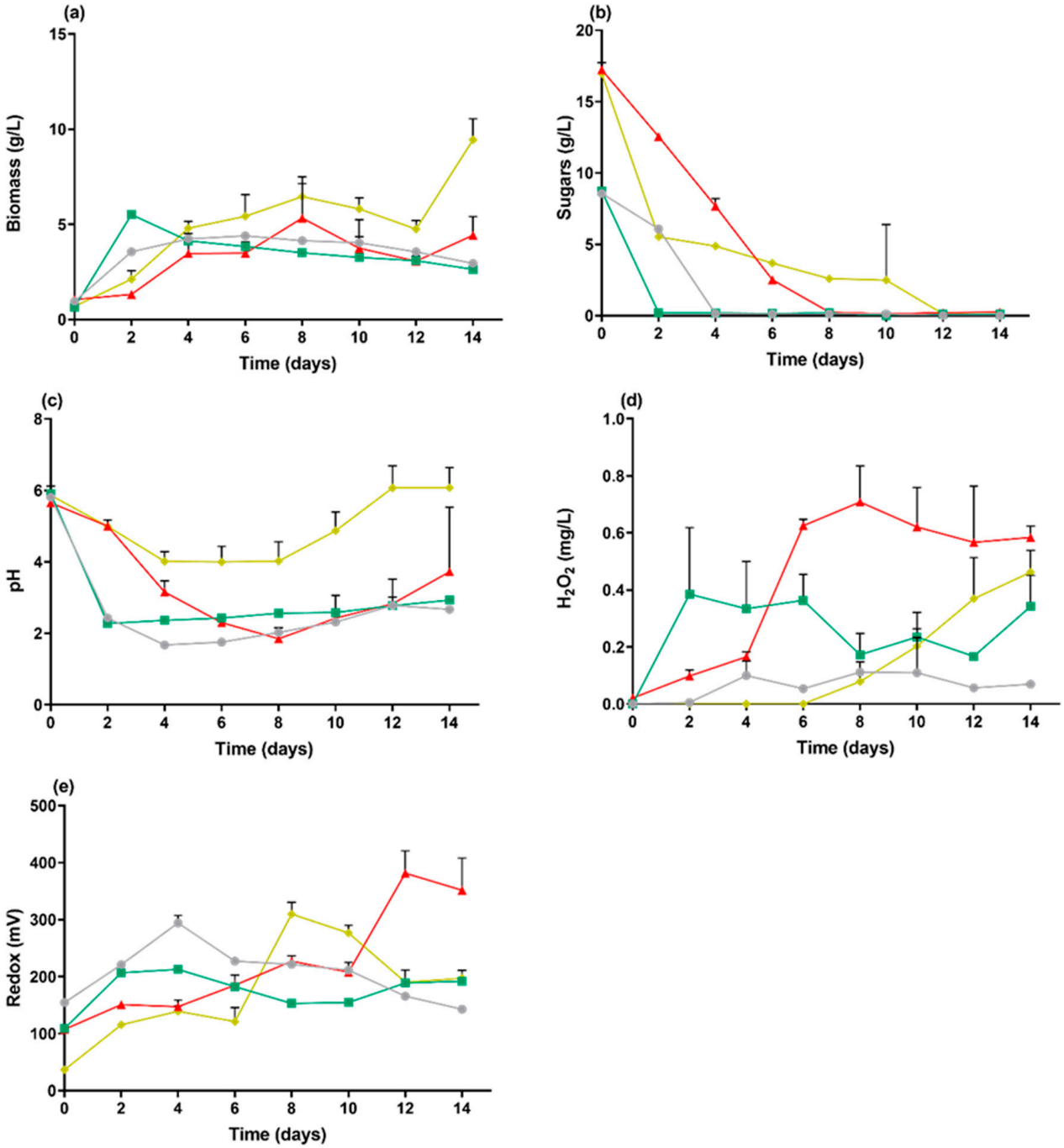

Figure 1. Fungal growth (**a**), dextrose consumption (**b**), pH (**c**), H_2O_2 concentration (**d**), redox potential, and Eh (**e**) for *A. niger* ●, *T. atroviride* ■, *P. placenta* ▲, and *P. chrysosporium* ◆. T = 28 °C for all fungi except *P. chrysosporium*, which was cultivated at 39 °C and 125 rpm.

The growth rates of the four fungi were tested using both the first-order model and the logistic model, with data collected after up to 8 days of cultivation. The results showed

that only *A. niger* was well-fitted to the logistic regression model (R^2 = 0.99) (Equation (1)). The R^2 value was lower for *P. chrysosporium*, at only 0.74.

$$dX/dt = \mu max\ (1 - X/Xmax) \times X \quad (1)$$

where X = biomass growth (g/L); μmax = maximum specific growth rate; $Xmax$ = maximum biomass concentration; and t = time (days).

The logistic model was found to be a better fit for the data compared to other models used to determine the growth rate of the fungi. The results indicated that the growth of fungal biomass (X) was correlated with μmax. However, to determine the kinetic parameters and their correlation with acidic and oxidizing conditions, a shorter analysis time may be appropriate for *T. atroviride* and *A. niger*.

Trichoderma atroviride presented a faster growth rate (Figure 1a) and greater sugar consumption (Figure 1c) than the other fungi. Suitable acidic conditions were generated for *T. atroviride* and *A. niger*, which produced the lowest pH values (2.20 and 1.8, respectively) (Figure 1c). These acidic conditions might be due to the organic acids produced during the secondary metabolism of fungi [24–26]. Organic acids, such as citric, oxalic, and gluconic acids, are known to be produced via the Krebs pathway [24]. These conditions were considered based on the bioscorodite produced with extremophile bacteria at a low pH [27].

Arsenic removal in the presence of Fe(II) under acidic conditions occurs naturally due to the presence of the organic acids produced by fungi, without the addition of chemicals. This has been exploited for As(V) and ferrous iron (Fe(II)) removal from acidic hydrometallurgical effluents [28], in which it has been reported that at pH > 5.5, As(V) and Fe(II) almost completely precipitate. These findings suggest that acidic environments and Fe(II) play significant roles in the removal and precipitation of As(V) and should be taken into consideration when treating As-bearing effluents.

The production of H_2O_2 (Figure 1d) can be attributed to glucose oxidase [29], which was detected in the culture experiments. Some oxidoreductases are activated in several white- and brown-rot fungi by H_2O_2 [29], such as the glucose-methanol-choline (GMC) oxidoreductase (AA3 family) or copper-radical oxidases (CRO: AA5 family) in a glucose medium. In our study, dextrose was used instead of glucose, which may have changed the metabolic pathway. The most frequently observed oxidases were from the AA3 family, such as aryl-alcohol oxidases (AAO), glucose oxidase (GOX), and alcohol oxidase (AOX), which are closely related phylogenetically. The next most abundant were pyranose 2-oxidase (P2O) and cellobiose dehydrogenase (CDH), which are GMC oxidoreductases [29].

These enzymes play an important role in white-rot fungi by providing lignin peroxidase enzymes with H_2O_2. In brown-rot fungi, such as *P. placenta*, these enzymes are not induced, and only oxidoreductases supply H_2O_2, which can then react with Fe(II) through a Fenton-type reaction [30,31]. This reaction is unique to this type of fungi and leads to the production of hydroxyl radical ($^{\bullet}OH$) from H_2O_2 (Figure 1d); *T. atroviride* produced this strong oxidant (2.8 V) [32] after a shorter time period (2 days), whereas *P. placenta* produced almost twice as much after a longer time period (8 days) [32].

This might be the reason for the considerable increase in the Eh of the brown- and white-rot fungi (Figure 1e) after longer time periods, which was also supported by the significant ($p < 0.002$; 0.0006) Pearson correlation between the H_2O_2 concentration (Figure 1c) and Eh (Figure 1e) for *T. atroviride* and *P. placenta*. Both H_2O_2 and $^{\bullet}OH$ are reactive oxygen species (ROS) that might be involved in the oxidation of As(III) into As(V) under acidic conditions [32].

3.2. Effect of As(III) and As(V) on Culture Parameters

Figure 2 displays the culture parameters of the brown-rot fungus *P. placenta*. No significant differences were observed between cultures amended with As(III), As(V), and the control, with the pH lowered from 5.48 to 3.68 and the Eh increased from 116 to

255.28 mV. No H_2O_2 was detected in any of the cultures, and glucose oxidase was only detected in the control; it was found to be inhibited mainly by As(III).

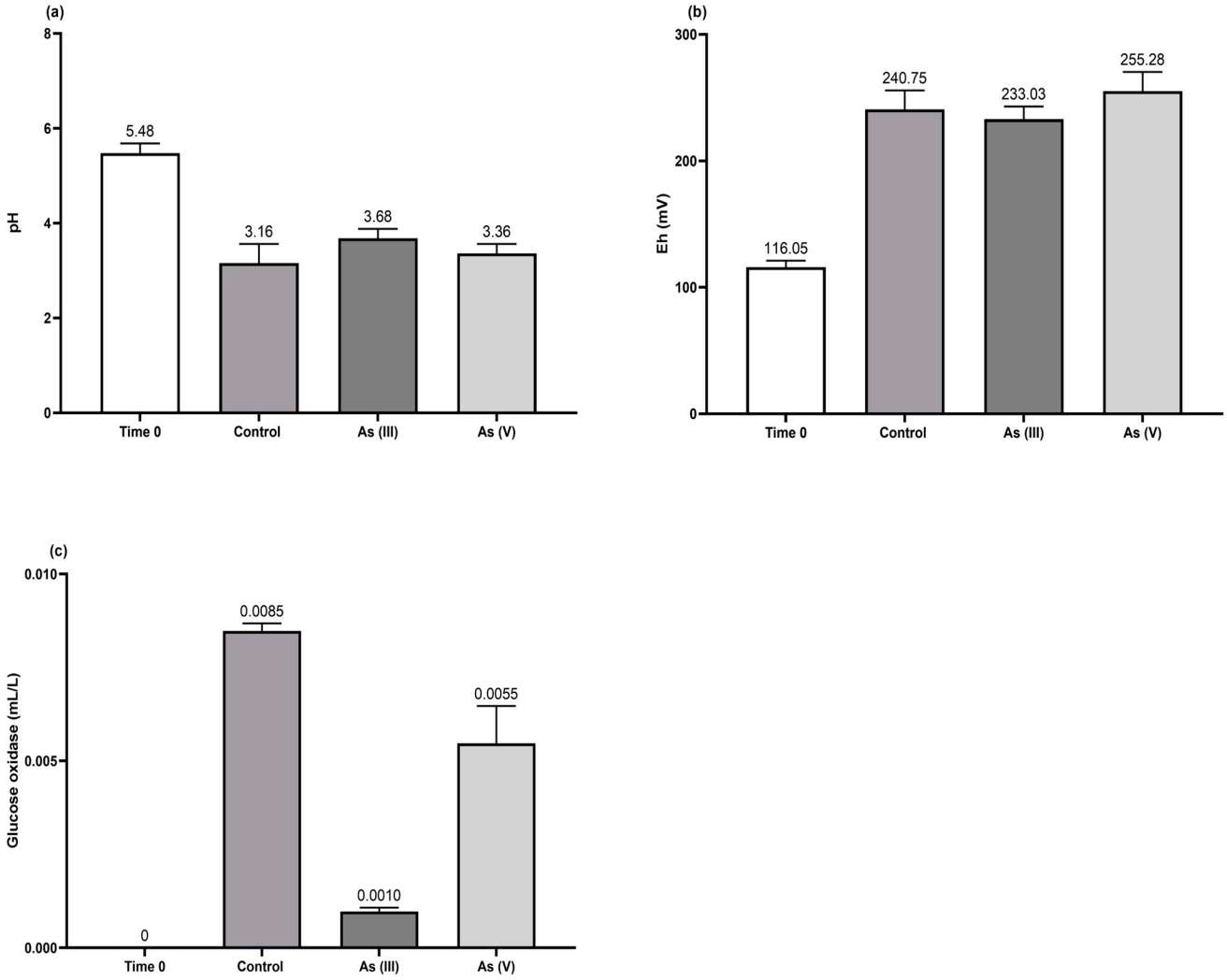

Figure 2. Culture parameters—pH (**a**), Eh (**b**), and glucose oxidase activity (**c**)—of *P. placenta* in Sivakumar medium with amendments of As(III) and As(V) salts at 28 °C, 125 rpm, after 12 days of cultivation.

In the culture of the white-rot fungus *P. chrysosporium*, no significant differences were observed in biomass production, pH, or sugar consumption between the cultures amended with the different As salts. The pH decreased slightly from 5.10 to 3.65 in the culture amended with As(V). The Eh increased as follows: control (146.2 mV) > As(V) (102.5 mV) > As(III) (55.5 mV). H_2O_2 was only detected in the culture amended with As(III) (0.5 mg/L) (Figure 3a,b).

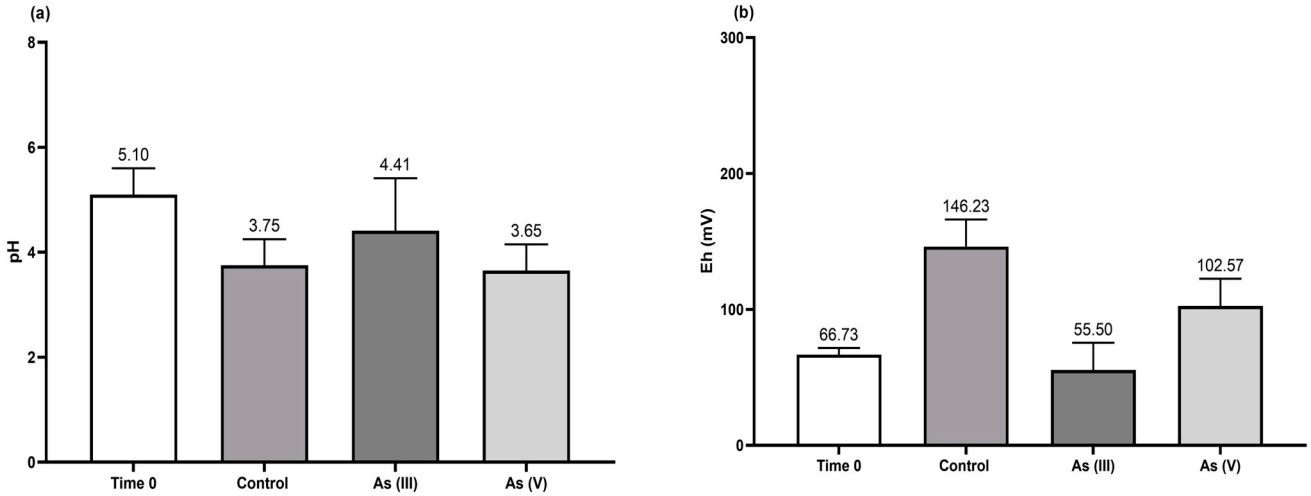

Figure 3. Culture parameters—pH (**a**) and Eh (**b**)—of *P. chrysosporium* in Sivakumar medium with amendments of As(III) and As(V) salts at 39 °C, 125 rpm, after eight days of cultivation.

No significant differences in biomass production were observed between the control and the cultures with As(III) and As(V). Neither the glucose oxidase activity nor the H_2O_2 production differed (Figure 4a,b). The pH decreased significantly ($p < 0.05$) from 5.7 to 1.9 in the culture amended with As(V), and the Eh increased from 141.80 to 170.80 mV in the culture supplied with As(III).

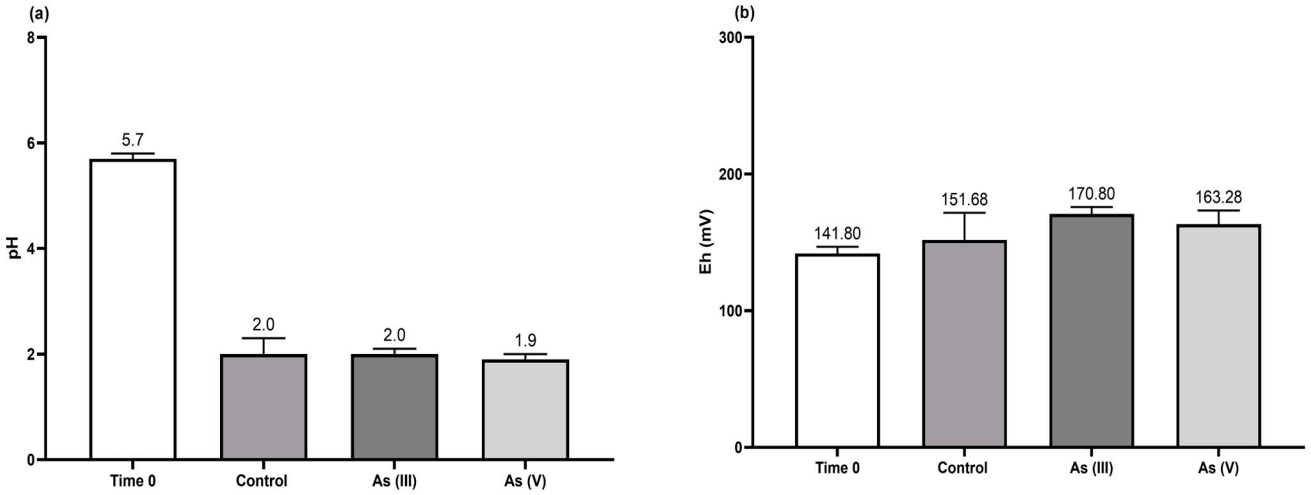

Figure 4. Culture parameters—pH (**a**) and Eh (**b**)—of *A. niger* in Wunder medium with amendments of As(III) and As(V) at 28 °C, 125 rpm, after four days of cultivation.

In the culture of *T. atroviride*, the fungus behaved similarly to *A. niger* in terms of biomass production, pH, and sugar consumption, with no significant differences between the control and the cultures amended with As(III) and As(V). The Eh was higher, and H_2O_2 production was detected only in cultures amended with As(III) and As(V) (Figure 5b,d), although there were no significant differences between them. The glucose oxidase activity was slightly higher in the presence of As(III).

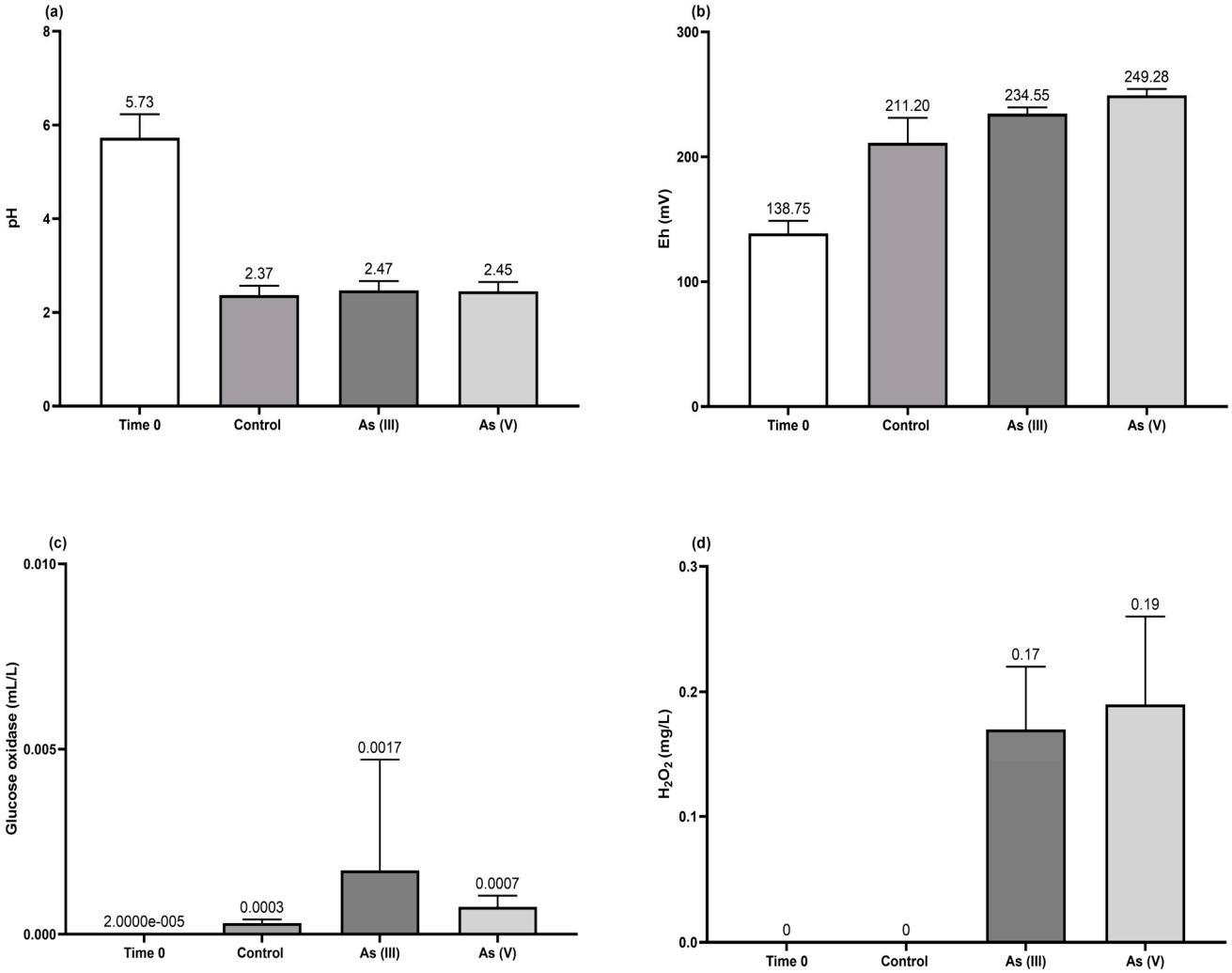

Figure 5. Culture parameters—pH (**a**), Eh (**b**), glucose oxidase activity (**c**), and H_2O_2 production (**d**)—of *T. atroviride* in Wunder media amended with As(III) and As(V) at 28 °C, 125 rpm, after four days of cultivation.

3.3. Improvement in the Oxidizing and Acidic Conditions of T. atroviride Using a 2^{3-1} FFD

The evaluation of fungal culture parameters in the presence of As(III) and As(V) to obtain acidic (low pH) and oxidizing (high Eh) conditions resulted in the selection of *T. atroviride* for further experiments. For *T. atroviride*, H_2O_2 production and glucose oxidase activity were detected in the broth culture at an earlier cultivation time than in the other fungi investigated. The parameters measured after 6 days of *T. atroviride* growth are shown in Table 2.

Table 2. The biomass production, pH, Eh, and H_2O_2 production for various C, Fe, and P sources evaluated through a 2^{3-1} FFD.

Treatment	Biomass (g/L)	pH	Eh (mV)	H_2O_2 (mg/L)
T1	2.9 ± 0.07	2.50 ± 0.04	286.20 ± 1.58	0.40 ± 0.13
T2	3.2 ± 0.05	2.30 ± 0.06	312.50 ± 1.88	0.65 ± 0.09
T3	4.4 ± 0.04	2.20 ± 0.02	324.80 ± 2.49	0.31 ± 0.03
T4	3.6 ± 0.30	2.40 ± 0.03	301.50 ± 0.99	0.12 ± 0.06

Note(s): SD = Standard Deviation.

The treatment parameters of the four culture conditions were established from the 2^{3-1} FFD (Table 2) with consideration of the biomass production (Figure 1a), low pH (Figure 1c), high redox value (Figure 1e), and high H_2O_2 production (Figure 1d), which were achieved after the shortest time period among the brown- and white-rot fungi that were investigated.

The culture conditions for treatment T3 (Table 2), which included lactose, $FeSO_4$, and K_2HPO_4, resulted in the highest biomass production (4.40 g/L) and lowest pH (2.20), and increased the Eh to 324.80 mV after the shortest time period. This was determined using ANOVA and media comparison ($p < 0.05$) and taking into consideration the factors of low pH (Figure 1c), high redox value (Figure 1e), and high H_2O_2 production (Figure 1d) achieved after the shortest time period among the brown- and white-rot fungi studied. Arsenic behaves as an anion, and As(III) is converted into As(V). This is important because the surface of the fungal cell becomes more negatively charged when the pH value decreases [33], which provides favorable conditions for the absorption of positively charged metals or cations [33].

Treatment T3 (with lactose, $FeSO_4$, and K_2HPO_4) resulted in the highest Eh (324.80 mV) (Table 2), which was approximately 78% higher than the value obtained in the previous liquid culture of *T. atroviride* after six days (182.30 mV) (Figure 1d). There was a significant increase in biomass and a non-significant decrease in pH ($p < 0.05$) in treatments T2 and T4. The biomass production, pH, and redox were affected by the Fe and P sources, while H_2O_2 production was only affected by the P source, for which the concentrations reached 0.65 and 0.31 mg/L in treatments T2 and T3, respectively. A H_2O_2 concentration of 0.19 mg/L was previously reported in a *P. placenta* culture, which used glucose as a C source [25]. The H_2O_2 production did not differ significantly between treatments T1, T2, and T3. The relationship between the three independent variables and the sources of C, Fe, and P followed a polynomial response model (Equation (2)):

$$Y = \beta_0 + \beta_1 x1 + \beta_2 x2 + \beta_3 x3 + € \quad (2)$$

where y is the response variable (biomass production, pH, redox, or H_2O_2 production), x represents the independent variable (x1 = C source, x2 = Fe source, x3 = P source), and € is the residual term that represents the experimental error. The parameter β_0 is the overall mean of the responses (biomass production, pH, Eh, and H_2O_2 production), which is a constant in the model; β_1, β_2, and β_3 are linear coefficients. The resulting data were adjusted to the general linear model, and, using a linear regression, the coefficients were determined. An analysis of variance (ANOVA) of the 2^{3-1} FFD and an estimation of the parameters from the regression analysis were performed, indicating the significance and adequacy of the experimental data to the model. The ANOVA of the FFD demonstrated that the independent variables were statistically significant ($p < 0.05$).

The regression analysis provided a first-order model for each of the variables:

$$\text{Biomass production (g/L)} = 3.53 + 0.035\ \text{Fe} - 0.30\ \text{P} \quad (3)$$

$$\text{pH} = 2.35 - 0.079\ \text{Fe} + 0.09\ \text{P} \quad (4)$$

$$\text{Eh (mV)} = 303 + 9.41\ \text{Fe} - 9.89\ \text{P} \quad (5)$$

$$H_2O_2\ \text{production (mg/L)} = 0.43 - 0.15\ \text{P} \quad (6)$$

where the C source (x1) was not significant for biomass production (Equation (3)), pH (Equation (4)), Eh (Equation (5)), or H_2O_2 production (Equation (6)). On the other hand, the Fe source (x2) at a high level ($FeSO_4$) and the P source (x3) at a low level (KH_2PO_4) were found to be significant ($p < 0.05$) for the decrease in pH and the increase in Eh. Based on these results, a residual analysis was performed to validate the model and verify its ability to predict pH, Eh, and H_2O_2 production with a minimum number of experiments.

An ANOVA and parameter estimation were conducted to support the significance and sufficiency of the regression analysis. The ANOVA and the first-order model demonstrated that the two independent variables, x2 and x3, were statistically significant ($p < 0.001$), mainly for the decrease in pH and the increase in Eh in *T. atroviride*.

3.4. Bioscorodite Production under the Selected Culture Conditions

All fungi were cultured at low temperatures (28 °C)—except for *P. chrysosporium*, which was cultured at 39 °C—for several days, depending on the fungus. Then, bioscorodite was produced under the acidic pH and oxidizing conditions obtained in the fungal broth. Further, the broth was amended with scorodite seed, Fe(III), and As(III) salts and maintained at 92 °C for 21 h. During bioscorodite generation, it is important to consider that the temperature needs to be controlled to remain near 92 °C, because upon heating, scorodite can decompose at 100–200 °C with the simultaneous release of both of its H_2O molecules to form anhydrous $FeAsO_4$ [28].

The use of fungi for As removal has several advantages, because fungi can tolerate and remove As through a diverse range of mechanisms [11]. Additionally, the pH is lowered by organic acids, and pH values of below 0.2 to 1.8 do not need to be achieved, because these would have little effect on the structure or composition of the scorodite. Fungi also produce the strong oxidant H_2O_2, enabling the oxidation of As(III) for bioscorodite production in effluents contaminated with this element. However, the concentration of As(V) needs to be considered, as concentrations greater than 15 mg/L are required to ensure a pure product.

During fungal cultivation, the culture parameters were varied. It was observed that after 2 days of cultivation of *T. atroviride*, the pH of the medium changed from pH 6 to 2 (Figure 1c), and the oxidative conditions changed, as the Eh increased from 149 to 319 mV, (Figure 1e). A low pH is suitable for bioscorodite formation because As behaves as an anion under these conditions. In contrast, when the pH is alkaline, the surfaces of the fungal cells become negatively charged, enhancing metal adsorption, and As behaves as a cation [33]. At an acidic pH, a Fenton-type reaction occurs [31,32], where $^{\bullet}O$ is produced from reactions between Fe(II) and H_2O_2 (Figure 5d), generated by glucose oxidase (Figure 5c) at a low pH (Figure 5a) in the broth culture. Additionally, the oxidizing conditions (Figure 5b) generated by H_2O_2 result in a change in As speciation from As(III) to As(V).

3.5. Characterization of the Calcium Sulfate Scorodite Seeds

Figure 6 presents an SEM image (a) and a XRD pattern (b) for the calcium sulfate (gypsum) used as a seed for scorodite formation. The SEM image revealed that the gypsum was not observed in clusters and only appeared as well-defined large crystals, with only a few smaller than 2 μm. The XRD analysis exhibited intensity peaks at 21.0, 23.4, 29.1, 31.5, and 33.4 (°), which were typical of calcium sulfate [34].

The calcium sulfate seed used in the study was selected from different concentrations of As(III), As(V), Fe(II), and Fe(III); periods of time; and experimental conditions (data not shown). Figure 7 shows the SEM (a) and XRD pattern (b) results for the calcium sulfate scorodite seed. The SEM micrograph revealed that the sample possessed heterogeneous clusters with an average size of 2 μm. Further analysis of the XRD pattern exhibited the typical characteristics of scorodite [29,34].

The XRD pattern revealed intensity peaks at 15.7, 17.6, 29.7, and 35.8 (°), all at the same angle (2θ). These results were comparable to those of previous studies [34], indicating the presence of specific mineral phases in the sample. Some of the intensity peaks (15.7, 17.6, 28.1, and 35.8 (°)) were different from those of calcium sulfate (Figure 6b), suggesting potential differences in the crystal structure and composition of the materials. Further analysis and comparison of these peaks may provide valuable insights into the mechanisms and effectiveness of As removal by the sample.

(**a**) (**b**)

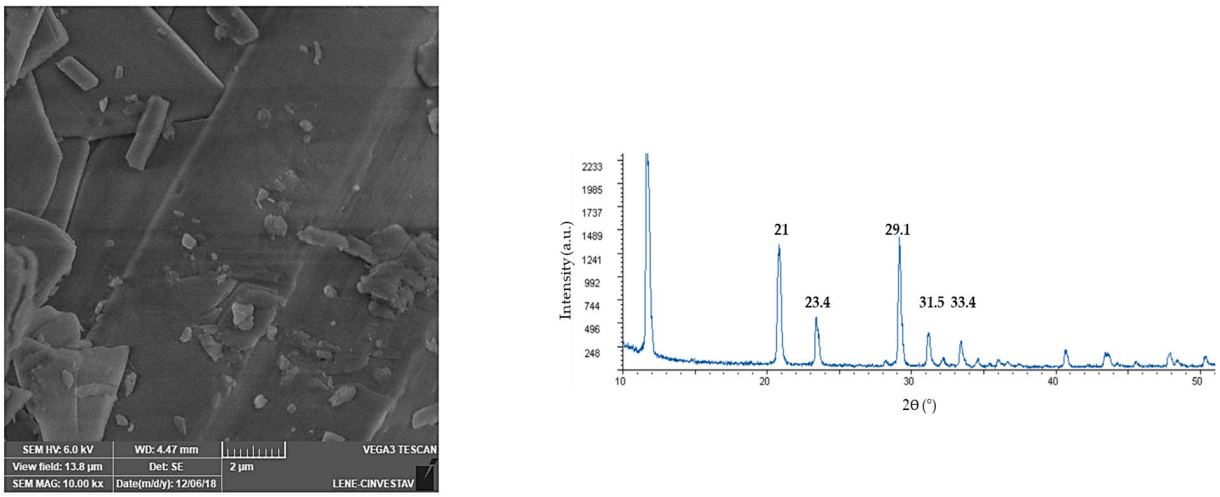

Figure 6. SEM image (**a**) and a XRD pattern (**b**) of calcium sulfate seed.

(**a**) (**b**)

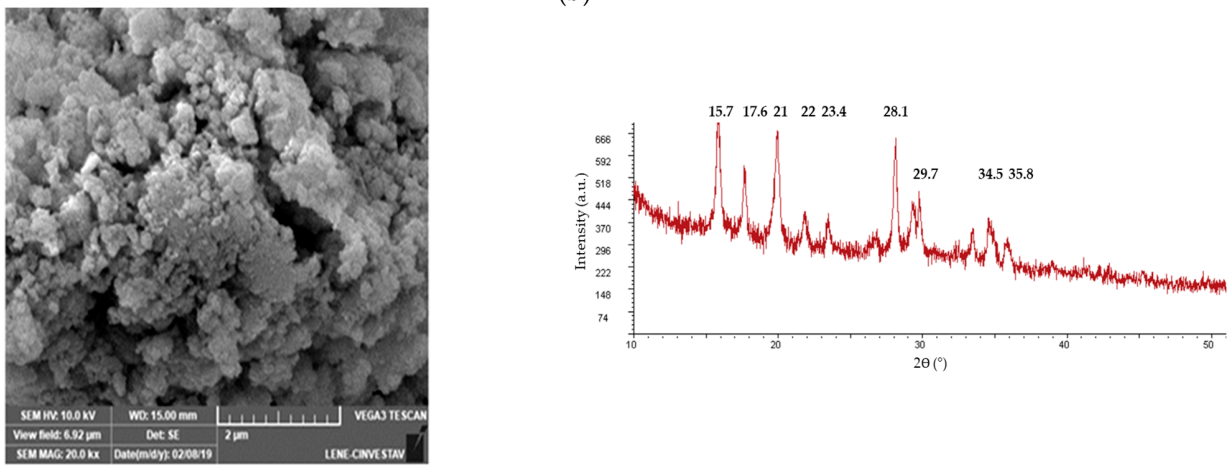

Figure 7. SEM imagen (**a**) and XRD pattern of solid phases (**b**) of calcium sulfate scorodite seed.

According to previous characterization analyses of the solids produced by *T. atroviride* and considering the reported information related to the chemical production of scorodite, the following mechanism is suggested:

Depending on the acidic and oxidizing conditions, bioscorodite may have been formed by the reaction of Fe(III) and the oxidized As(V), which produces crystals of biogenic scorodite. This is based on the following assumptions:

(a) The As(III) oxidation was proceeded by the H_2O_2 generated (Figure 5c) from the glucose oxidase enzyme detected in the broth culture of *T. atroviride* (Figure 5b). This implies the activation of a microbial detoxification mechanism when the fungus is exposed to trivalent As (see Equations (7)–(12)) (modified from [35]).

(b) The initiating reaction, Fe(III) with H_2O_2, produces Fe(II) and the anion radical superoxide ($O_2^{\bullet -}$) (Equation (7)).

(c) Fe(II) is oxidized by H_2O_2, which correlates positively ($p < 0.0006$) with Eh, making it the final electron acceptor in the respiratory chain to form hydroxylate Fe (Equation (8)).

(d) Further, Fe(III)$^{\bullet}$OH reacts with As(III) to produce As(IV) (Equation (9)).

(e) Once As(IV) is generated by H_2O_2 and Fe(III), it forms Fe(III)$^{\bullet}$OH and the oxidized As(V) (Equation (10)).

(f) As(IV) reacts with Fe(III)$^{\bullet}$OH to produce As(V) (Equation (11)).
(g) Fe(III) reacts with As(V) and precipitates as bioscorodite crystals (Equation (12)):

$$Fe(III) + H_2O_2 \rightarrow Fe(II) + O_2^{\bullet -} + 2H^+ \quad (7)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III)^{\bullet}OH + -OH \quad (8)$$

$$Fe(III)^{\bullet}OH + As(III) \rightarrow As(IV) + Fe(III) + -OH \quad (9)$$

$$As(IV) + H_2O_2 + Fe(III) \rightarrow Fe(III)^{\bullet}OH + As(V) + -OH \quad (10)$$

$$As(IV) + Fe(III)^{\bullet}OH \rightarrow As(V) + Fe(III) \quad (11)$$

$$Fe^{3+} + AsO_4^{3-} + 2H_2O \rightarrow FeAsO_4 \cdot 2H_2O \text{ (Scorodite)} \quad (12)$$

3.6. Bioscorodite Characterization

Solid-green color bioscorodite was produced from the broth culture of *T. atroviride* grown at T = 28 °C, 125 rpm over 4 days, with an acidic pH (1.5) and oxidizing conditions (Eh = 324.80 mV). It was produced by precipitation with bioscorodite seeds, Fe(III), and As(III) salts at 92 °C for 21 h.

The SEM image is presented in Figure 8a and the XRD pattern for bioscorodite is presented in Figure 8b, displaying a similar pattern to the calcium sulfate scorodite seed (Figure 7b). The SEM image of bioscorodite shows crystals of 0.07–0.3 µm; the particles formed by the reaction exhibited clusters of lower size than those of calcium sulfate scorodite seed (Figure 7a), indicating a potential difference in the morphology of the resulting materials. Further characterization and analysis of these clusters may provide insights into their implications for the effectiveness of As removal and the possible mechanisms involved. The signal intensity of the XRD pattern was lower, which might be due to the crystallization conditions [36]. This is likely, because bioscorodite was prepared under different conditions, and the best conditions were found to be with 0.24 g/L $Fe_2(SO_4)_3 \cdot nH_2O$ + 0.28 g/L $Na_2HAsO_4 \cdot 7H_2O$ and 0.56 g $CaSO_4 \cdot 2H_2O$ (gypsum as a seed). Bioscorodite was prepared from the filtered broth of fungus grown at 28 °C for 4 days, amended with 0.85 g/L $Fe_2(SO_4)_3 \cdot nH_2O$ + 0.1 g/L $NaAsO_2$ and 0.2 g of scorodite seed, at pH = 1.5, Eh = 324.80 mV and 92 °C, and for 21 h.

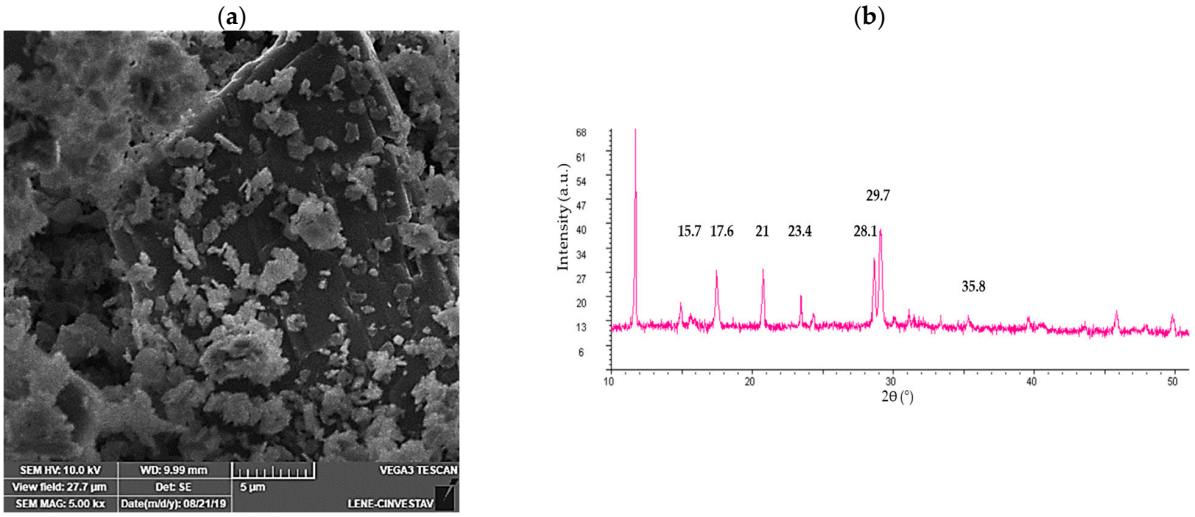

Figure 8. SEM image (**a**) and XRD pattern (**b**) of bioscorodite obtained from the broth of *T. atroviride*, amended with scorodite seeds and As(V)/Fe(III), at pH = 1.5 and 92 °C for 21 h.

The XRD diffractogram showed relatively low signals (Figure 8b) compared to calcium sulfate scorodite seed (Figure 7b). However, an AAS analysis after the reaction indicated a 73% As removal. This could be due to the possible complexation between metabolites produced by the fungus and As, as reported by other authors [11]. They reported a high As removal in fungal cultures attributed to As biotransformation, which allows for the decrease in As toxicity to soil-dwelling microorganisms and plants.

4. Conclusions

The *T. atroviride* fungus was selected for further investigation after initial tests with two ascomycetes, *T. atroviride* and *A. niger*, and two basidiomycetes, *P. chrysosporium* and *P. placenta*. The optimum acidic and oxidizing broth culture conditions required for bioscorodite production were determined with Fe(III) and As(III) salt amendments at 92 °C and pH 1.5 for 21 h. Under these conditions, the As(V) concentration decreased to 73% during bioscorodite generation at pH 2.2 with an Eh of 324.80 mV. The oxidizing conditions were first determined in the broth culture of *T. atroviride*, which showed a Pearson positive correlation ($p < 0.0006$) between Eh and H_2O_2 production. This oxidant and the glucose oxidase activity were detected at the earliest time (after 4 days) in the broth culture, with lactose or dextrose, $FeSO_4$, and K_2HPO_4 as sources of C, Fe, and P, respectively, selected using a 2^{3-1} FFD. Under these conditions, As(III) was oxidized into As(V).

The scorodites were Identified by SEM and XRD, and they were found to have the same characteristics as previously reported in the literature. The benefit of using fungi is the low temperature (28 °C) required to obtain the acidic and oxidizing conditions. This contrasts with the use of extremophile microorganisms, where the temperature must be kept high and inorganic acids are needed to lower the pH. However, more research is needed to understand the fungal mechanisms for As removal, since they have a high potential for the detoxification of water contaminated by this metalloid. Other options involving oxygenation and the use of fungal-colonized organic residues will be investigated in future studies to improve As removal from drinking water and make the process more competitive at a larger scale. The production of bioscorodite is potentially a sustainable way to remove arsenic from water.

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