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Cleansing Tannery Effluent with *Pleurotus opuntiae*: A Green Solution for Environmental Restoration and Toxicity Evaluation

Priyanka Yadav [1], Vartika Mishra [1], Tejmani Kumar [2], Umesh Kumar Singh [3], Emanuel Vamanu [4,*] and Mohan Prasad Singh [1,*]

[1] Centre of Biotechnology, University of Allahabad, Prayagraj 211002, India; priyankayadav@allduniv.ac.in (P.Y.); vartikamishra@allduniv.ac.in (V.M.)
[2] Department of Physics, University of Allahabad, Prayagraj 211002, India; tejmani38@gmail.com
[3] Centre of Environmental Studies, University of Allahabad, Prayagraj 211002, India; uksingh@allduniv.ac.in
[4] Faculty of Biotechnology, University of Agricultural Sciences and Veterinary Medicine of Bucharest, 011464 Bucharest, Romania
* Correspondence: emanuel.vamanu@gmail.com (E.V.); mpsingh.16@gmail.com (M.P.S.)

Abstract: Heavy metal contamination has emerged as a global environmental concern, with tannery effluents serving as a significant source of these pollutants. The discharge of tannery effluents (TEs) into natural ecosystems has given rise to a spectrum of catastrophic risks, exacerbating concerns related to public health, safety, and environmental integrity. This current study focuses on the mycoremediation of the heavy metals present in TE, employing the mycelia of *Pleurotus opuntiae*, an environmentally sustainable solution. The toxicity of TE was rigorously characterized by evaluating a range of physicochemical parameters in accordance with the American Standard and Testing Methods. Subsequently, various diluted concentrations of effluent (25%, 50%, 75% and 100%) were incorporated into MDA media to assess the tolerance index (TI) of *P. opuntiae*. Notably, the highest TI was observed in the 25% and 50% TE concentrations, while no growth was observed in the 75% and 100% groups due to the exceptionally elevated heavy metal content. *P. opuntiae* demonstrated remarkable efficacy in heavy metal removal, with the most substantial reductions recorded in the 25% diluted effluent (91.3% Pb, 72.2% Cr and 66.5% Zn), closely followed by the 50% diluted effluent. The highest intracellular bioaccumulation was observed for Pb (17.2 µg/g), outperforming Cr (14.5 µg/g) and Zn (8.5 µg/g) in mycelia grown in 25% diluted effluent. To elucidate the detoxification mechanisms underlying metal removal, various characterizations of the mycelium were conducted, including SEM, FTIR, and XRD analyses. Furthermore, LC–MS analysis shed light on the pivotal role of metabolites in regulating heavy metals within the physiological metabolism of *P. opuntiae*. Moreover, an upsurge in the concentration of the stress marker, metallothionein, and augmented activity of antioxidant enzymes, like SOD, CAT, LPO and GSH, collectively suggested the significant role of antioxidants in mitigating reactive oxygen species (ROS) and heavy metal toxicity. These comprehensive findings provide a solid foundation for understanding the mechanisms responsible for heavy metal removal by *P. opuntiae* and pave the way for the development of effective remediation strategies for decontaminating the effluents discharged by the leather industry, contributing to the preservation of our environment and to public well-being.

Keywords: heavy metals; tannery effluents; mycoremediation; tolerance index; bioaccumulation; metallothionein; antioxidant enzymes

Citation: Yadav, P.; Mishra, V.; Kumar, T.; Singh, U.K.; Vamanu, E.; Singh, M.P. Cleansing Tannery Effluent with *Pleurotus opuntiae*: A Green Solution for Environmental Restoration and Toxicity Evaluation. *Water* **2024**, *16*, 1313. https://doi.org/10.3390/w16091313

Academic Editor: Alexandre T. Paulino

Received: 8 April 2024
Revised: 25 April 2024
Accepted: 2 May 2024
Published: 6 May 2024

1. Introduction

Environmental pollution is one of the most significant issues facing the world today, and this problem is worsening every day as a result of industrial activity [1,2]. The tannery industries (TIs) are crucial to Indian trade and employ those from economically underprivileged populations [3]. India is the second most populated nation in the world, and

tannery industries account for around 15% of its overall economy [4]. Due to the release of massive amounts of toxic contaminants and hazardous wastes, TIs are also regarded as being among the most polluting industries [5,6]. Nevertheless, the most challenging issue facing the global community is how to adequately handle these toxins. The tanning process in the TIs produces large amounts of toxic and highly colored effluents and also utilizes enormous amounts of water and other chemicals [7,8]. Many hazardous metals, like nickel, zinc, lead, and chromium, are present in the wastewater released from Tis, along with other toxic chemicals and phenolic derivatives that can have a deleterious effect on living things [9]. Thus, the elimination of heavy metals from wastewater is an environmental and public necessity. Accordingly, several physicochemical treatment methods, namely chemical precipitation, inverse osmosis, coagulation, ion exchange, membrane filtration, and adsorption processes, have been employed to mitigate environmental pollutants [10]. Nevertheless, these traditional approaches might result in the release of secondary pollutants during the repair process, have significant operative expenses, or be inefficient in the exclusion of heavy metals at ppm levels [11,12]. Therefore, an alternative, easy, environmentally benign, and economical cleanup solution is urgently needed. The bioremediation of toxic substances via different microorganisms or their metabolites has received a lot of attention. Algae, bacteria, and fungi [13–17] are amongst the microorganisms employed in the process of bioremediation. However, fungi are preferred in this investigation as they are the most significant bioremediation agents. Fungi are proficient in waste removal because of their adaptability in hostile environments, the metal-binding capabilities of their cell walls, their high tolerance to metals, and their ability to produce huge amounts of biomass [18]. Macrofungi or mushrooms can function as a highly efficient biosorbents alternative to plants and other microbes in terms of eliminating toxic heavy metals from the soil and wastewater, and this eco-friendly process is referred to as mycoremediation [19–21]. Additionally, some mushrooms contain extremely potent enzymatic machinery that secretes extracellular enzymes and is capable of biodegrading and bioremediating recalcitrant and resistant contaminants with great efficacy [22].

Pleurotus opuntiae is a typical filamentous fungus with a high level of environmental adaptation [23]. Although many studies have concentrated on the effectiveness of heavy metal removal, more research is still needed to fully comprehend its accumulation and tolerance mechanisms. *Pleurotus opuntiae* was used as a test strain in this study. Numerous physiological changes were measured, along with the effectiveness of its development and its elimination of heavy metals. Furthermore, to facilitate the groundwork for creating fungal wastewater treatment technology, the current effort sought to investigate the tolerance potential and accumulation capacity of heavy metals by fungi and their underlying mechanisms.

Considering the qualities of mushrooms led to their selection for use in the decontamination of tannery effluent (TE) obtained from a leather industry situated in Kanpur, Uttar Pradesh, India. Different physicochemical characteristics (such as pH, turbidity, electrical conductivity, BOD, alkanity, and total hardness), as well as various heavy metal concentrations including Mg, Pb, Cr, and Zn, were also measured in TE. In order to perform bioremediation, various concentrations of medium containing TE were inoculated with *Pleurotus opuntiae* mycelium under in vitro conditions, and the fungus' mycelia were assessed following TE treatment.

2. Materials and Methods

2.1. Collection of Sample

In the present study, samples were obtained from the leather industry in Kanpur, Uttar Pradesh, India. The tannery effluent (TE) was carefully collected in sterile plastic containers. Subsequently, the effluent was promptly brought to the laboratory under cold conditions to facilitate further analysis.

2.2. Characterization of Effluent

The TE samples were examined to determine several physicochemical parameters and heavy metal concentrations. Our work followed the standard methods prescribed by the American Public Health Association, the American Water Works Association, and the Water Pollution Control Federation for the examination of water and wastewater [24]. Different parameters, such as pH, turbidity, and electrical conductivity, were measured via a portable pH meter, nephelometer, and digital conductivity meter, respectively. To assess alkalinity and total hardness, we employed titration methods with the help of methyl orange and Eriochrome black-T indicators, respectively. The biological oxygen demand (BOD) was also calculated using the conventional APHA procedure. The level of total solids, encompassing both suspended and dissolved solids, was assessed by subjecting the sample to a hot air oven, with temperatures ranging from 100 °C to 105 °C. Further, the concentrations of sulphate, ammonia, chloride, nitrate, and phosphate were measured via the established standards described in APHA regulations [24]. The heavy metal concentrations present in the effluent were analyzed by utilizing an atomic absorption spectrophotometer (AAS) (A–Analyst 700, PerkinElmer, Waltham, MA, USA).

2.3. Fungal Culture Maintenance

The *Pleurotus* species culture was acquired from the Directorate of Mushroom Research (DMR), Solan, India, and then maintained on malt dextrose agar (MDA) media at pH 6–6.5 and 25 ± 2 °C and periodically sub-cultured.

2.4. Establishing Tolerance Index of Pleurotus opuntiae

The tolerance potential of *P. opuntiae* towards different diluted concentrations of tannery effluent (TE) was demonstrated via the plate assay method. In order to perform the plate assay, MDA media were first inoculated with 0.5 cm diameter fungal mycelium plugs and then mixed with varying concentrations of effluent (25%, 50%, 75%, and 100%). Conversely, MDA plates with no effluent concentration were represented as the control. Subsequently, the plates were incubated at 25 ± 2 °C for 9 days to examine the fungal growth and photographed on each day throughout the incubation's time frame. The screening was conducted in triplicate for each diluted concentration. The tolerance index (TI) was then calculated as the ratio of fungal growth under the exposure of effluent to the control (with no effluent) over the same time interval [25].

$$TI = Rm/Rc \quad (1)$$

where *TI* = tolerance index; *Rm* = radius of the fungal hyphae grown under the exposure of effluent; and *Rc* = radius of control fungal hyphae.

2.5. Experimental Design for Mycoremediation of Tannery Effluent

To perform mycoremediation, TE was diluted with distilled water (DW) in several ratios, including 25% TE + 75% DW, 50% TE + 50% DW, 75% TE + 25% DW, and pure or 100% TE. This was performed in Erlenmeyer flasks of 250 mL, containing 100 mL of malt dextrose broth (MDB) media. In each medium containing a varied percentage of TE, an agar plug of the strain with a 0.5 cm diameter was excised from the periphery, inoculated, and then permitted to grow for 28 days at 25 ± 2 °C and 180 rpm. For this study, each percentage of TE was assessed in triplicate. In addition to pure and different diluted effluents, a control group (without TE) was also constructed.

After 28 days of incubation, mycelium and the culture media were collected from each concentration of TE groups and from the control group for heavy metal analysis. The mycelia were recovered via a filtration method, utilizing Whatman filter paper no. 1, and the dried biomass was quantified following oven drying at 60 °C for 24 h. Thereafter, the mycelium obtained from each group was stored at −80 °C for further analysis. To analyze heavy metal removal and bioaccumulation, mycelia and culture medium were digested

on a hot plate at 130 °C through a mixture of diacid in 3:1 ratio of HNO_3 and H_2SO_4. The concentration of heavy metals was measured by an atomic absorption spectrophotometer (AAS) (A- Analyst 700, PerkinElmer, Waltham, MA, USA) by following standard protocols [26]. The removal rate (R%) and the bioaccumulation efficiency (B) of fungus were calculated via the equations given below. These are (2) and (3), respectively.

$$R\% = (Ci - Cf)100/Ci \quad (2)$$

$$B = (Ci - Cf)V/M \quad (3)$$

where, *Ci* and *Cf* represent the initial and final heavy metal concentration (mg/L); *V*: volume of the solution (L); and *M*: Fungal biomass's dry weight (g).

2.6. SEM, FTIR, and XRD Analysis

The influence of heavy metal stress on the external morphology of fungi was studied by employing scanning electron microscopy (SEM) [27]. The fungal hyphae were scraped off from the edge and fixed with a solution containing 0.1 M of phosphate buffer (pH 7.4) and 3% glutaraldehyde before being incubated for 12 h at 4 ± 2 °C. Following the performance of a 15 min dehydration sequence in ethanol solutions at 70%, 80%, 90%, and in three times at 100%, the samples were then rinsed thrice with 0.1 M of phosphate buffer (pH 7.4). The samples were then dried in a desiccator at 25 ± 2 °C, coated with carbon, and characterized via Jeol-EPMA scanning electron microscopy (JXA-8100, Tokyo, Japan).

The type of functional groups that facilitate the uptake of heavy metals onto the surface of *P. opuntiae* has been assessed using the Fourier Transform Infra-Red (FTIR) approach, following the method elucidated by Gola et al. [28]. At the end of the experiment, the fungal mycelia were taken out and freeze-dried at −85 °C under strong vacuum conditions by utilizing a freeze dryer. Thereafter, 100 mg of KBr powder was combined evenly with 1 mg of finely ground, lyophilized fungal mycelia. All the tests were conducted utilizing a PerkinElmer (USA) FT-IR/FIR frontier spectrometer operating in the 500–3500 cm^{-1} range.

The crystalline structure of fungal biomass under exposure to heavy metal stress was identified through X-ray diffraction (XRD). For this, the obtained fungal mycelium was freeze-dried and then crushed into a fine powder for use in an XRD examination (Rigaku, Tokyo, Japan, SmartLab 3KW).

2.7. LC-MS Analysis

After treatment, the mycelium was subjected to metabolite analysis using an ACQ-TQD#QBB1152 triple-quadrupole tandem mass spectrometer. LC was conducted using a C18 reverse-phase column (150 × 4.6, 2.6 µm), and the mobile-phase solvents of acetonitrile/water (5:95, *v*/*v*), 5 mM ammonium acetate (95:5 H_2O: Acetonitrile, pH 6.5), acetonitrile, and methanol were used for elution for 40 min. The injection volume of the sample was 5 µL, and the mobile-phase flow ramp rate was maintained at 0.30 mL min^{-1}. The desolvation temperature was adjusted to 350 °C, the source temperature to 120 °C, and the cone voltage to 30 V. Both the ES^+ and ES^- ionization modes were used to record MS data in the mass range of *m*/*z* 150–2000 from 0 to 40 min. At various retention durations, all eluted peaks were noted. Following that, mass spectrometry was used to characterize the fractions. This study was conducted at the Sophisticated Analytical Instrumentation Facility (SAIF) of the CSIR-Central Drug Research Institute (CDRI), Lucknow, India.

2.8. Analysis of Metallothionein Concentrations in Fungal Mycelium

The metallothionein concentration present in the fungal mycelium was measured using Ellman's reagent via a spectrophotometric method following the procedure of Viarengo et al. [29]. For this, mycelium was homogenized in three volumes of homogenization buffer, containing 0.55 mol/L sucrose, 0.01% β-Mercaptoethanol, 0.02 mol/L Tris-Hcl buffer, and 5×10^{-3} mol/L PMSF. It was then centrifuged at 10,000× *g* for half an hour at 4 °C to obtain a supernatant comprising metallothionein. Thereafter, we added 1 mL of chilled

ethanol (100%) and chloroform (80 µL) for every 1 mL of the resultant supernatant. After centrifuging the samples for 10 min at 6000× *g*, three volumes of chilled ethanol were mixed into the supernatant and the solutions were kept at −20 °C for 60 min. Again, the supernatant was centrifuged at 6000× *g* for 10 min. The obtained pellets were rinsed with chloroform (1%) and ethanol (87%), and then centrifuged one more time at 6000× *g* for 10 min. The pellets were dried and reconstituted in Tris HCl and EDTA (pH 7). We combined 5,5-dithiobis (nitrobenzoic acid) with the precipitated metallothionein fraction. The product was then kept at an ambient temperature for 30 min. A UV spectrophotometer was used to measure the concentration of reduced sulfhydryl at 412 nm of absorbance. Also, a standard curve, using glutathione (GSH) as a standard, was used to calculate the amount of MTs present in the samples.

2.9. Analysis of Antioxidants Enzymatic System in HM Containing Mycelia

The antioxidant enzymes were examined by homogenizing the fungal mycelium in 0.1 M of chilled phosphate-buffered saline (pH 7.0) using a glass homogenizer (Potter-Elvehjem) outfitted with a Teflon pestle. The homogenized mycelium was centrifuged at 2500 rpm for 10 min at 4 °C, and the obtained supernatant was centrifuged again at 12,000× *g* for 20 min at 4 °C. The supernatants were then kept at −80 °C for future examination. The total proteins were estimated using the method described by Lowry et al. [30]. The activity of superoxide dismutase (SOD) in fungal mycelium was determined using the procedure described by Das et al. [31]. The SOD activity unit was specified in the U/mgprotein.

Catalase (CAT) activity was calculated using the Aebi [32] method, which showed that the enzyme could break down H_2O_2 to produce H_2O and molecular oxygen. The unit of the CAT assay was indicated in Pkat/mg of protein.

The reduced glutathione (GSH) assay was carried out using the method described by Moron et al. [33], and the lipid peroxidation (LPO) assay was carried out using the method provided by Zhu et al. [34]. The units of LPO were expressed in nmoles/mg protein.

2.10. Statistical Analysis

All the experiments were conducted in triplicate to enhance the analytical accuracy of the variables, the data were analyzed statistically using OriginPro 9.8 software (Version 2021) with a one-way analysis of variance (ANOVA), and the mean values were compared by performing a Tukey test ($p \leq 0.05$).

3. Results and Discussion

3.1. Physicochemical Analysis of TE

The numerous physicochemical parameters employed revealed that the TE was highly hazardous, as shown in Table 1. The pH of the TE was slightly acidic (6.5), which might have been due to the chemicals employed in the processing of skins and hides. Also, the presence of fine leather flakes in the effluent resulted in a high concentration of suspended solids, one which was above the permissible limits set by USEPA [35] and CPCB [36]. The suspended particles also impacted the reproduction process of aquatic life, resulting in rising aquatic organism mortality. According to a report [37], the high chloride concentrations in the effluent may be attributed to the fact that chlorides are heavily infused into tannery effluent, entering in huge quantities in the form of sodium chloride for hide and skin preservation. The TE had significantly greater concentrations of nitrate and phosphate. Increased levels of nitrate and phosphate are key factors in the growth of algal blooms, which lower the content of dissolved oxygen in water. Also, algal blooms increase ammonia and CO_2 production, which leads to negative impacts on aquatic life [38]. The presence of several minerals and harmful metals is indicated by high TDS values. This presence can impair aquatic life by lowering the water clarity, which lessens the amount of sunlight received by water bodies, thus decreasing photosynthetic activity [39].

Table 1. Physicochemical characterization of TE.

Physicochemical Parameters	Tannery Effluent	USEPA (2002) [a]	WHO (2008) [b]	CPCB (2013) [c]
pH	6.5 ± 0.42	5.0–9.0	6.0–9.0	6.0–9.0
Total solids (mg/L)	2097 ± 43.86	-	1500	-
Total suspended solids (mg/L)	473.33 ± 23.02	35	-	100
Total dissolved solids (mg/L)	658.33 ± 6.80	-	-	2100
Turbidity (NTU)	27.43 ± 0.70	-	-	-
Electrical conductivity (S/cm)	1.23 ± 0.25	-	-	-
Alkanity (mg/L)	540.96 ± 30.0	-	-	-
Total hardness (mg/L)	1410 ± 15.52	-	-	-
Phosphate (mg/L)	28.17 ± 0.34	1.0	-	5
Nitrate (mg/L)	505.89 ± 10.90	10	50.0	10
Ammonia (mg/L)	126.5 ± 1.67	1.0	1.50	-
Sulphate (mg/L)	36.95 ± 0.17	-	-	1000.0
Chloride (mg/L)	947.2 ± 23.4	-	-	600
BOD (mg/L)	751.13 ± 21.61	-	-	30.0
Mg (mg/L)	149.25 ± 0.88	-	-	-
Cr (mg/L)	58.2 ± 0.07	-	0.05	2.0
Pb (mg/L)	7.06 ± 0.032	0.05	0.01	0.1
Zn (mg/L)	3.52 ± 0.005	2.0	0.05	5.0

Notes: [a] U.S. Environmental Protection Agency, Act 2002; [b] World Health Organization, Geneva, 2008; [c] Central Pollution Control Board, 2013.

Additionally, various heavy metals, including lead (Pb), chromium (Cr), and zinc (Zn), were observed in TE after AAS analysis. All the metals present in TE were found in extremely high concentrations, surpassing the permitted limits recommended by the USEPA in 2002, the WHO in 2008, and the CPCB in 2013. This rendered TE extremely dangerous. Moreover, chromium is generally found in high concentrations in tannery effluent because it is used for marking and surfacing leathers and also utilized for dying leather products [40]. Higher Cr concentrations induce catastrophic consequences such as anemia, lymphocytosis, eosinophilia, renal, and bronchial diseases. Its high concentration may damage fish gills and lead to genotoxic and mutagenic effects in living systems [41,42]. TE has high concentrations of toxic metals and is accountable for their toxicity. Thus, it becomes necessary to decontaminate the effluent before discarding it into water bodies. Herein, we tried to eliminate toxic heavy metals from the TE via a cost-effective and sustainable process i.e., mycoremediation utilizing the macrofungi known as *P. opuntiae.*

3.2. Effect of TE Concentrations on P. opuntiae Growth

The fungal mycelia grown with different percentages of TE have shown varying levels of growth. It is obvious from Figure 1a that mycelium could not grow on the media containing 100% (raw) effluent. A high concentration of various hazardous metals might have been present in raw effluent, having a negative impact on the mycelial growth and not allowing mycelia to develop and survive (Figure 1a). However, the mycelia that were cultured on the media containing 50% TE (Figure 1c) and 25% TE (Figure 1d) exhibited robust and continuous growth when compared with the mycelia cultured on 75% TE (Figure 1b) (growing at a much slower speed). The dilution of TE resulted in a decrease in the concentration of various metals present in effluent, which allowed mycelia to reach their normal growth rates in the diluted effluents, such as those with 25% and 50% TE concentrations, and this also permitted mycelia to effectively accumulate metal from the TE and assisted in their normal growth. The presence of a higher metal content in the 75% TE solution in comparison to the 50% and 25% diluted effluent might be responsible for the limited growth of mycelia with distorted hyphae morphology; however, the maximum growth was recorded in the control group.

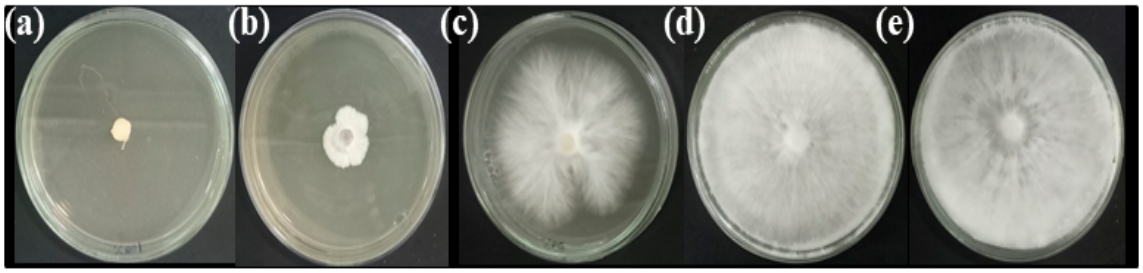


Figure 1. Growth of *P. opuntiae* in media containing different dilution percentage of effluent (**a**–**e**). These represent 100%, 75%, 50%, 25%, and control solutions, respectively.

Further, the impact of heavy metals on fungal growth was mapped via a tolerance index. When the TI value ranged to 0, it showed that growth was completely inhibited by metals; TI < 1 displayed high tolerance; a TI value of 1 showed a level of growth that was similar to that of the control; however, TI > 1 showed an absolute fungal growth that was greater than that of the control [26]. Figure 2 displayed the tolerance index (TI) of *P. opuntiae* under varying concentrations of TE. The maximum TI value of 0.95 was observed in a 25% TE concentration at 72 h of the incubation period, while the TI of *P. opuntiae* in 50% TE concentration was observed to be only 0.58 at 72 h. After 72 h, the TI value declined slightly as it reached a climax for the rapid phase in both the 25% and 50% TE concentrations.

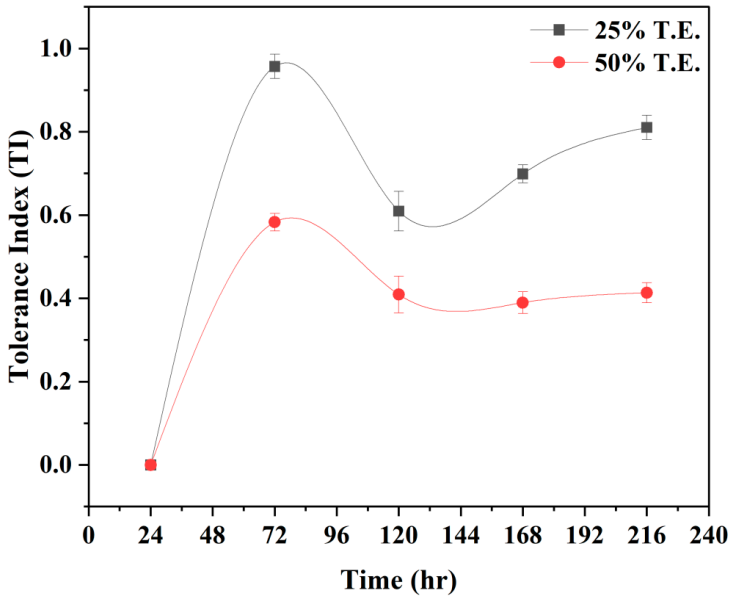


Figure 2. Representing the TI of *P. opuntiae* in 25% and 50% tannery effluent (TE), mycelium of *P. opuntiae* were grown in liquid media for 9 days at 25 ± 2 °C and 180 rpm.

3.3. Quantifying Removal Rate and Bioaccumulation of Heavy Metals Using P. opuntiae

The removal rate of heavy metals when using *P. opuntiae* was established under aqueous culture conditions. In Figure 3a, the results show that the highest removal rate for Pb (91.3%) was observed when the solution exposed to a 25% TE concentration, whereas the rates were 72.2% and 66.5% for Cr and Zn, respectively. However, in a 50% TE concentration, the removal rates for Pb, Cr, and Zn were recorded as being 62.3%, 53.2%, and 55.8%, respectively. Since the control mycelium was grown without metal, the control data were not given.

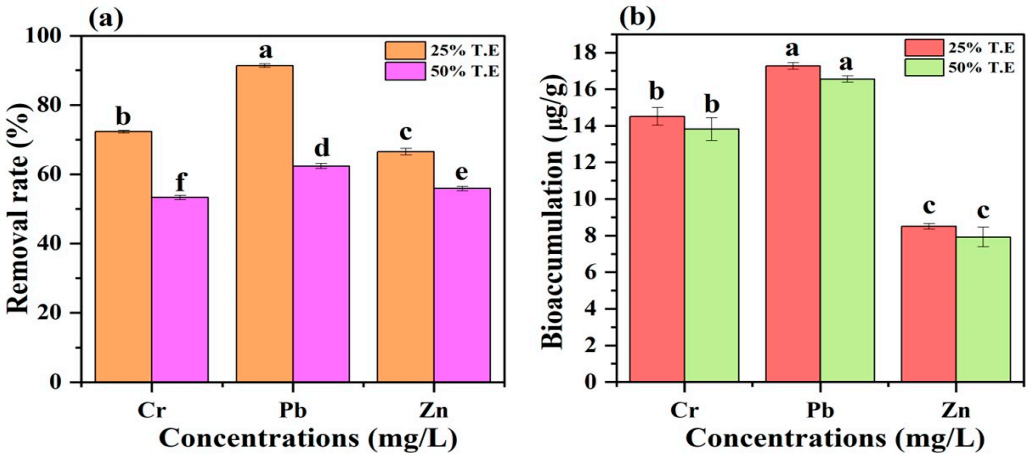


Figure 3. Removal (**a**) and bioaccumulation (**b**) of heavy metals by *Pleurotus* species grown in liquid media containing different percentages of TE for 28 days at 25 ± 2 °C.

Further analysis revealed that the removal of heavy metal was primarily facilitated via an intracellular bioaccumulation mechanism. Hence, the bioaccumulation of heavy metals by the fungal mycelium was depicted in Figure 3b. In the current study, the maximum degree of bioaccumulation was observed for Pb (17.2 µg/g), rather than for Cr (14.5 µg/g) or Zn (8.5 µg/g), in the mycelium grown in solutions with 25% TE concentrations. A similar trend of bioaccumulation was also observed in the fungal mycelium when exposed to a 50% TE concentration. i.e., the uptake of Pb and Cr by the fungal mycelium was statistically significant in comparison to the uptake of Zn metal ions. A decrease in Zn uptake was observed, which was likely due to the presence of lower concentrations of Zn in the media.

Furthermore, after mycoremediation, it was found that all the metals present in TE were successfully eliminated from the diluted TE by employing *P. opuntiae*. However, effective removal and bioaccumulation were observed only in 25% and 50% TE concentrations. This could have been because of the reduced metal concentration in the diluted effluent, making the mycelium more effective at accumulating the metals from the effluent and aiding in its development and physiological function. In contrast, higher metal concentrations in other diluted TEs (75%) and at the raw concentration might have hindered the growth of the fungal mycelium, thus reducing its bioaccumulation potential. Similar results were also obtained by employing *P. ostreatus* to remove heavy metals from coal washery effluent [43]. Macrofungi respond to metal stress in the environment by generating stress compounds that are both proteinous and non-proteinous in nature. The mycelium has been reported to release stress factors that aid in sequestering the accumulated metal ions in their vacuoles. Metallothionein (MT), phytochelatins (PCs), glutothionine (GSH), and plastocyanin are the stress components most frequently generated by fungi. Several reports have also suggested that whether the organisms have the capacity to accumulate heavy metal ions mainly depends on the heavy metal concentrations that occur in their growing environments [44–46]. The uptake of different heavy metals by fungi has also appeared to vary with metal ion type. According to Sazanova et al. [47], the high concentrations of many metals appeared to prevent fungal growth due to a number of physical abnormalities, including cell membrane destruction, the induction of lipid peroxidation, the production of ROS, the suppression of respiration, the alteration of enzyme activity, and damage to protein structures and DNA [48]. The results of the current investigation indicated that the amount of multi-metal concentration and the kind of metal ion had a substantial impact on the multi-metal accumulation and its removal by *P. opuntiae.*

3.4. Analysis of Surface Morphology of Pleurotus opuntiae Mycelium after Mycoremediation

The surface morphologies of the fungal mycelia that appeared with and without TE exposure were examined under a scanning electron microscope. Figure 4a–c represent the image of control mycelium (without TE exposure). Figure 4d–f depict the image of the

mycelium grown in a 25% TE concentration whereas, Figure 4g–i represent the image of the mycelium exposed to a 50% TE concentration. In Figure 4a–c, the control micrograph showed an uneven meshwork of elongated, ribbon-like hyphae, with entangled branches that were made of porous structures without any physical damage. Similar morphology was also observed in the 25% TE exposed *P. opuntiae* mycelium. On the cotnrary, *P. opuntiae* grown in a 50% TE concentration exhibited swollen hyphae that are undetectable, densely packed, lack voids, and provide a smooth surface (Figure 4g–i). This could be a detoxification mechanism since mycelia aggregation minimizes the surface area of cells exposed to harmful metals. The aggregated mycelia might have lessened direct contact between the toxic metal and the cell, reducing their vulnerability to adverse effects. This might have led to increased accumulation in the mycelium, thus promoting the mycoremediation of TE. Similar conclusions have been drawn from numerous investigations where alterations were seen, in contrast to the control group [23,49].

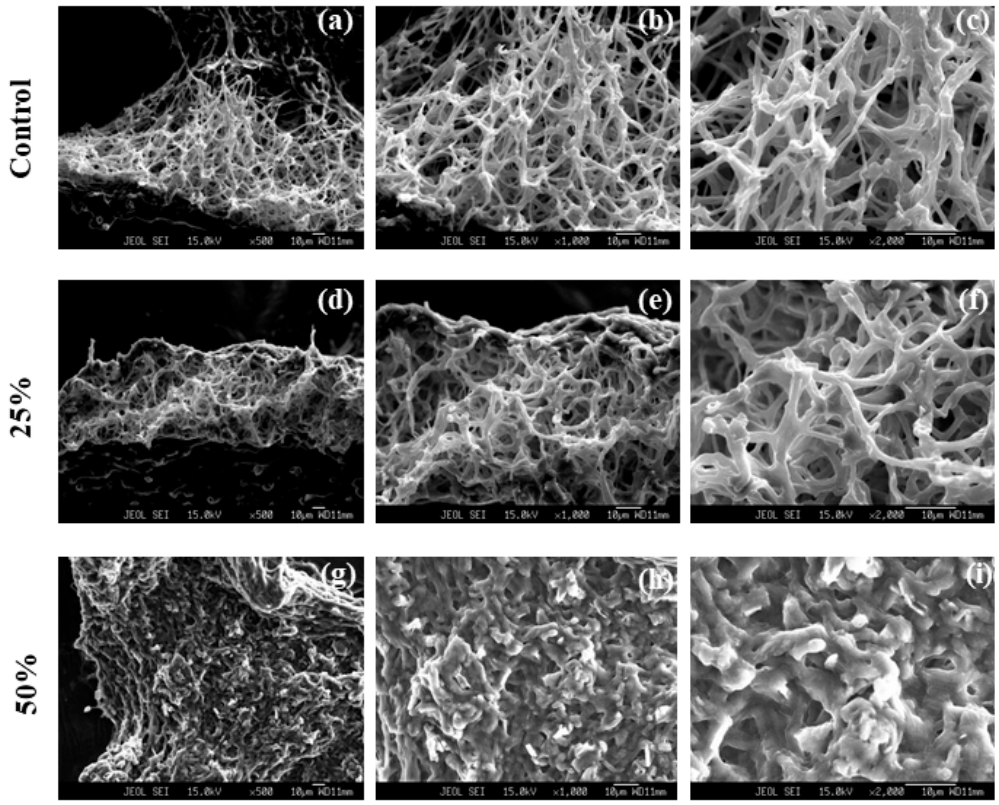


Figure 4. SEM micrograph of *Pleurotus opuntiae* cultivated in control (**a–c**), 25% TE (**d–f**), and 50% TE (**g–i**).

3.5. FTIR Analysis

The type of functional groups that occurred on *P. opuntiae* cell walls before and after TE exposure were examined using FTIR analysis in order to ascertain the influence of heavy metal on cell wall properties. The alterations in the vibrational frequencies of FTIR spectra unveiled the role of cell walls in the elimination of heavy metals. Figure 5a–c showed the FTIR spectra of *P. opuntiae* after exposure to different concentrations of TE (i.e., 50% and 25% TE) and control, respectively. In control spectra, the band ranges between 3500 cm^{-1}–3000 cm^{-1} displayed a sharp absorption peak of N-H and O-H stretching [50]. This absorption peak was also observed in mycelium grown in 25% TE, but N-H stretching was absent in 50% TE mycelium. Further, the inclusion of C-H stretching vibrations at 2926 and 2852 cm^{-1} in the metal ion adsorption process was not very apparent. Only a weak shift was observed at 780–782 cm^{-1}, 1314.7–1313.8 cm^{-1}, and 1399–1401 cm^{-1} when comparing the level before to that after the treatment of TE, specifying the interference of amino, carboxyl, and hydroxyl groups during absorption process. The absorption peak at

1100 cm^{-1}, representing C-O and C-O-C groups of polysaccharides, shifted slightly after exposure to TE. Two new sharp peaks were identified within the 500–1000 cm^{-1} regions of the PbO group and Cr=O group in both the diluted groups (Figure 5b,c).

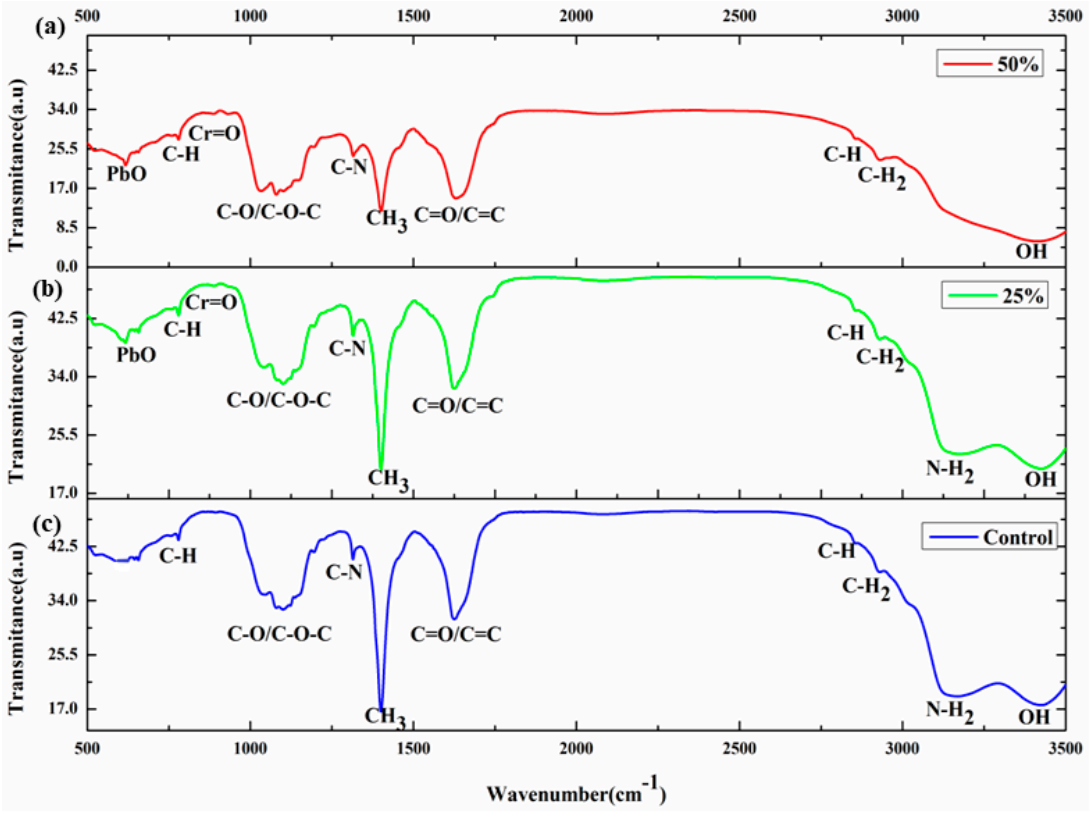


Figure 5. FTIR spectra of *Pleurotus opuntiae* cultivated in 50% TE (**a**), 25% TE concentrations (**b**), and control (**c**).

The shifting of absorption peaks indicated the involution of many functional groups present in fungal biomass during mycoremediation [51]. The type of functional groups involved varied on the fungus species, the origin of isolation, the method of processing the biomass, and solution chemistry [49,52]. Hence, it could be concluded that the inclusion of functional groups with negative charges (such as hydroxyl and carbonyl groups) provides electrostatic interactions for the binding of positively charged metal ions to cell walls for heavy metal removal [53].

3.6. XRD Analysis

XRD was employed to identify the mineral compositions that occur as a precipitate on the fungal mycelium. Moreover, XRD analysis enabled the ascertainment of the structure of the sample, i.e., whether it was amorphous or crystalline. Figure 6c shows the XRD spectra of the control fungal mycelium. Before TE treatment, the spectra showed only one large peak at about 20°, indicating that the mycelium was amorphous before treatment. However, in Figure 6a,b, the mycelium showed a crystalline structure due to the appearance of diffraction peaks after exposure to different concentrations of effluent. In this study, we found that the heavy metals present in the effluent, such as Pb and Cr, were biomineralized to PbO and CrO_3, respectively, by *Pleurotus opuntiae*. Further, the high-intensity peaks of PbO and CrO_3 were observed in the mycelium grown in 50% TE rather than in the 25% diluted group due to the presence of high concentration of heavy metals. Similar findings were also observed when Cr(VI) ion was reduced to Cr_2O_3 using *A. flavus* CR500 and *A. fumigatus* [54,55]. Further, the diffraction peaks of Pb complexes corresponded to

lead oxalate and lead hydroxyphosphate when utilizing *P. polonicum* and *P. chrysoporium*, respectively [56,57].

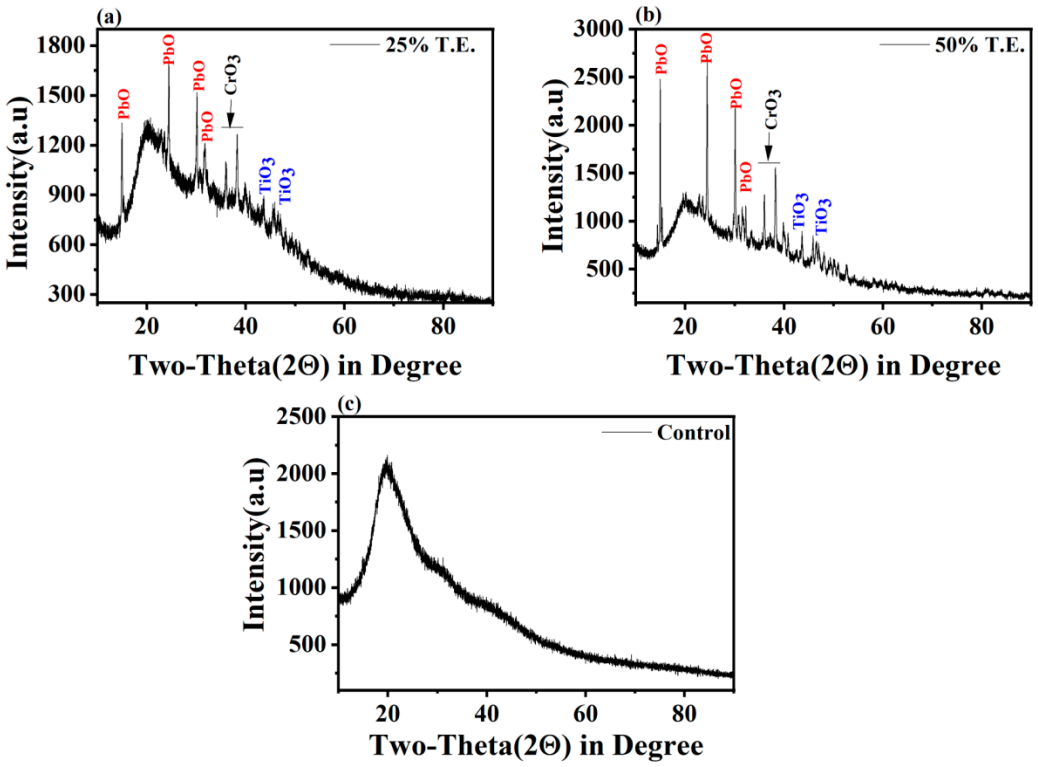
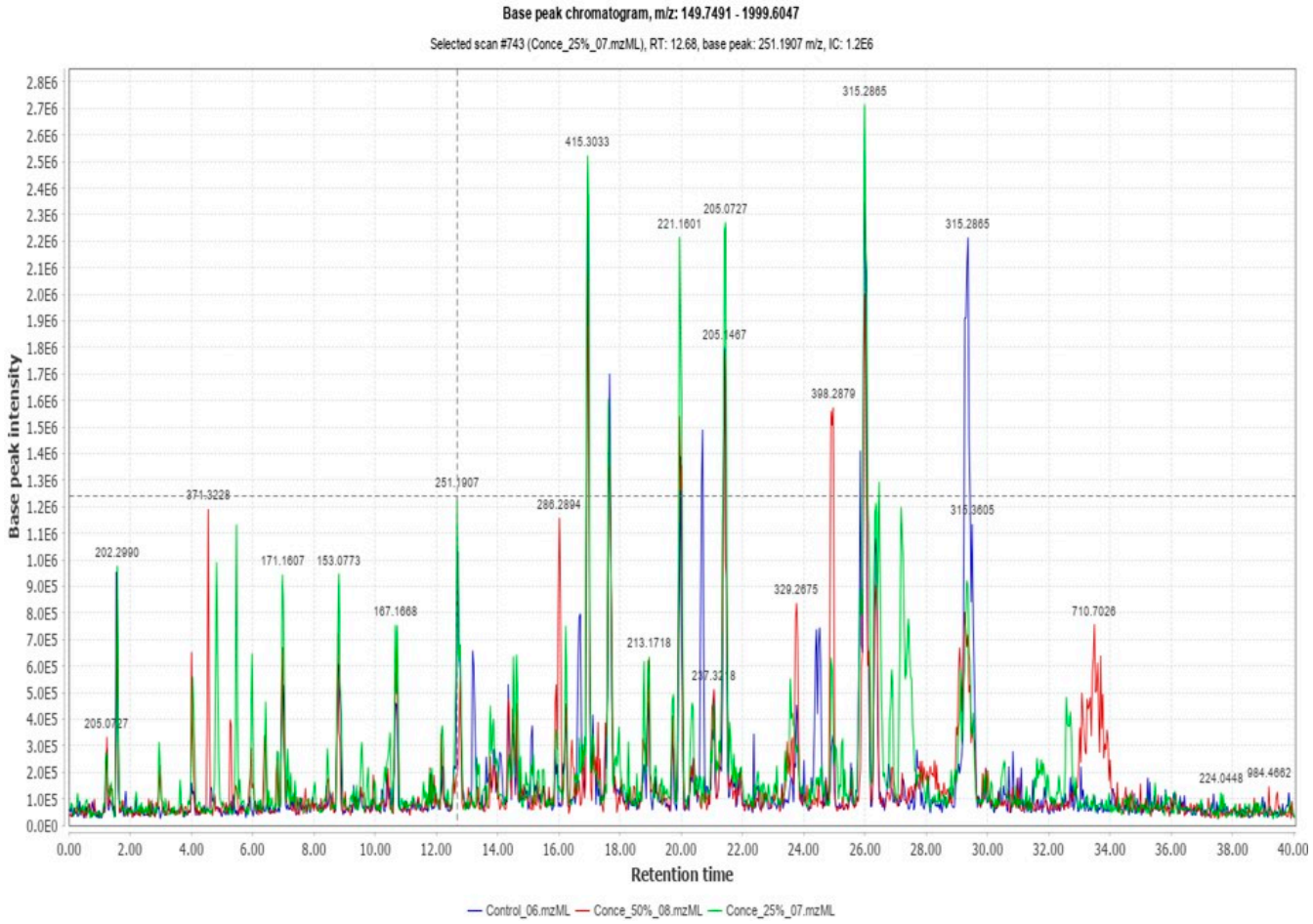


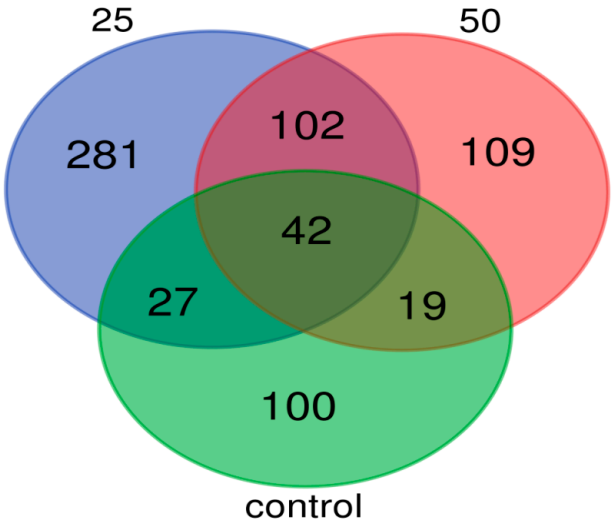
Figure 6. XRD analysis of *Pleurotus opuntiae* cultivated in 25% TE (**a**), 50% TE concentration (**b**), and control (**c**).

3.7. LC-MS Analysis

The LC-MS chromatographs of *P. opuntiae* are shown in Figure 7a, which represents the retention time in minutes of each metabolite present in the fungal mycelium. The peaks seen in the chromatograms were studied using the yeast database in order to determine their components. By comparison, Figure 7a shows that there was a total of 7938 peaks in the control mycelium, while there were 22,132, and 11,653 peaks in the mycelium grown in 25% and 50% effluent concentrations, respectively. Figure 7b shows the amount of some specific metabolites in *P. opuntiae* mycelium, and these decreased or increased in comparison to the control and the effluent-exposed mycelium. This was likely due to the regulatory effect of heavy metals on the physiological metabolism of *P. opuntiae*. In particular, the retention time of 32.0–34.0 min demonstrated a higher degree of relative ionic strength intensity in the positive ionization mode in the mycelium after exposure to effluent concentrations of 50%. At the retention duration of 34.7 min, a molecule with m/z value of 710.77 was observed. Moreover, most of the molecules actively appeared in the mycelium that was exposed to the 25% dilution concentration of effluent, where four molecules with m/z values 315.28, 415.30, 205.07, and 221.16 indicated a strong intensity of ionic strength at 25.9 min, 16.9 min, 28.8 min, and 19.9 min, respectively. Further, a molecule with an m/z value of 315.28 was observed in the control mycelium, with higher ionic intensity seen at a retention time of 29.3 min. Some studies have concluded that phytochelatins (PC) play a pivotal role in heavy metal detoxification, and the mechanism of heavy metal bioaccumulation via PC has been observed in many metal-resistant fungal kingdoms. Additionally, several studies have also reported that PCs bind to heavy metals and facilitate the transport of excess metal ions into the vacuole or periplasmic space for storage, thereby reducing cellular toxicity. This phenomenon is essential for maintaining cell homeostasis in the presence of heavy metal stress. Thus, the mechanism of metal accumulation might therefore be summarized from the findings of this current study [58].



(**a**)



(**b**)

Figure 7. (**a**) LC-MS chromatograph of *Pleurotus opuntiae* at various retention times after exposure to effluent and control. (**b**) Venn diagram representing the number of metabolites detected via LC-MS in the mycelium of *P. opuntiae* when exposed to different effluent concentrations and the control.

3.8. Metallothionein Concentration in P. opuntiae after Mycoremediation

The metallothionein concentration was measured in the fungal mycelium after exposure to different diluted concentrations of effluents at various points in time (i.e., on the

8th, 13th, 18th, 23rd, and 28th days). Metallothionein is a metal-binding protein with a high cysteine content that protects against heavy metal toxicity [59]. Figure 8 demonstrates that the metallothionein levels increased in fungal mycelia cultured in 50% TE as well as in the 25% diluted group. However, the maximum concentration was found in the 50% dilution group, which could be ascribed to the presence of excessive levels of heavy metals. Furthermore, as the exposure time increased, the concentration reached its peak on the 28th day. A defense mechanism against metal accumulation in the fungal mycelium could be the cause of the elevated metallothionein concentration. The greatest level of accumulation of most heavy metals was seen in mycelia that were exposed to 50% TE, possibly due to these samples having the highest metallothionein protein synthesis rate in order to shield the cells from the detrimental effects of heavy metals. The concentrations in the control groups did not change significantly, remaining consistent until the end of the day. According to a report, the presence of Cu and Cd increased the transcription level of the metallothionein genes in *Hebeloma cylindrosporum* [60]. Similar kinds of outcomes were also observed in the fungus *Beauveria bassiana* after exposure to heavy metals [61].

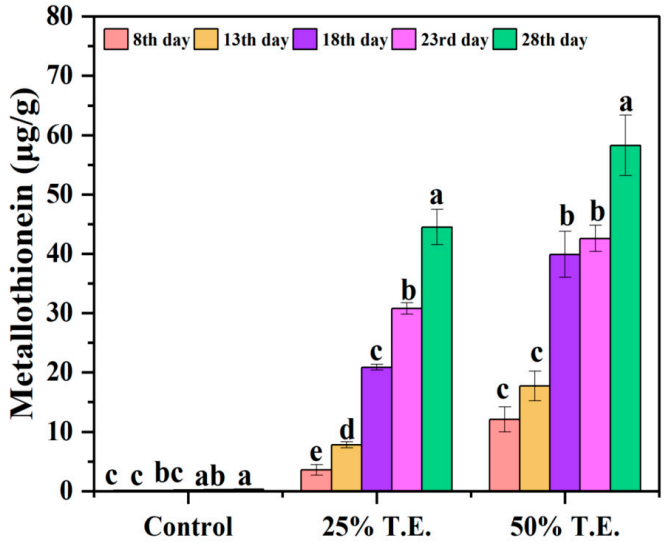


Figure 8. Concentration of metallothionein in the fungal mycelium grown in media containing different dilutions of effluent at several time intervals. Values followed by different alphabets in the same figure differ significantly ($p < 0.05$).

3.9. Effect of TE Stress on Antioxidant Enzymatic System of Fungi

Antioxidants are a crucial part of the cellular immune system due to their capacity to scavenge reactive oxygen species (ROS) [62]. SOD, LPO, and CAT are associated with the class of induced enzymatic antioxidant systems that lessens the effects of oxidative stress [63], whereas GSH is an essential non-enzymatic antioxidant protein that detoxifies non-essential metals via the production of distinct, low-molecular-weight chelators [64,65]. In the present study (Figure 9a–d), increased activity of SOD, CAT, and GSH was observed in the fungal mycelium grown at a 25% effluent concentration. However, the activity of SOD reached a maximum value of 5.8 U/mg protein, and the activity of CAT reached a maximum value of 1.59 Pkat/mg, in the mycelium grown in a 25% effluent concentration on the 28th day. Moreover, the mycelium grown in 50% effluent displayed the enhanced activity of SOD, CAT, and GSH during the initial exposure period. Thereafter, the activity of these enzymes lessened, falling at the end of the period. In the case of LPO, the mycelium that was subjected to effluent concentrations of 50% and 25% exhibited fluctuation and reached its highest level on the final day of the exposure period.

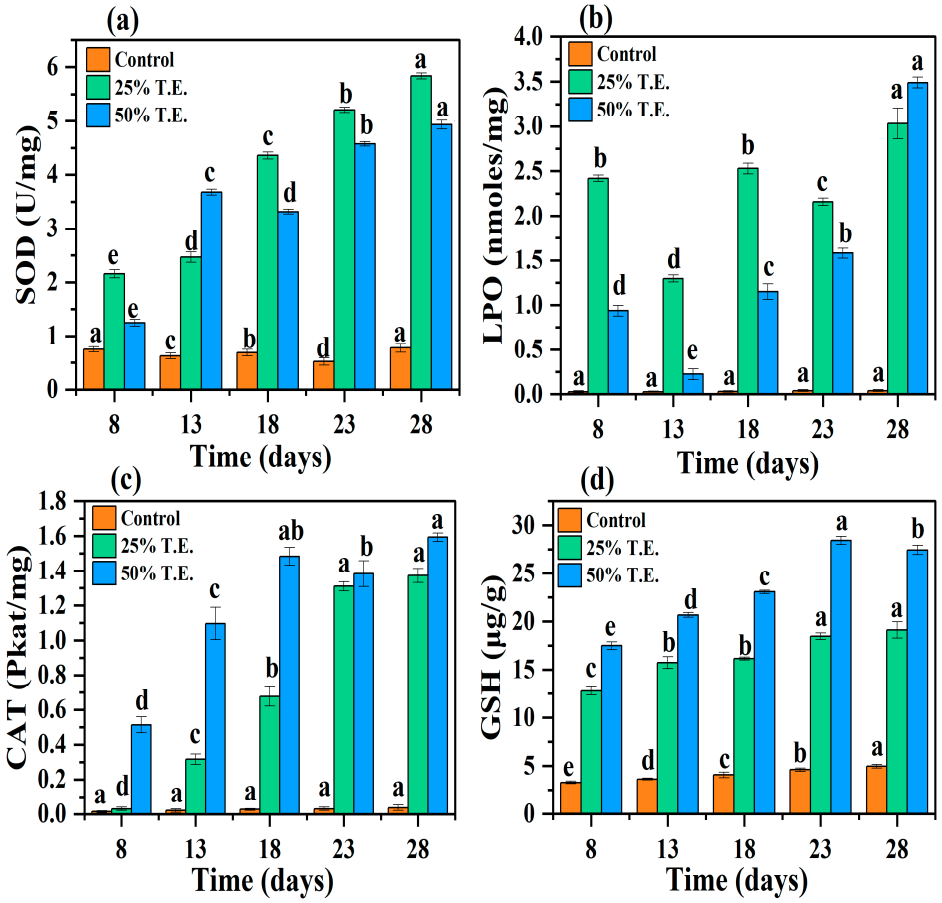


Figure 9. Antioxidant enzyme activity in fungal mycelia grown in media containing different dilutions of effluent at several time intervals. These are (**a**) SOD, (**b**) LPO, (**c**) CAT, and (**d**) GSH, respectively. Values followed by different alphabets in the same figure differ significantly ($p < 0.05$).

4. Conclusions

The mechanisms for the removal and accumulation of multiple heavy metals by *P. opuntiae* were systematically studied under the exposure of tannery effluent at different dilution concentrations. The stain could show maximum tolerance index values at the 25% and 50% diluted groups and removed a maximum of 91.3% of Pb and 72.2% and 66.5% of Cr and Zn, respectively, from the 25% diluted effluent. Notably, Pb showed the highest degree of bioaccumulation, followed by Cr and Zn. SEM, FTIR, and XRD analysis revealed the detoxification mechanism involved in metal removal, such as accumulation, adsorption onto the cell wall via functional groups, and surface precipitation. However, bioaccumulation proves more advantageous than adsorption in wastewater treatment, as it circumvents the issue of desorption. Moreover, LC-MS analysis revealed the role of the metabolites involved in the regulation of heavy metals in the physiological metabolism of *P. opuntiae*. In addition, the elevated concentrations of stress markers, namely metallothionein, and active antioxidant enzymes such as SOD, CAT, LPO, and GSH withstood the oxidative stress brought on by heavy metal toxicity. The current study emphasizes the negative effects of tannery effluent owing to the presence of numerous heavy metals above their permissible levels, while also highlighting that the efficacy of remediation could also be boosted by diluting the effluent. Thus, these findings suggest that mycoremediation could be a sustainable approach for decontaminating an effluent by utilizing *Pleurotus opuntiae.*

Author Contributions: Conceptualization, M.P.S. and P.Y.; Methodology, P.Y. and V.M.; software, T.K.; Investigation, P.Y. and T.K.; Writing—Original Draft Preparation, P.Y., V.M. and T.K.; Writing—Review & Editing, M.P.S., U.K.S., E.V. and P.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data presented in this study are available in article.

Acknowledgments: I would like to express my heartfelt thanks to Shraddha Vishwakarma (Research Scholar), Centre of Bioinformatics, University of Allahabad for fruitful discussions on LC-MS analysis.

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

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