Wastewater treatment and Mycoremediation by *P. ostreatus* mycelium

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Abstract

Wastewater from industries contains a considerable amount of chemicals and is characterized by high biological oxygen demand (BOD), heavy metals, intense color and are thus toxic to the environment. White-rot fungi such as Oyster mushrooms are comprised of high biomass and various degradative enzymes that add to another dimension of use, such as mycoremediation. The mushroom was maintained *in vitro* in mycelial form. Remazol Brilliant Blue R (RBBR), Methyl Red (M.R.), and Malachite Green (M.G.) dyes, which are recalcitrant dyes that are often used for the preparation of many polymeric dyes, and textile dyeing, were used to test the decolorization potential of natural isolates of oyster mushroom. Mycelium was grown in the presence of RBBR, MR, and M.G. at a concentration of 100µM. It was found to decolorize more than 70%, 95%, and 80% of RBBR, MR, and M.G. dye, respectively, within 6 days. Mushroom mycelium was also tested for its lead tolerance and heavy metal biosorption potential. Lead tolerance was studied up to 500mg/L, which shows species' latent growth with fungal morphology changes. Biosorption ability was observed by the use of Inductively Coupled Plasma spectrometry, which shows lowered concentrations of heavy metals (Pb, Cr, Ni) after 5 days of mycelial growth in the presence of these metals. The isolated strain was also found to have significant decolorizing and degrading potential for synthetic dyes and phenolic compounds in industrial effluents. This study focuses on edible oyster mushrooms' ability to develop environmentally less aggressive processes in the treatment of industrial effluents.

Keywords- oyster mushrooms, heavy metal biosorption, wastewater treatment, dye decolorization, mycoremediation, fungi.

1. Introduction

White rot fungi are known for their outstanding ability to produce extracellular oxidative enzymes, which can be used to deteriorate a wide variety of materials and compounds that contaminate the environment. [1][2] Treatment of our water bodies has always been a challenge. It requires physical labor and economic input, and it is essential to find a natural remedy for the cause.

Mycoremediation stems from my (Greek for fungus) words and remediation (reversing environmental damage), a term used to describe fungi' use in degrading contaminants and toxins from the environment. Environmental cleaning up by using practices related to bioremediation yields remarkable results. [3] There are various bioremediation processes amongst which mycoremediation is one such step that is important in treating contaminated soils and wastewaters, thus restoring naturally occurring habitats [4][5]. Mycoremediation has plenty of advantages over other cleaning practices; naturally occurring, non-toxic, requires zero maintenance, recyclable, profitable, less invasive, labor unintensive, and secure. One of the edible mushrooms that can also be used industrially for mycoremediation purposes is the oyster mushroom. [6][7][8] Oyster mushrooms work for the environment by decomposing the dead logs, thus revitalizing the soil and returning minerals to the environment in a reusable form.

During these times, the success load of the industrialization boom is suffered by our water bodies. Though these bodies are considered a renewable energy source, grueling conditions wouldn't allow them to be the same for long. Industries discharge millions of liters of untreated effluents, most of which contain recalcitrant dyes such as azo-dyes. [9]–[11] Wastewater treatment methods, namely filtration, activated carbon, specific coagulation, and chemical flocculation, have been used and are found to be effective but are quite expensive. Flocculation and adsorption require different treatments before disposal, and they also generate large amounts of sludge and waste, thus incurring more labor and cost.[4][12] Synthetic dyes have antroquinoid, indigoid, and azo aromatic structures that are not easy to degrade. Biological systems can bring about the degradation of the target chemicals primarily due to their enzymes, highly specific, and extremely efficient catalysts. [13], [14] Enzymes are being investigated as means of effluent cleaning as they can significantly reduce target toxic chemicals without affecting the other useful materials in the effluent.[15], [16] Hence, treatments for such discharge containing vast amounts of the recalcitrant materials and enzymatic methods are most favorable. Edible mushrooms can be employed to evolve remediation processes less vigorous to the environment than traditional chemical techniques. [17]. Even in low volume and different reaction conditions, their versatility and efficacy give them an edge over other treatment procedures.

2.Material and methods

2.1 Materials

Oyster Mushrooms were collected from barks of trees and dead wooden logs from the Indian Institute of Technology, Varanasi campus, easily from April to November. The dyes Malachite green and Methyl red were procured from Hi-media, India. Textile dye, Remazol brilliant blue R, was bought from Sigma. PDA was purchased from SRL, India.

2.2 Isolation and cultivation of mushroom mycelia

Oyster mushrooms were collected from the bark of dead trees. The mushrooms were washed in running tap water to remove any dirt, and then they were washed with 0.1% $HgCl_2$ for 1 minute. Mushrooms were immersed overnight in autoclaved water for spore release, and 2-3 drops of water with spores were used to spread onto solidified potato dextrose agar (PDA) plates. Distinct fungal colonies were isolated from plates and characterized on their morphological basis.[18]. The pure strain of oyster mushroom was isolated by taking a square piece of agar from the periphery of the growing mushroom mycelia and keeping it upside down over a fresh PDA plate kept at 28ºC.

The spores of oyster mushroom were also allowed to grow in a liquid medium – potato dextrose broth (PDB) by transferring a small PDA piece containing mycelial growth.[19][20] PDB preparation was done by boiling sliced potatoes approximately 20g in weight in distilled water for 30 minutes, then

filtering it through a muslin cloth, making up the volume to 100 ml, and dextrose (2g) added as a carbon source.

2.3 Decolorization of synthetic dyes

Decolorization of dyes in liquid medium was identified by loss of pigment from the liquid medium. Dye decolorization in the broth was measured using a spectrophotometer in the wavelength range of 200–800 nm and calculating the absorbance corresponding to the dye's absorbance wavelength maxima. All reactions and control samples that contained no dye were done in triplicates and incubated at 28º C for 6 days. The percentage of decolorization was noted by monitoring the decrease in its optical density and was determined by using the formula:

$$D = 100(A_{in} - A_{obs})/A_{in}$$

D is decolorization (in %), $A_{in,}$ initial absorbance, and A_{obs}, observed absorbance.

50mg/L stock solution of the dyes Remazol Brilliant Blue R (RBBR) [21][22], Methyl Red (M.R.), Malachite Green (M.G.) were prepared in 10ml of water or DMSO[3]; [23] 100µM concentration of this stock was added to two flasks of Potato Dextrose Broth media of 250 ml. Experiment Flask had media with 5% mycelial inoculum and dye. The control flask had media with dye but no mycelial inoculum. Absorbance was taken at 595nm, 617nm, 410nm for RBBR, MR, and M.G. respectively at regular time intervals (24 hours) until visible decolorization was observed [24]–[26]. Absorbance was noted and was compared to control samples. Growth of fungus was also calculated in terms of dry mass to know if growth and decolorization were co-occurring.

2.4 Rapid detection of degradative enzymes:

The rapid identification for the presence of enzymes in the supernatant fluid plate assay was performed; different substrates were dissolved in agarose wherein a well was created that contained 50 µl of extracellular fluid. The visible color development around the wells was considered as a positive test for degradative enzymes.

Laccase was detected by taking 25 ml of pure agarose (1%) media that contained 0.5mM of ABTS [2,29-azinobis(3-ethylbenzathiazoline-6-sulfonic acid); Sigma Chemical per ml in 100 mM sodium acetate buffer (pH 3.5) was placed in a clean petri dish. A well was made in the gel with the help of a sterile cork borer; 50 µl of extracellular fluid was pipetted in the well.[27] Similarly, for catechol oxidase and tyrosinase plate assay, the Petri dishes contained pyrocatechol (Sigma –Aldrich) in 0.1M concentration and L-tyrosine (Hi-media) in 0.1M concentration respectively in 100 mM phosphate buffer along with 25 ml of sterile agarose (1%).

2.5 Lead tolerance and heavy metal biosorption

For the evaluation of lead's tolerance, the solid PDA media plate containing 7-day old growing mushroom species was taken, and an agar section was cut out from the margin of the growing fungus, and it was inoculated on similar media that contained 50mg /L Pb. After 7 days of growth, another agar section was cut out from the periphery and was inoculated on a fresh PDA plate containing 100 mg /L Pb. The process was repeated many times (data not shown), and the Pb concentration was raised gradually up to 500 mg/L until the species was incompetent to grow. [6]

In general, white-rot fungi possess great adsorption capacities due to their high biomass for heavy metal removal. Heavy metals used to check biosorption by mushroom species were lead, chromium, and nickel with the maximum permissible limit in drinking water 0.01 mg/l, 0.05 mg/l, and 0.02 mg/l, respectively. The biosorption analysis sample was prepared by making a stock solution of heavy metals (Pb, Cr, and Ni compounds). Lead acetate, Potassium dichromate, and Nickel nitrate hexahydrate were used to prepare stock by dissolving 1.5g of lead acetate, 1.5 g of Potassium dichromate, and 4.05g of nickel nitrate hexahydrate in 1L of distilled water.

2 mL of this stock was mixed with 100ml PDB media, and mushroom species were inoculated with it (5% inoculum). The zero-day sample was kept aside for evaluation, and after 5 days of incubation (28ºC, 200rpm) of mushroom, it was also removed from the shaker and refrigerated for evaluation. [28] All the determinations were done with **Inductively Coupled Plasma** spectrometry (ICP). Standards were prepared by diluting commercially available NIST traceable standard solutions.[29] Before ICP evaluation, fungal biomass was removed by centrifugation and was filtered 4-5 times to remove any fungal cells as it may choke the nebulizer. ICP analysis was carried out by Thermofischer scientific.

3. Results and discussion

3.1 Growth of mushroom mycelia and morphological characterization:

Figure 1 shows fungal growth kinetics in liquid medium (PDB). The fungal hyphae observation was done under the phase-contrast microscope, 40X magnification of (Olympus CKX53) staining was done with lactophenol cotton blue (Fig. 2c). The mushroom collected had a wide oyster-shaped cap with varying (5–25 cm) diameter; naturally occurring oyster mushrooms vary from white to grey and dark-brown in color; the margin is inrolled in a young specimen, and was smooth and often lobed/wavy in older more oversized mushrooms. The mushroom tissue was white, firm, and varied in thickness. The gills of the mushroom were creamish and subsided on the stalk. These observations show that the strain belongs to the oyster mushroom category. A similar type of findings was also made by A. Mishra *et al.* [18]

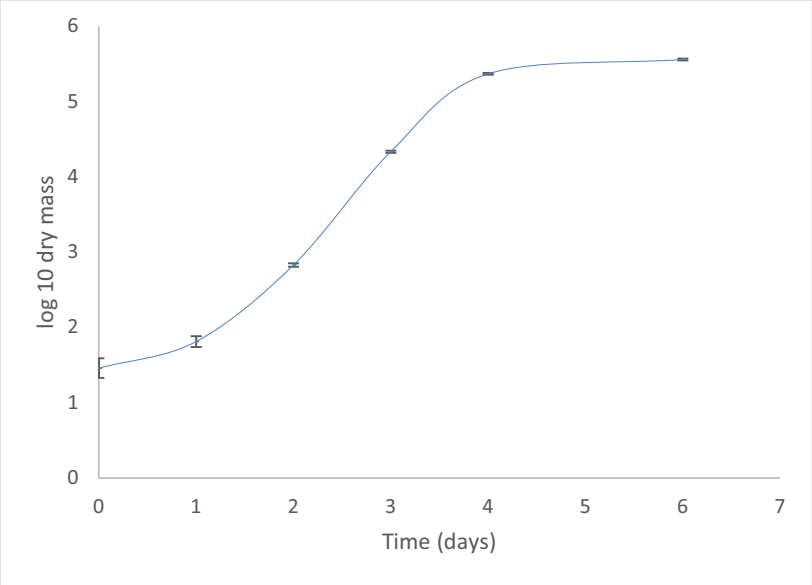


Figure. 1: The growth of mushroom in PDB (liquid medium) at an initial pH 5.8, RPM 220 and temperature 28ºC

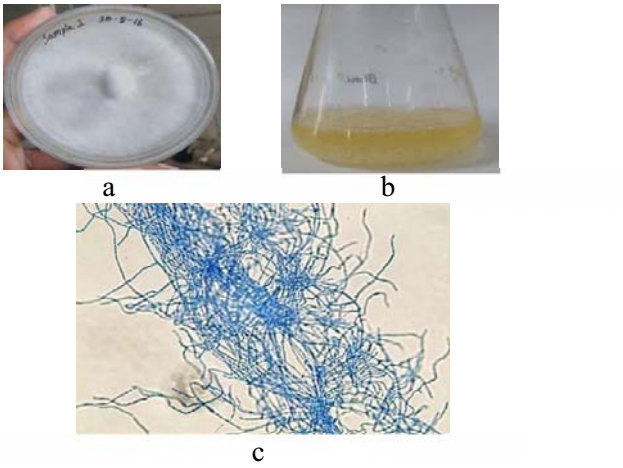


Figure. 2**:** Mycelial growth of mushroom (a) on PDA, (b) in PDB. (c) Fungal hyphae under phase contrast microscope (40X), stained with lactophenol cotton blue.

3.2 Decolorization of synthetic dyes

The results show that mushroom was able to decolorize more than 70% of Remazol Brilliant Blue R [30], 95% of Methyl Red and about 80% of Malachite Green within 5 days at 100µM concentration.[12], [23], [31], [32] [33] After 2 days, no visible changes in color were observed in malachite green; fungus stopped growing in the experiment, which might be because of the antifungal nature of the dye. It was also observed that fungal biomass decreased when inoculated with dye compared to the growth without dye; this might be due to stress to the fungus caused by dye and lowered its growth rate. Malachite green is an antifungal agent, shows a fungicidal effect on mushroom mycelia.[34] Effective decolorization can be observed in 48 hours by fungal mycelia. After that, no observable changes were seen (Fig. 3, 4, and 5).

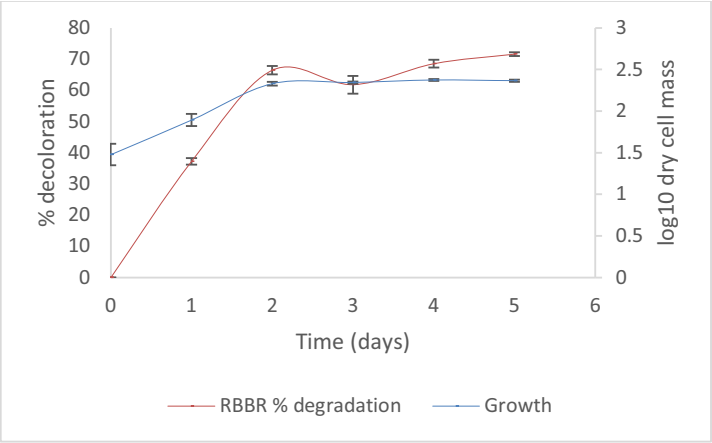


Figure 3: RBBR % decolorization by fungus during fermentation at an initial pH 5.8and temperature 28ºC.

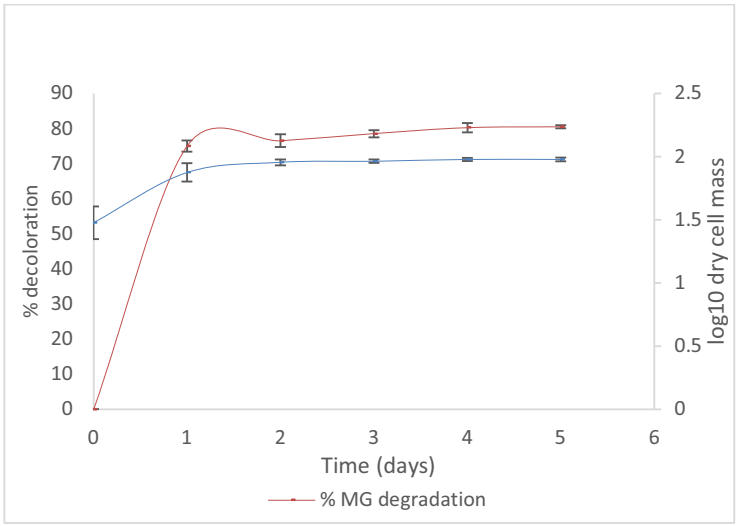


Figure 4: Malachite green % decolorization by fungus during fermentation at an initial pH 5.8and temperature 28ºC.

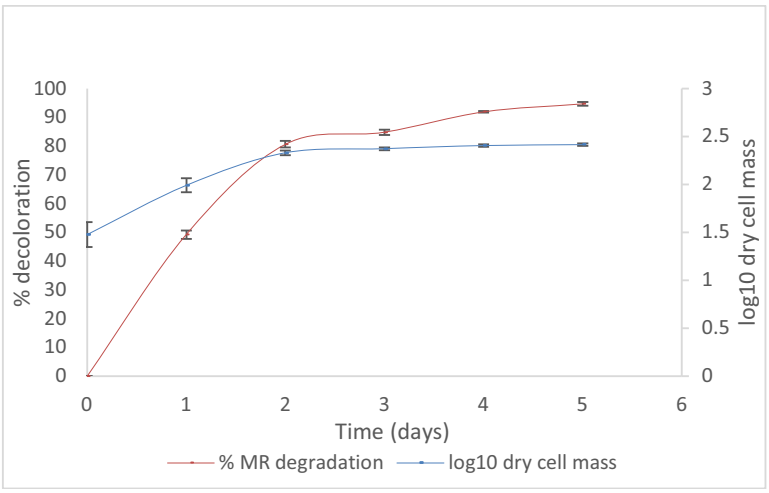
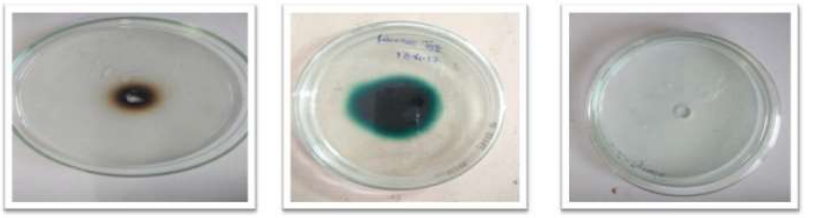


Figure 5: MR % decolorization by fungus during fermentation in liquid culture medium at an initial pH 5.8and temperature 28ºC.

3.3 Rapid detection of degradative enzymes

Figure6 (a) shows the occurrence of brownish color around the well, which is considered a positive test for catechol oxidase enzyme activity[35]; similarly, the development of an intense greenish-blue color around the well of figure6 (b) was considered a positive test for laccase activity.[36] In figure6(c), no zone was observed, and hence it was a negative test for tyrosinase activity. The inference was made from similar findings by Sunil S. Moore *et al.*[27]

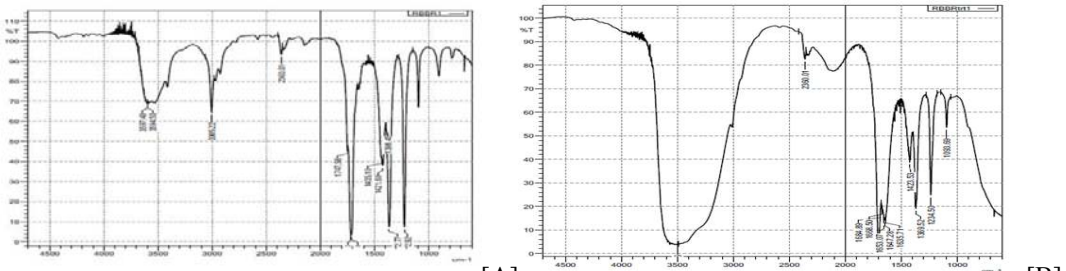


(a) (b) (c)

Figure 6: Rapid Detection assay for enzymes (a) catechol oxidase- positive, (b) laccase- positive, and (c) tyrosinase test –negative.

3.4 FTIR analysis of degradation products

FTIR spectra of the treated RBBR sample (Fig. 7) shows the extinction of C.N. bond (853 cm^{-1}) and CX groups, and a shift in 2150 cm^{-1} N.H. groups stipulate the degradation of RBBR chromophore. 800-600cm^{-1} are compatible with previous studies reporting the deamination and dehalogenation. Broad peak around 3600-3000cm^{-1} indicates N.H. stretching that states the formation of primary and secondary amines.



[A] [B]

Figure.7: The FTIR spectra of control RBBR dye [A] and the fungal growth in the presence of RBBR [B] after 4 days of cultivation in liquid culture medium at 220 rpm, temperature28 ºC and pH 5.8

M.R. sample spectra (Fig. 8) shows the presence of N.H. stretching at 3500 cm^{-1} that accounts for the emergence of 2-ABA, 1643 cm^{-1},1422 cm^{-1} (C.H. makes crooked CH3), 1236 cm^{-1} (O.H. deformation from primary or secondary alcohols), and COH stretching at 1093 and 802 cm^{-1} represents the existence of aromatic structure.[37]

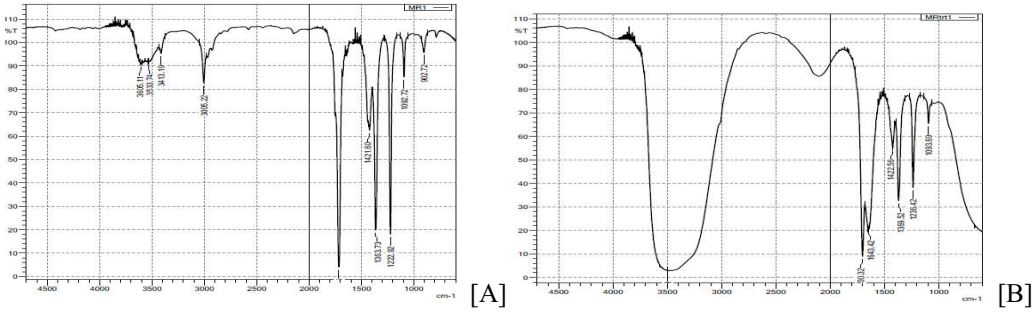


Figure 8: The graph represents the FTIR spectra of [A] control dye MR and the fungal growth in the presence of MR [B]after 4 days of cultivation in liquid culture medium at 220 rpm, temperature 28 ºC and pH 5.8

Also, in treated M.G. samples (Fig. 9), the presence of 1643 cm^{-1} indicates C=C stretching of the benzene ring, 1093 cm^{-1} shows aromatic C.N. stretching. Around 700 cm^{-1} indicates the bending of ring hydrogens. 2358cm^{-1} shows C-H asymmetric stretching and free NH_2 group, and 3425 cm^{-1} have antisymmetric vibration.[28] Results confirm with the findings of Punaveshwari N *et al.* [14] and D.C. Kalyani *et al.* [38] on FTIR studies of dyes degradation and it was reported that the degraded product is non-toxic.

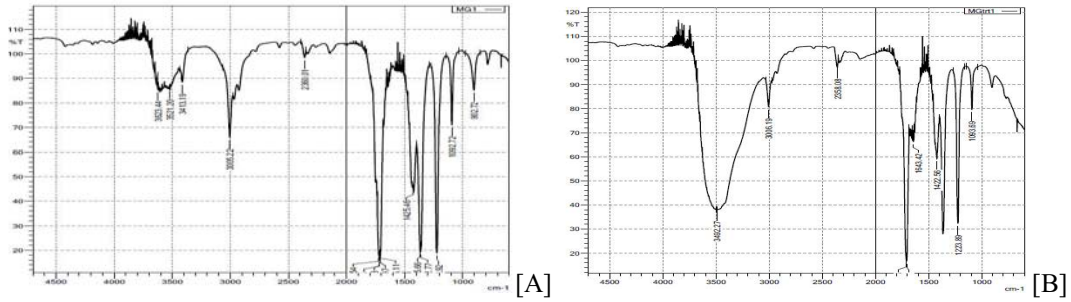


Figure 9: The above graph represents the FTIR spectra of [A] control dye MG and the fungal growth in the presence of MG [B]after 4 days of cultivation in liquid culture medium at 220 rpm, temperature 28 ºC and pH 5.8

3.5Lead tolerance

As the lead concentration was increased, the growth was observed to be latent, but the tolerance to Pb was improved considerably. Some deformations in mycelia structure were also observed. Mycelium, which was high in density and abundant earlier in media containing no Pb, was less dense and scarce in Pb concentration's gradual increase. With increasing Pb concentration, growth of the fungus was ceased. In figure 10 (a) Pb (50 mg/l), growth was observed after 5 days of incubation with dense mycelium covering the whole plate. Figure10 (b) Pb (100 mg/l) having high heavy metal content shows scarce growth with deformities in the mycelium. Figure10 (c), having a maximum Pb (500 mg/l), shows the least growth in 10 days. From the results, it was evident that latent growth in heavy metal presence was due to the fungus acclimatizing itself to the stress and its increasing tolerance to lead.

Figure. 10: Effect of Pb concentration on fungal growth at temperature 28ºC and pH 5.8 after 15 days of incubation.

3.6 Heavy metal biosorption and bioaccumulation

Biosorption by the fungus was studied by growing fungus in heavy metals, namely Pb, Cr, and Ni. Concentrations of heavy metals were determined before and after fungal growth using inductively coupled plasma spectrometry (ICP). [37][28]The following results were obtained Day 0 sample 1, when the fungus was just inoculated into the heavy metals containing medium, shows a higher amount of metals, whereas sample 2, which was taken on the 5th day after the fungus inoculation, shows a relatively lower concentration of metals. Thus it can be inferred that heavy metals were absorbed into the fungal biomass. Biomass was separated by centrifugation, and the supernatant was used to test the remaining heavy metal concentration in it. In Figure11, ICP results confirmed 55%, 25%, 89% of Cr, Pb, and Ni metals were biosorbed respectively by fungal biomass. [29][39]

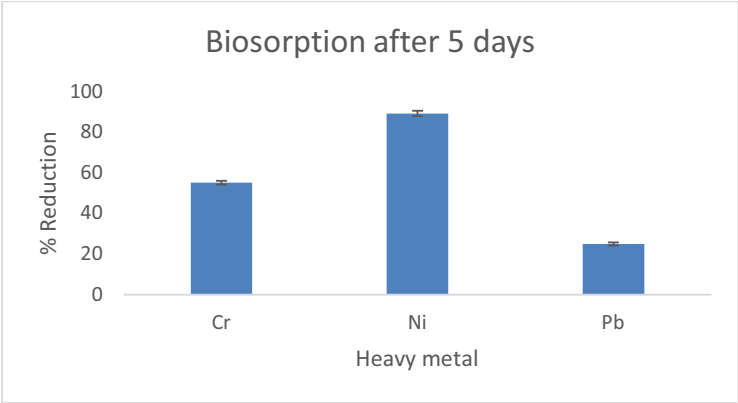


Figure 11: Effect of Heavy metal Biosorption of Pb,Cr and Ni during fungal growth in the liquid medium at temperature 28ºC and pH5.8 and RPM 220.

4. Conclusion

This research focused on economic and widely available edible oyster mushroom sources and studied its potential in dye decolorization, Pb tolerance, and heavy metal biosorption. It displayed positive potential in all of the above. It was observed that this mushroom's working conditions are relatively easy to maintain and economically feasible. The species' culturing and maintenance did not display the requirements for any additional substance to function. Therefore, the potential of using this oyster

mushroom as a sustainable alternative for mycoremediation purposes can be explored in future research. Mushroom species such as *Galerinavittiformis* have been shown to have good multimetal biosorption capability, accumulating heavy metals (Pb, Zn, Cd, Cu, and Cr) from soil. [40] Experiments could be outlined to decipher the tangled mycoremediation processes globally and know how the impact of environmental factors occurs on degradation**.**

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Conflict of interest disclosure statement-

Authors declare no competing financial conflicts of interest.

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