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MULTIPLE METAL BIOSORPTION BY A FILAMENTOUS FUNGUS (*DALDINIA STARBAECKII*) ISOLATED FROM LANDFILL LEACHATE CONTAMINATED SOIL

Auwalu Hassan[a,b], Azman Azid*[a], Fauziah Shahul Hamid[c], Agamuthu Pariatamby[d], Saiful Iskandar Khalit[a], Innocent Chukwunonso Ossai[c], Jayanthi Barasarathi[e], Suzanne Christine Aboudi-Mana[c], Mohd Zuhairi Zakaria[f]

[a]Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22000 Besut, Terengganu Darul Iman, Malaysia
[b]Department of Biological Sciences, Faculty of Science, Federal University of Kashere, Gombe State, Nigeria
[c]Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[d]Jeffrey Sachs Center on Sustainable Development, Sunway University, Sunway, Malaysia
[e]Faculty of Health and Life Sciences (FHLS), INTI International University, Pesiaran Perdana BBN, Nilai, Negeri Sambilan, Malaysia
[f]HZN Associates (M) Sdn Bhd, No. 13 (1st Floor), Jalan Majistret U1/26, Seksyen U1, Hicom Glenmarie Industrial Park, 40150 Shah Alam, Selangor, Malaysia

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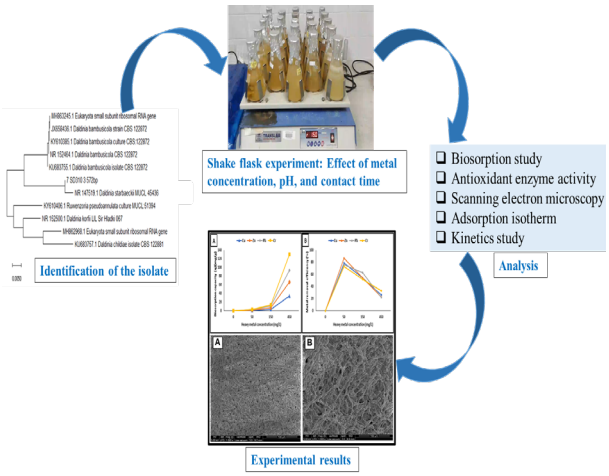
*Corresponding author
azmanazid@unisza.edu.my

Graphical abstract



Abstract

The objective of this research was to investigate the ability of an environmentally benign fungal strain to deal with multiple metal stress and to accomplish their bioremoval from the contaminated liquid medium. *Daldinia starbaeckii* was used as the biosorbent material for this research. The effects of metal concentration (0, 50, 150, 450 mg/L), pH (4.5, 7, 8), and contact time (0, 24, 48, 72, 96, 120 hours) on the metal biosorption were determined. The maximum biosorption (36.77 mg/g) was observed at 450 mg/L. Biosorption increased with the increase in pH and contact time and the removal ranged between 43 – 76% and 3 – 80%, respectively. The SEM images revealed that in metal-treated mycelia, deformed, tightly packed with shortened length hyphae were observed, while long ribbon-like hyphae which are loosely packed and broad were noted in the control. The Freundlich isotherm model best described the biosorption data. This fungus can serve as a potential biosorbent for the bioremediation of metal-contaminated media.

Keywords: Daldinia starbaeckii, Biosorption, Heavy metals, Liquid medium, Adsorption isotherm

Abstrak

Objektif penyelidikan ini adalah untuk menyiasat keupayaan strain kulat jinak alam sekitar untuk menangani tekanan logam berganda dan untuk mencapai penyingkiran bio mereka daripada medium cecair yang tercemar. Daldinia starbaeckii digunakan sebagai bahan biosorben untuk penyelidikan ini. Kesan kepekatan logam (0, 50, 150, 450 mg/L), pH (4.5, 7, 8), dan masa sentuhan (0, 24, 48, 72, 96, 120 jam) ke atas biosorpsi logam telah ditentukan. Biosorpsi tertinggi (36.77 mg/g) diperhatikan pada 450 mg/L. Biosorpsi meningkat dengan peningkatan pH dan masa sentuhan dan kecekapan penyingkiran masing-masing antara 43 – 76% dan 3 – 80%. Imej SEM mendedahkan bahawa dalam miselia yang dirawat logam, cacat, padat padat dengan hifa panjang yang dipendekkan diperhatikan, manakala hifa seperti reben panjang yang lebar dan padat longgar diperhatikan dalam kawalan. Model isoterma Freundlich menerangkan dengan terbaik data biopenyerapan. Kulat ini boleh berfungsi sebagai biosorben yang berpotensi untuk bioremediasi media tercemar logam.

Kata kunci: Biosorpsi, Daldinia starbaeckii, logam berat, medium cecair, isoterma penjerapan

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1.0 INTRODUCTION

The increasing rate of urbanization and industrialization has resulted in large production of municipal solid waste (MSW) [1]. The landfill system is one of the most widely used options for MSW disposal in Malaysia. However, this option has led to metal contamination of the environmental compartments from the production of leachate [2, 3]. Leachate is polluted water that contains many compounds such as acids, alcohols, aromatic hydrocarbons, hydroxybenzene, alkanes, esters, alkenes, amides, ammonia, and heavy metals [4]. Among the pollutants found in the leachate, toxic metals are of serious concern. Reports show that a significant amount of heavy metals is released through landfill leachate which eventually ends up in the soil and water [5-8].

Exposure to toxic metals results in a lot of serious illnesses which include digestive disorders, autoimmune disorders, kidney, liver, and heart disorders, as well as lung and liver cancer [9-12]. Heavy metals are generally highly reactive and have a strong propensity for sulfhydryl groups little biological molecules and proteins. As a result, many biological functions can be severely affected through enzyme inhibition or disturbance of the pathways, leading to many lethal disorders [13]. Hence, the clean-up of metal from contaminated media is imperative. Many techniques have been used conventionally to clean up metals from a contaminated liquid medium, however, these technologies are associated with various drawbacks [14, 15]. During the past few years, an intriguing, cost-effective, and environmentally friendly method for removing metal pollutants has come to light. Biosorption is an intriguing method that uses biological systems to remove metals from contaminated environments [13, 14, 16-18]. Biosorption operates contingent upon the association between metal ions and organisms [19-21]. Microorganisms can bind metals in a liquid media and perform a critical part in the elimination of metal contaminants [22-24]. This research investigated the biosorption of metals in a liquid culture medium using a filamentous fungus isolated from landfill leachate-polluted soil.

Fungi are versatile biosorbents that are readily available, cheap, and have strong adsorption power [25, 26]. Fungi have anionic functional groups on their surfaces which are negatively charged and act as active sites for binding of the positively charged metallic ions. The functional groups include alcohol, amine, ester, hydroxyl, thiol, carboxyl, thioester, phosphoryl, sulfonate, and sulfhydryl groups [27-30]. Fungi possess the biochemical capability and ecological adaptability to tolerate metal toxicity and reduce the threat of metal contaminants by affecting their bioavailability or modification of their chemical structure [31, 32]. Many strategies are employed by fungi to thrive in heavy metal-contaminated environments [33, 34].

Several fungal organisms are found to significantly clean-up metals from contaminated media such as leachate, industrial wastewater, broth media, and other metal-polluted liquid media [35-37]. However, to our knowledge, no such research was conducted using *Daldinia starbaeckii*. Furthermore, many research on biosorption have concentrated on the decontamination of aqueous solutions contaminated with single or at most binary metals. Only scanty information is available on the biosorption of multiple-metals using a single microbe, likely because microorganisms fail to efficiently deal with multi-metal pollution simultaneously. This is for the fact that metals

when in the consortium may interact antagonistically, synergistically, or in a non-interactive way to result in toxicity. Moreover, in a multiple metal system, the removal of the metal contaminants varies depending on the metal affinity for the biomass where some of the metals are removed better than other metals in the solution. Metal clean-up is a complicated procedure that relies on various factors such as cell wall composition, metal chemistry, physicochemical variables like pH, temperature, metal concentration, ionic strength, duration of exposure or contact, as well as, physiology of the microorganism [38]. Given the above, the objectives of this study are to investigate the ability of *D. starbaeckii* to tolerate multiple metal stresses and to accomplish their removal from the contaminated broth medium. In this research, the multiple metals (Cu, Zn, Pb, and Cr) biosorption ability of *D. starbaeckii* isolated from heavy metal-contaminated landfill soil was examined. Moreover, the influence of operational parameters that govern the metal biosorption specifically the metal concentration, pH, and contact time was determined.

2.0 METHODOLOGY

2.1 Sampling Area and Sample Collection

The landfill Taman Beringin (JinJang Utara, Kuala Lumpur, Malaysia) was chosen as the sampling area. Since the landfill was non-engineered, a liner to protect groundwater and soil from leachate penetration was not included in the design. The landfill was in use between 1992 and 2005, and during that time, 1800 to 2000 tons of MSW were dumped daily for a complete disposal of 8,541,000 to 9,490,000 tons over thirteen years. Most of the MSW disposed of at the site was commercial and residential waste. The facility was still producing leachate and methane gas. Both physicochemical and biological (oxidation pond) methods were used to treat the leachate. The generated methane gas was passively delivered to the atmosphere via the gas vent [39]. Soil contaminated by landfill leachate was cored from 0 to 30 cm depth using a soil corer [40]. The soil was randomly sampled from leachate-contaminated locations. After being cored, the soil was put in a sterile container and brought to the laboratory for analysis and other experiments.

2.2 Characterization of isolate

2.2.1 Morphological Identification

One gram of soil was placed in a flask containing 10 mL of sterile water and agitated for 10 mins. From this solution, 1 mL was transferred into a separate tube carrying 9 mL of sterile distilled water and strongly shaken. The procedure continued till the 10^{-7} dilution was attained. 0.1 mL of 10^{-7} was added to the surface of a prepared PDA plate. The added was dispersed onto the agar with a sterile spreader and incubated for 6 days at 28 °C. Visual examination of the colonies was used to read the incubated plates. The isolated fungus was first microscopically identified by suspending the mycelia on microscope slides, then stained with lactophenol cotton blue. The identification was done using microscope at 40x in careful conjunction with the accepted identification manual [41].

2.2.2 Molecular Identification of Fungi

The molecular examination of the isolate was performed after culturing the conserved isolate onto malt extract agar (MEA), which was then incubated at 30 °C for 7 days. The fresh isolate's DNA was isolated using the method described in [42]. Amplification of the ITS segment was accomplished using primers ITS 1F and ITS-4 [43]. PCR buffer, magnesium chloride, deoxyribonucleotide triphosphates (dNTPs) (Ampliqon Company, Odense, Denmark), and Taq polymerase were used in the polymerase chain reaction. The reaction solution (25 µL) contained 10 pmol/µL of each forward and reverse primer, as well as 10 ng gDNA. The PCR products were analyzed on a 1.5% agarose gel dyed with ethidium bromide. The DNA products were cleansed and sequenced using ITS 1F and ITS 4 primers. The acquired sequences were contrasted to those of recognized species using blasting in the GenBank database.

2.3 Heavy Metal Tolerance Assay

The identified *D. starbaeckii* was tested on different heavy metal concentrations to assess its tolerance capacity. Metal concentrations (10 mg/L, 20 mg/L, 30 mg/L, and 40 mg/L) were generated by combining metal salts (Cr, Pb, Zn, and Cu) (Table 1) with distilled water. Diluting the stock solutions yielded varying metal concentrations. The PDA medium was made using the previously obtained metal concentrations. The fungal strain was cultured into the PDA and incubated for 6 days at 28 °C. All petri plates were incubated in triplicate. The medium was not treated with metals for the control (only sterile distilled water was used), and the control plates were inoculated using the same procedure as the treatment plates. After plate incubation, the radial growth diameters were determined with a meter rule [1]. Each plate had at least three distinct readings taken and recorded. The tolerance index was calculated by dividing the radial diameters of metal-treated *D. starbaeckii* by those of the untreated control (Equation 1). A high tolerance index indicates that the fungus is quite tolerant [44]. The rating of fungal tolerance in classes was adapted from [45].

$$T_i = {Dt}/{Du} \quad (1)$$

T_i stands for tolerance index,
D_t stands as the radial growth diameter on treatment plate,
D_U stands as the radial growth diameter on control plates.

Table 1 Heavy metals used in tolerance assay

S/N	Metal Salt	Molecular formula	Molecular wt (g/mol)	Atomic wt (g/mol)	Product Brand
1	Cu	$CuSO_4$	159.60	63.55	Bendosen
2	Zn	$ZnSO_4.7H_2O$	287.55	65.38	AnalaR
3	Pb	$Pb(NO_3)_2$	331.20	207.20	AnalaR
4	Cr	$Cl_3CrH_{12}O_6$	266.436	52.00	Aldrich

2.4 Determination of Antioxidants Enzyme Activity

2.4.1 Peroxidase Activity

A 3 mL reaction solution made up of 200 µL of 200 mM guaiacol, 100 µL of crude extract, and 2.5 mL of 0.05 mM sodium phosphate buffer (pH 7) was used to measure the activity of POD. 200 µL of hydrogen peroxide (H_2O_2) (30%) was introduced in a cuvette as an electron acceptor. The addition of H_2O_2 initiated the process. At 475 nm and 25 °C, the decline in absorbance was observed for 2 mins. For the control, the mixture was constituted without the addition of H_2O_2. According to [46-47], the enzyme's activity was measured in mg of absorbed protein per min.

2.4.2 Ascorbate Peroxidase Activity

The techniques outlined by [48] and [49] were used. The experimental solution contains 100 µL of extract, 200 µL of 0.1 mM EDTA, 200 µL of 0.1 mM H_2O_2, 300 µL of distilled water, and 2 mL of 50 mM potassium phosphate buffer (PPB) (pH 7). This brought the entire volume to 3 mL. By observing the reduction in OD at 290 nm for 2 mins, it was possible to determine that ascorbate was being oxidized. The quantity of enzyme required to break down 1 mM ascorbate/min was measured in units of enzyme per gram of fresh material.

2.4.3 Catalase Activity

A cuvette holding 2 mL of 50 mM PPB (pH 7), 100 µL of crude enzyme extract, 500 µL of water, and 400 µL of 12.5 mM H_2O_2 was used to evaluate the activity of CAT. After supplementation of H_2O_2, the reaction was started, and then the decline in absorbance at 240 nm was observed for 2 mins. By quantifying the amount of H_2O_2 that has broken down and recording it as Units (mol of H_2O_2 decomposed/min) per g fresh weight, the enzyme's activity was ascertained [49, 50].

2.5 Biosorption Study

2.5.1 Effect of Initial Metal Concentration, pH, and Contact Time on Heavy Metal Biosorption

Before determining the metal uptake, the growth condition of the fungus was enhanced. The isolate was cultivated for four days at 28 oC, pH 5.0 on a PDA medium. Following incubation, the organism was suspended by placing roughly five plugs of fungal colonies into 100 mL of sterile PDB in a flask and incubating for an additional four days at 28 °C. For the influence of metal concentration on the fungus's metal uptake, 100 mL of PDB was treated with metals at varied concentrations (450 mg/L, 150 mg/L, and 50 mg/L) at pH 5. 50 mg/L modified PCB medium maintained at varied pH levels 8.0, 7.0, and 4.5 was employed for the biosorption, whereas PDB modified with 50 mg/L of each metal at pH 5 was utilized for the influence of contact duration (120, 96, 72, 48, and 24 hours). In each setup, a 1 mL suspension of the fungal culture was inoculated into flasks holding 100 mL of the metal-treated PDB medium, and the combination was incubated at 28 °C at 150 rpm on a rotary shaker. The time of incubation for the effect of metal concentration and pH was fixed at 96 hours, whereas for contact time it was fixed at 120 hours, with subsamples taken and tested every 24 hours [51]. The inoculated untreated medium was used as the control experiment for each setup. The content was filtered, the trapped mycelia were cleansed with distilled water and further filtered, and the liquid component was retained in beakers. The rinsed mycelia were dried in an oven at 110 °C overnight and then weighed to estimate the dry weight of the biomass. The liquid component was digested, filtered, and the metal content was determined using AAS machine [42, 51].

2.6 Metal Biosorption by the Fungus

Equations 2 and 3 [52] were employed to calculate the biosorption (Q_e) and removal efficiency (E).

$$Q_e = \left(\frac{Ci - Ce}{m}\right) X\ V \quad (2)$$

Where Q_e stands as biosorption capacity (mg/g), C_i stands as initial metal concentration (mg/L), C_e stands as final metal concentration following biosorption (mg/L), V stands as entire volume of reaction mixture (L), and m stands as dry mass of mycelia (g).

$$E = \left(\frac{Ci - Ce}{Ci}\right) X\ 100 \quad (3)$$

2.7 Scanning Electron Microscopy of mycelia

For the SEM investigation of the fungal organism, the mycelia in the liquid medium was spun at 500 rpm for 10 mins. Following centrifugation, the liquid was removed, and the condensed bottom mycelia were re-suspended in 5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.2). The contents were held for 30 mins before being centrifuged and the supernatant discarded. The pellet was again immersed in 0.1 M PB two times for 10 mins each, with the supernatant removed. Following treatment with PB, the pellet was immersed in 0.1 M PB containing osmium tetraoxide (1%) and kept for 1 hour. The content was spun and

decanted. Dehydration was carried out in a sequence of ethanol concentrations of 35%, 50%, 75%, 95%, and 100% for 10 mins each. The ethanol fraction was removed, and the pellet was dried in hexamethyldisilazane (HMDS). Upon discarding the HMDS, the pellets were maintained in a desiccator overnight. The mycelia were eventually examined using a scanning electron microscope. The SEM examination was carried out in a low vacuum with an HV of 5.00 kV [10].

3.0 RESULTS AND DISCUSSION

3.1 Fungal Organism

Daldinia starbaeckii is a novel fungal species belonging to the division *Ascomycota* in the fungal kingdom. The identification is depicted in Figure 1.

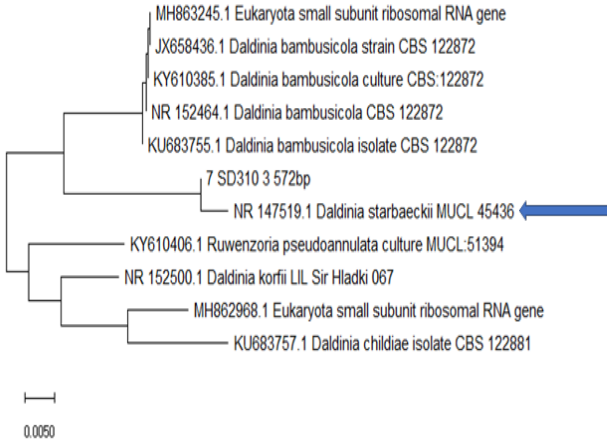

Figure 1 The evolutionary relationship of the fungus. The analysis was carried out using MEGA X

3.2 Heavy Metal Tolerance Assay

In Figure 2a, a decreasing order of growth was observed from the control (0 mg/L) to the highest concentration (40 mg/L). Even though there was a decrease in the radial growth, however, for each metal, the growth was maintained at some stages of the concentrations. For instance, under Cu exposure, the fungal growth was maintained at 30 and 40 mg/L, meanwhile, under Zn concentrations, after a slight growth decrease from the control, there was no change in the radial diameter between 20 and 30 mg/L. However, a further increase in Zn level (40 mg/L) leads to further inhibition of the growth. On the other hand, a strong resistance was demonstrated towards Cr. This is from the viewpoint that inhibition was only observed from 0 mg/L to 10 mg/L, beyond that, the fungus resisted exposure till 40 mg/L. The overall growth diameter ranged from 5.1 cm at 40 mg/L for Pb to 8.0 cm at 0 mg/L for Cr, likewise, the maximum radial growth diameter at the highest concentration (40 mg/L) was 7.6 cm for Cr. Statistical analysis revealed a significant difference in fungal growth on most metal-amended medium ($P < 0.05$). However, the exception was between Zn and Pb ($P = 0.733$). The tolerance disparity exhibited by the fungus towards the metals may be connected to the properties of each metal and their behaviors toward the fungus. This is on the basis that heavy metal tolerance by microorganisms depends on ionic types that associate with extracellular proteins, chitins, polysaccharides or cell exterior [53].

On the other hand, *D. starbaeckii* had a tolerance index ranging from 0.7 to 1.0, with the highest reported against Cr (Figure 2b), this signifies a considerable tolerance against the heavy metals. This assertion is based on the proposed tolerance rating index by [45]. The tolerance attributes displayed by the fungus to the metals can be related to the release of the antioxidant enzymes (POD, APX, and CAT) by the fungus which has been documented to lower the toxic effect of metals against microbes. Supporting literature has shown that at an elevated concentration of Cu, *R. mucilaginosa* maximized the rate of CAT and superoxide dismutase (SOD) production to alleviate the oxidative stress due to Cu concentration [54]. This is in agreement with the previous results which affirmed that CAT, POD, and SOD were all found to protect fungal cells from heavy metal-induced stress [55]. Current findings are further supported by other related findings [37, 56, 57]. Furthermore, many antioxidant enzymes that can remove ROS and their derivatives, repair damages induced by heavy metals, and detoxify metals have been reported in fungi [58, 59]. Other supporting facts for the tolerance are related to the opinion that isolated microbes from environments naturally polluted with heavy metals are sometimes resistant to multiple metal pollutants because of their adaptation to such conditions [21]. In the same vein, fungi exhibit various other defense strategies to overcome heavy metal toxicity; such consisting of vacuolar sequestration, metal uptake reduction, chelation by metal-binding polypeptides and proteins, as well as enhanced extrusion [60]. It was reported that cellular responses to heavy metals employed various strategies such as exportation from the cell or compartmentalization into organelles like vacuoles which is one of the most efficient mechanisms of detoxification [61]. Other defense mechanisms that are thiol-mediated involving metallothionine, glutathione, or phytochelatin are also used by fungi to chelate metals and buffer the reactive oxygen species (ROS). For example, metallothioneins can bind to heavy metals for detoxification and storage. Similarly, peptides like phytochelatins and other thiol-containing compounds such as oxidized glutathione, glutathione disulfide, and glutathione have efficient binding characteristics towards heavy metals [62]. These help in rendering the metals less harmful to the fungi. To a wider extent, transcriptional, cytologic, and metabolic modifications performed a substantial function in the tolerance of fungi to metal toxicity [63].

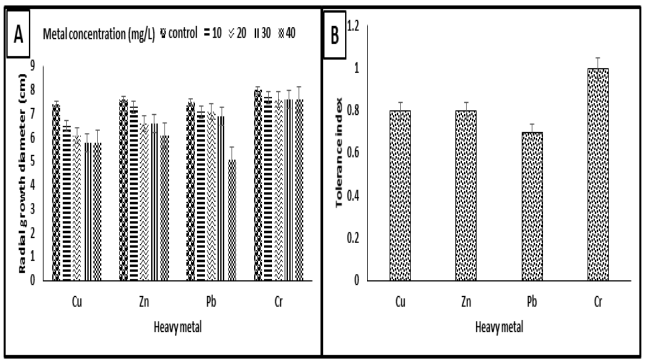

Figure 2 a) Radial growth diameter of *D. starbaeckii* under heavy metal concentration, b) tolerance index of *D. starbaeckii* against the heavy metals

3.3 Antioxidant Enzymes Activity

Antioxidant enzymes play an important role in avoiding oxidative stressors, the majority of which are caused by ROS [64]. The activity of POD increased from 0.08 (at 0 mg/L) to 0.35 U mg^{-1} protein (at 540 mg/L) (Figure 3a). A Similar pattern was witnessed for APX and the least activity was 0.22 U mg^{-1} protein. While the maximum was 2.24 U mg^{-1} protein (at 450 mg/L) (Figure 3b). In the case of CAT (Figure 3c), an initial increased activity was observed from 0 mg/L to 150 mg/L, however at 450 mg/L, a decreased activity was noticed, and the range was 0.02 to 1.05 µmol H_2O_2 min^{-1} mg^{-1} protein. The findings highlighted that the up-regulation of APX at 450 mg/L surpassed that of POD and CAT.

The findings revealed that the antioxidant enzymes were very active. This can be likely due to the metals releasing ROS (hydrogen peroxide, hydroxyl radical, etc.), which may have prompted the fungus' protective reaction. *D. stabaeckii* was able to produce substantial levels of antioxidant enzymes, which may have contributed to scavenging ROS and minimizing the destruction of nucleic acids, proteins, and cell membranes [65]. For example, the isolate's interaction with metals may have caused the build-up of H_2O_2, which could have boosted POD and CAT. The enzymes converted the generated H_2O_2 water and other compounds. This assumption is confirmed by [66] and [67], which state that an elevated level of metals can cause the creation of ROS and free radicals, causing significant destruction to many components of cells. As a result, the equilibrium between ROS generation and scavengers may be skewed, resulting in excessive generation, also known as oxidative stress at such elevated metal concentrations [68, 69]. On the contrary, [70] stated that excessive ROS generation may be the cause of increased entire protein and antioxidant enzyme activities (CAT, SOD, and POD). Comparably, [69] revealed that antioxidant systems like SOD and CAT reduced oxidative damage and improved cell tolerance. The build-up of antioxidant enzymes following cell interaction with heavy metals has also been described [71-73]. In a similar vein, the present findings are supported by [46]'s conclusion that the elevated activity of antioxidant enzymes at elevated concentrations is related to enhanced ROS generation or overexpression of antioxidant-coding genes.

In another finding, CAT activity was shown to be reduced at 450 mg/L, which may be explained by the argument that at 450 mg/L, the created H_2O_2 may be above that of the CAT's tolerance level [73]. The decreasing activity of CAT at elevated levels is consistent with [70], who discovered that CAT's activity declined from 30% to 10% at 75 ppm with increasing contact time. Likewise, this is centred on the belief that POD and CAT have distinct propensity levels for transforming H_2O_2 to oxygen and water [65, 74]. It is assumed that the higher CAT, POD, and APX in the present study indicated the reduction of oxidative stress produced by the metals.

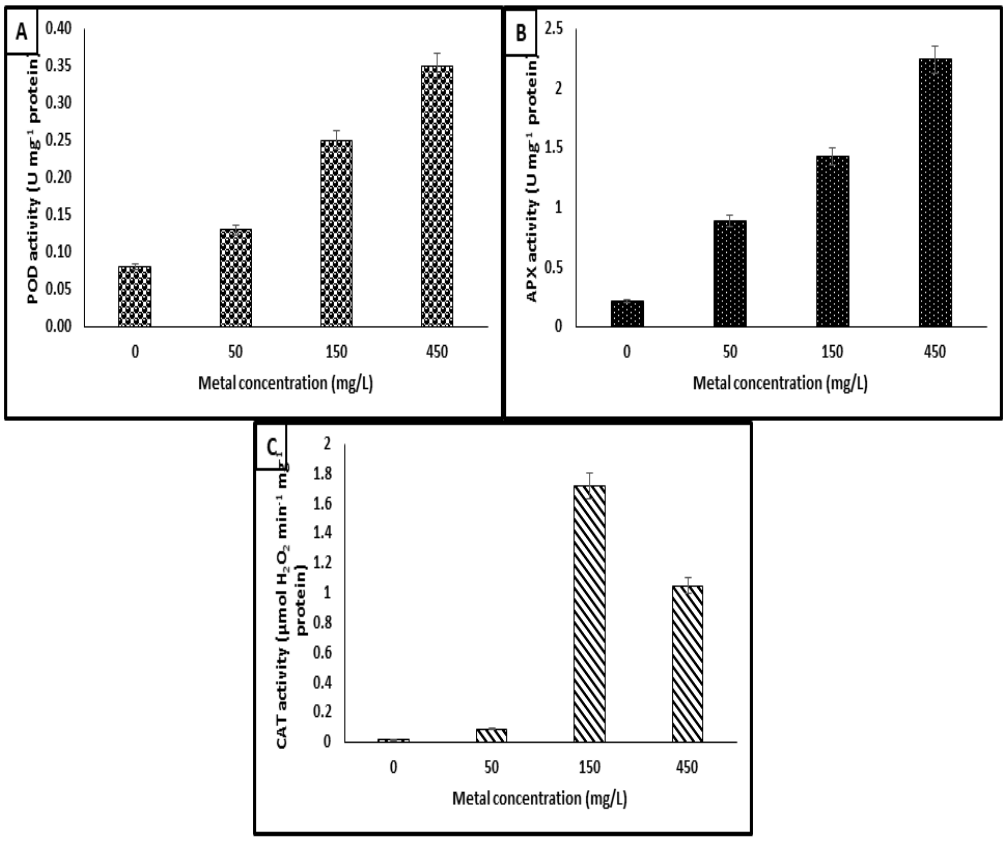

Figure 3 a) POD activity, b) APX activity, c) CAT activity of *D. starbaeckii* in response to interaction with a consortium of heavy metals (Cu, Zn, Pb, and Cr)

3.4 Heavy Metal Biosorption

3.4.1 Effects of Metal Concentration on Biosorption

In Figure 4a, an increasing trend of biosorption was noticed from the control (0 mg/L) to the highest concentration (450 mg/L) for all the metals.

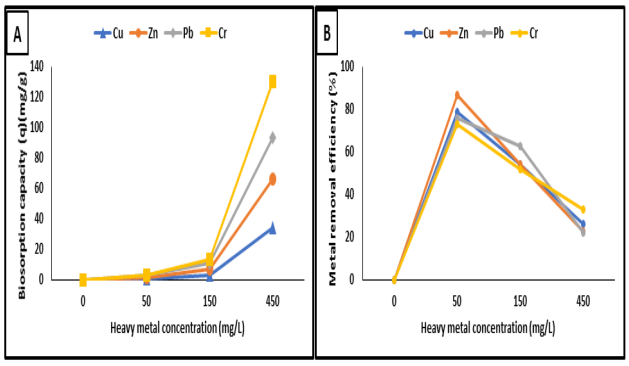

Figure 4 a) Effect of metal concentration on biosorption, b) Heavy metal removal efficiency of *D. starbaeckii* under various metal concentrations

No biosorption was recorded for the control experiments. The biosorption recorded in heavy metal amended broth medium ranged from 0.45 mg/g to 36.77 mg/g for Cu, Zn, Pb, and Cr. The lowest biosorption capacity (0.54 mg/g) was at 50 mg/L for Cr, and the maximum (36.77 mg/g) was recorded at 450 mg/L for Cr. However, in Figure 4b, the metal removal efficiency of the fungal biomass followed the opposite trend to that of Figure 4a with the maximum removal efficiency (87%) at 50 mg/L and the least (22%) at 450 mg/L. Contrarily, the maximum removal achieved was for Zn while the least was for Pb.

The boost in biosorption due to higher metal concentration indicated that the higher metal concentrations offered a greater accelerating power to conquer the mass transfer resistance of metal ions between the solid and aqueous phases, leading to contraction between the biosorbents and the metal ions [75-78]. It has been shown that at elevated metal levels, the amount of ions adsorbed on biosorbents is greater than at low concentrations when binding sites are readily available [79]. Contrarily, because the pH of the broth medium was acidic (5.0), the metals may have been bioaccumulated within the cells. Some of the metals may have been adsorbed by the fungus via the cell plasma membrane and into the cytoplasm, where they bind to cell organelles. This argument can be based on the fact that a low pH increases metal solubility, which promotes metal mobility through the plasma membrane and subsequent accumulation

into the fungus' cells [81, 82]. Additionally, [83] believes that pH could impact the metal-fungal interaction by affecting cell metabolism, metal speciation, and physiology, implying that fungal biosorption could eliminate metals via acid secretion, increasing metal solubility in the medium [83].

The present results are comparable with those of [84], who showed that the biosorption capacity of *A. flavus* increased from 20.75 to 93.65 mg/g for Cu and 3.25 to 172.25 mg/g for Pb at 200 to 1400 ppm. Nevertheless, the maximal removal efficiency measured in this study is less than that of [85]. Contrarily, variations in metal removal can be explained by a variety of factors, including metal toxicity to the fungus, atomic mass, electronegativity, ionic or atomic radius, and the existence of anionic functional groups on the fungal surface, all of which affect the entire efficiency of metal biosorption [10]. The present findings are corroborated by [86], who discovered that Pb biosorption was greater than that of Cd and Cu largely as a consequence of Pb's bigger ionic radii when contrasted to other metals. Similarly, [87] revealed that *Rhizopus arrhizus* removed more Cr than other heavy metals (Cd and Cu). Additionally, [88] demonstrated that *T. atroviride* uptake was stimulated, as evidenced by a doubling of some metal uptake in a three-metal system experiment.

3.4.2 Effects of pH on Biosorption

The solution pH has an impact on both physiological and metabolic activity. Figure 5a shows the biosorption capacity of *D. starbaeckii* under the influence of pH ranging from pH 7 to pH 8, and a clear distinction was noted between the pH values, in which an increasing pattern of biosorption was observed with the increasing solution pH. The ranges of the biosorption are as 0.96 – 1.68 mg/g for Cu, 1.23 – 1.58 mg/g for Zn, 1.52 – 1.65 mg/g for Pb, and 1.37 – 1.60 mg/g for Cr. In Figure 5b, the lowest (43%) and highest (76%) metal removal efficiencies were observed at pH 4.5 and pH 8, respectively, and were all for Cu. Meanwhile, the maximum removal efficiencies were in the order Cu > Pb > Cr > Zn. The increasing trend in biosorption witnessed with the increasing pH was in line with much existing literature [19, 90-92]. The finding of raised biosorption at higher pH could be explained by the physicochemical interactions between metal cations and anionic functional groups [33, 55]. In addition, metals can attach to fungal cell walls or create precipitates like phosphates, oxalates, or sulfates [62]. As a result, with raised pH, H^+ dissociates from the active sites, allowing cationic metals to associate with the free sorption sites [92], resulting in increased biosorption of metal pollutants. Moreover, it was noted that nitrogenous groups, phosphates, hydroxyls, carboxyl, etc. which are the principal sites that can bind to heavy metals, will become fully carboxylic acids or partially amine and phosphate groups when pH increases [93]. This will result in greater binding of the cationic metals.

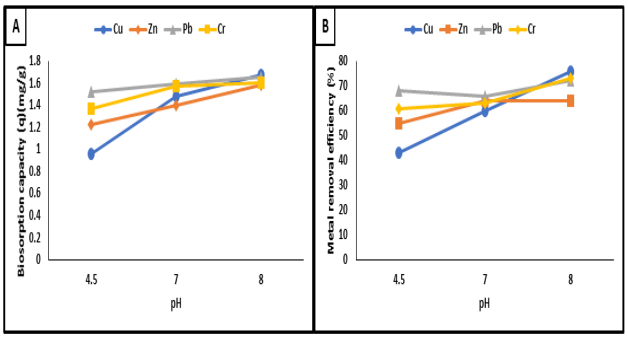

Figure 5 a) Effect of pH on metal biosorption, b) Heavy metal removal efficiency of *D. starbaeckii* under various pH

On the contrary, at an initial pH of 4.5, intracellular metal precipitation/accumulation may have occurred. This is partly because, at lower pH, excessive production of H^+ at the sorption sites by a significant amount of produced protons may have eventually reduced the negative charge on the binding sites, resulting in a decreased or full blockage of metal binding. This could result in the dissolution of metals, which are then absorbed into the cell by the activity of a plasma membrane enzyme known as H^+-ATPase, which generates an electrochemical gradient that aids metal entry [22, 94]. Also, previous studies have connected low pH to inadequate ionisation of acidic functional groups in the fungal cell wall, as well as a weak complex attraction between fungal cell walls and metal ions [95, 84].

3.4.3 Effect of Contact Time on Biosorption

Time profile for the biosorption of Cu, Zn, Pb, and Cr by *D. starbaeckii* revealed an initial rapid increasing trend with an increase in hours. The biosorption ranged from 1.0 to 2.74 mg/g within 24 to 120 hours. There was no biosorption at the initial phase of the experiment (0 hours), however, as the duration increased to 72 hours, the biosorption increased for all metals except Cr (Figure 6a). This suggests that at the initial phase, there was a lack of enough mycelia to remove the metal ions from the media, however, with an increase in mycelial generation, more biosorption sites were available on the fungal cell wall, and this has led to the increased biosorption of the metals. Further, the increase in contact duration to 96 hours and beyond, has led to a marked decline in the biosorption, even though, the metals were biosorbed, however, they were biosorbed minimally to the level that the difference in the biosorption at 96 hours and 120 hours (the longest duration) was negligible. This decline in biosorption at extended duration can be associated with the fact that the binding sites were gradually becoming occupied to the level beyond further sorption, this highlighted that

an equilibrium has been approached [92]. Contrarily, the removal of the metals varies depending on the metal type. For instance, Cu was maximally biosorbed (2.74 mg/g) compared to the other metals. This might be attributed to the surface chemistry of the fungal biosorbent and to the chemistry of the metals in the solution [33]. Meanwhile, the removal efficiencies followed an increasing pattern that ranged from 7% – 80% (Figure 6b). The lowest removal (7%) was recorded for Pb at 24 hours, while 80% removal was for Zn at 120 hours.

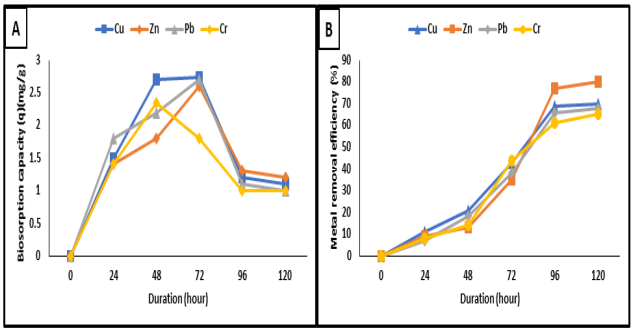

Figure 6 a) Effect of contact time on metal biosorption, b) heavy metal removal efficiency of *D. starbaeckii* in respect to different contact times

The enhanced removal efficiency witnessed was in line with the longer contact time resulting in the increased generation of mycelia as observed in the results which ultimately resulted in higher metal removal due to the available binding sites on the extended surface of the fungal mycelia [42, 96]. Considering the trend in the current results, it is presumed that these findings verify two phases of biosorption (surface bioadsorption and intracellular bioaccumulation) as explained earlier. Our assertion can also be supported by [97] who stated that first the stage of metal removal is completely reliant on bioadsorption to the cell wall.

3.5 Adsorption Isotherm

An adsorption isotherm provides significant details on binding affinity, adsorption capacity, and biosorbent surface features, all of which aid in determining the process of adsorbate binding with biosorbent or adsorbent. Two adsorption isotherms (Temkin and Freundlich) were employed to clarify the balance of the adsorption features. The Freundlich isotherm model is appropriate for a multilayer adsorption procedure with a non-uniform distribution of heat and affinity across a heterogeneous surface [98]. Equation 4 shows Freundlich's linearized equation.

$$logQe = InK_F + \frac{1}{n} logCe \quad (4)$$

K_F denotes adsorption capacity, 1/n denotes adsorption intensity, n denotes adsorption intensity constant, C_e denotes equilibrium concentration (mg/L), Q_e denotes the quantity of metal ions adsorbed at equilibrium (mg/g), and 1/n is calculated by plotting $LogQ_e$ versus $LogC_e$. 1/n defines the kind of isotherm: irreversible if $1/n < 0$, favourable if $0.1 < 1/n < 0.5$, and unfavourable if $1/n > 2$ [99]. The Temkin isotherm takes into account the indirect interaction of adsorbate and adsorbent in the adsorption process. In this model, it is believed that the adsorption heat of all molecules in the layer decreases linearly as surface coverage increases [100, 101]. Equation 5 provides the linear version of Temkin's isotherm.

$$Qe \frac{RT}{b} InA_T + (\frac{RT}{b}) InCe \quad (5)$$

Q_e is the adsorption capacity at equilibrium (mg/g), C_e is the equilibrium concentration (mg/L), b is the Temkin isotherm constant (J/mol), A is the Temkin isotherm equilibrium binding constant (L/g), R is the universal gas constant (8.314 J/mol/K). T is the absolute temperature (K). The slope $(\frac{RT}{b})$ and intercepts $(\frac{RT}{b})$ InA_T of the Q_e versus InC_e plot were used to compute R_T/b and A_T.

The linear plots for Freundlich and Temkin isotherms are presented in Figures 7a-d and Figures 8a-d, respectively. For the Freundlich isotherm, the values for 1/n, K_F, and R^2 ranged between 1.2913 – 1.7117, 0.2035 – 0.4110 mg/g, and 0.9876 – 0.9895, respectively. The order of magnitude of K_F for the metals is Pb < Zn < Cr < Cu. Meanwhile, in the case of Temkin isotherm, the ranged values for B_T, K_T, and R^2 were 10.5908 – 18.1314 J/mol, 0.0239 – 0.0473 L/mg, and 0.8476 – 0.9551, respectively (Table 2). Based on the obtained data, Freundlich isotherm best described the uptake of Cu, Zn, Pb, and Cr onto the biosorbent (fungal mycelia). This is for the fact that the values for the linear regression coefficient (R^2) of Freundlich were greater than those of Temkin isotherm for all metals. This suggests that the adsorption was onto a heterogeneous surface with a non-uniform dispersion of affinity and heat. In addition, since the 1/n was less than 2, the adsorption was favourable.

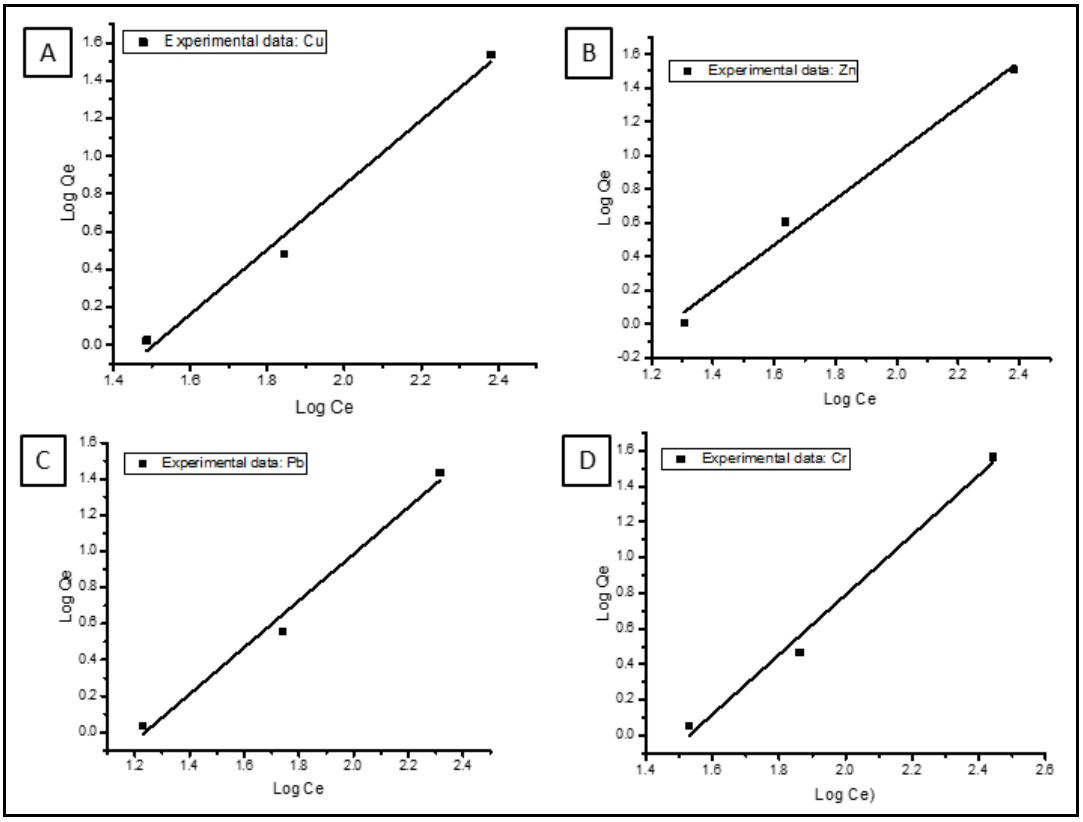

Figure 7 Linearized Freundlich isotherm for a) Cu, b) Zn, c) Pb, and d) Cr biosorption by live fungal mycelia at concentrations of 50 mg/L, 150 mg/L, and 450 mg/L. The contact temperature was 28 °C, contact duration was 96 hours, and the pH was pH 5.0

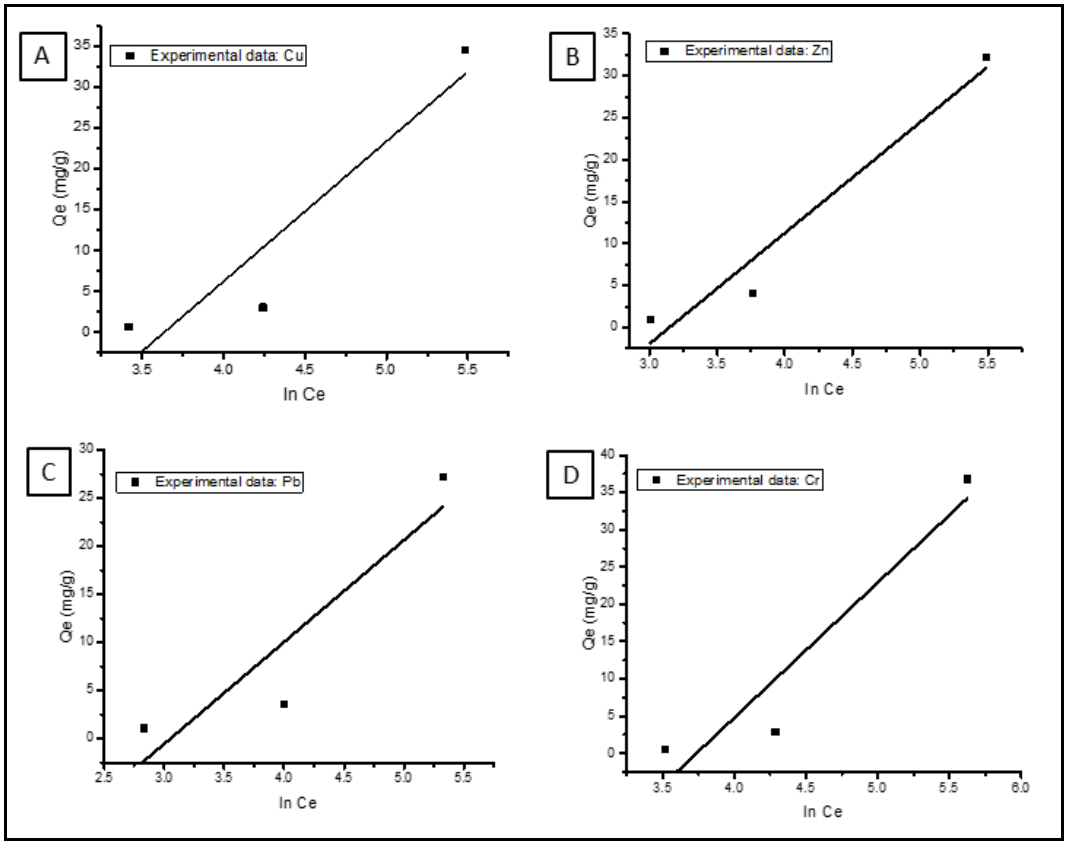

Figure 8 Linearized Temkin isotherm for a) Cu, b) Zn, C) Pb, and d) Cr biosorption by live fungal mycelia at concentrations of 50 mg/L, 150 mg/L, and 450 mg/L. The contact temperature was 28 °C, contact duration was 96 hours, and the pH was pH 5.0

Table 2 Calculated parameters from Freundlich and Temkin adsorption isotherm models

Model	Index	Heavy metals			
		Cu	Zn	Pb	Cr
Freundlich isotherm					
	1/n	1.7117	1.3579	1.2913	1.6785
	N	0.5842	0.7364	0.7744	0.5958
	K_f (mg/g)	0.4110	0.2310	0.2035	0.4093
	R^2	0.9882	0.9889	0.9876	0.9895
Temkin isotherm					
	B_T (J/mol)	17.1509	13.2031	10.5908	18.1314
	K_T (L/mol)	0.0263	0.0430	0.0473	0.0239
	R^2	0.8856	0.9551	0.8476	0.9072

3.6 Kinetics Study

The first-order kinetic equation (Equation 6) was used to calculate the pace of metal removal from the liquid medium.

$$K = -\frac{1}{t}\left(In\frac{Ce}{Ci}\right) \quad (6)$$

K stands as the first-order kinetic rate constant, C_e stands as equilibrium metal concentration (mg/L), C_i stands as initial metal concentration in the medium (mg/L), and *t* = contact time (hours). The half-life of a biosorption data was calculated using Equation 7.

$$\text{Half-life } t_{1/2} = \frac{In(2)}{K} \quad (7)$$

The results for the metal biosorption were further supported by determining the kinetic variables of the metal removal. The kinetic parameters are contained in Table 3. The metal removal rate constant ranged between 0.0088/hour to 0.0133/hour for all the metals. Meanwhile, the corresponding half-lives were in the range of 52 to 79 hours. The maximum K value (0.0133/hour) was noted for Zn, followed by 0.0100 for Cu. Contrarily, Cr had the maximum half-life, followed by that of Pb. These values implied that Zn and Cu would be bioremoved faster looking at the fact that they had lower half-lives as compared to Cr and Pb. The variation in the metal removal might likely be from the discrete ability of the fungus to remove the metals [102]. Current results varied from those reported by [103], in which the rate constant and the half-life reported by [103] were lower and higher, respectively than those of the current research. Variations in experimental settings, type of metals, type of microorganisms, and concentrations of metals might have been the reasons for the variation in the results.

Table 3 Kinetics parameters and half-life of bioremoval of heavy metals by *D. starbaeckii* for 120 hours

Metal	K(hour^{-1})	R^2	$t_{1/2}$ (hours)
Cu	0.0100	0.979	69
Zn	0.0133	0.883	52
Pb	0.0095	0.981	73
Cr	0.0088	0.964	79

On the other hand, linear regression analysis was performed to fit Equation 4 with the help of the origin software 2015 SR2 version b9.2.272. The findings revealed that the first-order kinetic model fitted well with the kinetic data with high coefficients of determination (R^2 = 0.979, 0.883, 0.981, 0.964 for Cu, Zn, Pb, and Cr, respectively) (Figures 9a-d). Furthermore, the results are supported by Fisher's F-statistics with the F values as 90.36, 38.57, 102.14, and 103.18 for Cu, Zn, Pb, and Cr, respectively. Current findings are in concordance with those recorded by [104].

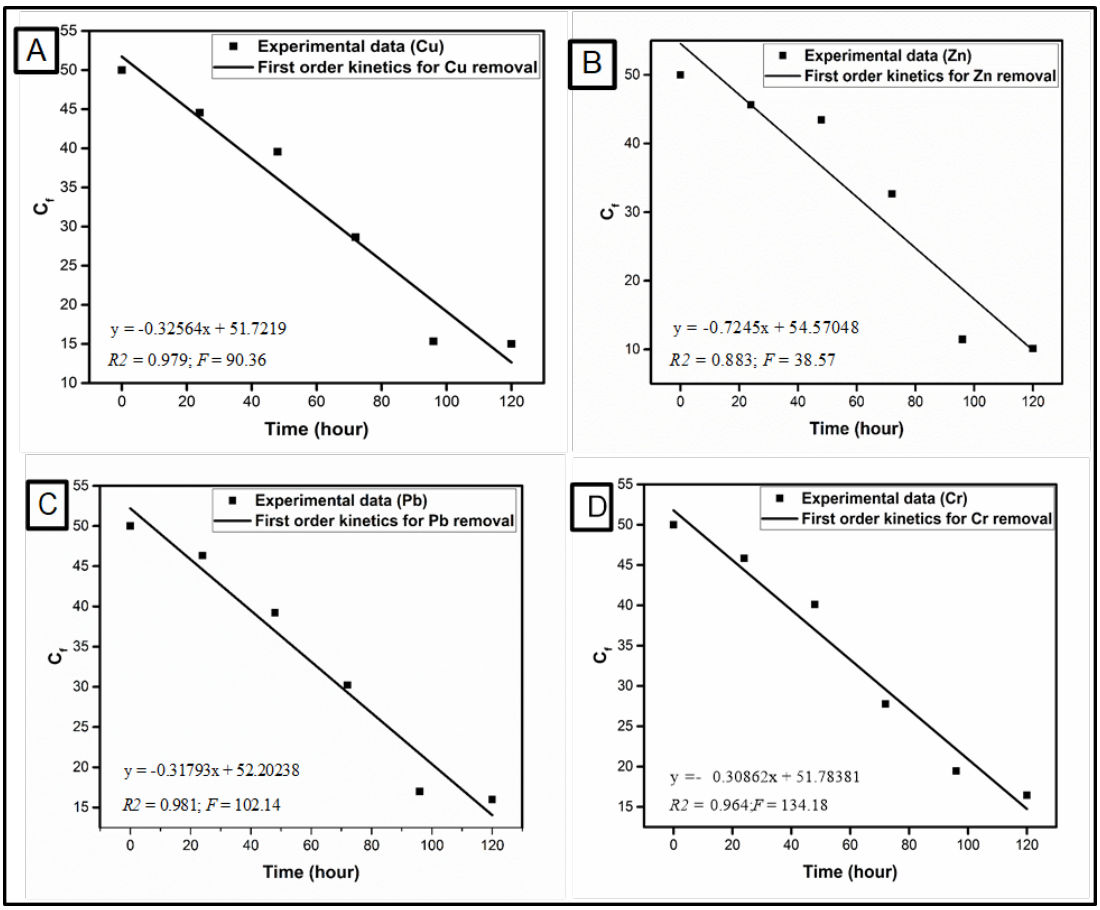

Figure 9 Linear plots of first order kinetic model for bioremoval of a) Cu, b) Zn, c) Pb, and d) Cr by *D. starbaeckii*

3.7 SEM

SEM images revealed a strong contrast between metal-accumulating fungal mycelia (Figure 10a) and control mycelia (Figure 10b).

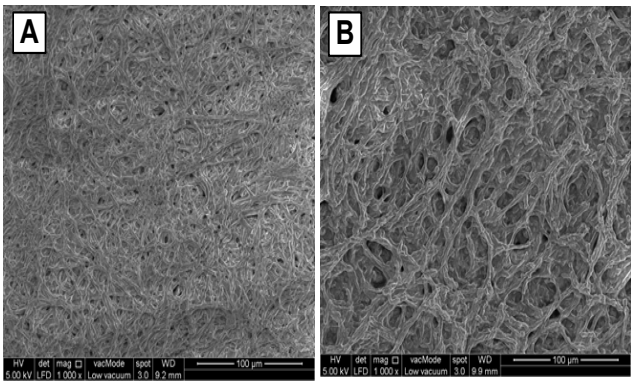

Figure 10 a) SEM image of the multiple metal-treated mycelia, b) SEM image of the metal-free mycelia

Multiple metal-amended fungal mycelia showed a distorted, densely packed appearance with shorter lengths. Individual hyphae were also observed to loop and twist. In the meantime, for control, elongated ribbon-like hyphae that are big and loosely packed were seen. The dense mycelia and rough exterior in the metal-amended mycelia indicated efficient adsorption. Nonetheless, the stunted mycelia may suggest amalgamate metal toxicity, which activated a defense mechanism by the fungus. As stated by [105], under the circumstances of metal exposure, agglomeration of fungal hyphae can be used to tolerate harmful pollutants like heavy metal via reducing the fungal hyphal surface area. Supporting observations by [106] found twisting and looping of hyphae at high metal concentrations. In a comparable finding, [85] discovered similar effects, as well as the creation of interconnected hyphal structures in multi-metal-treated fungal hyphae.

4.0 CONCLUSION

The findings showed that *D. starbaeckii* had the greatest tolerance index of 1.0. The maximum biosorption of 36.77 mg/g occurred at the maximum concentration (450 mg/L), whereas the greatest removal efficiency was 87%. The removal efficiency was shown to decrease with increasing metal content. Biosorption was observed to rise with

increasing solution pH. The data fit well to the Freundlich isotherm model, with the greatest R^2 value of 0.9895. Additionally, the data was best represented by the first-order kinetic model, which had the maximum R^2 of 0.981. It concludes that *D. stabaeckii* can tolerate the toxicity of several metals and remove them from the aqueous media. As a result, it could potentially be used in the treatment of wastewater contaminated with many metals.

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Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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