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Introduction

Environments are o en contaminated with both metals and organic toxicants [1]. In such mixed pollution, metal tolerance plays an important role in growth and metabolic functioningof biodegrading microorganisms, and is a desirable trait in bioremediationapplications. Hence, those organisms that can tolerate and grow in presence of metals while degrading the organic toxicants are the potential bioremediation agents of choice. Filamentous fungi have been investigated for their metal tolerance and utilized in bioremediation due to the ease of growing and handling these organisms and their versatility in biodegradation of normally recalcitrant organopollutants. However, little is known on their ability to tolerate heavy metals in presence of organic toxicants. Heavy metals such as cadmium (Cd) and polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene (Phe) , pyrene (Pyr) and benzo(a)pyrene [B(a)P] are commonly occurring toxicants, that are frequently encountered in various contaminated environmental matrices [1]. Bioremediation of suchcontaminated media requires the organism to tolerate both metal such as Cd and PAHs. White rot fungi are known to possess extraordinary ability to oxidize a broad range of organic toxicants. Limited studies have also shown their ability to tolerate certain metals [2]. Our group has focused on the model white rot fungus Phanerochaete chrysosporium for investigating biodegradation of recalcitrant pollutants including PAHs [3,4]. However, metal tolerance of this organism in presence of PAHsin a mixed pollution scenario is not yet understood. In light of the above background, we investigated Cd tolerance of this PAH-degrading white rot fungus. e study involved monitoring of tolerance at varying

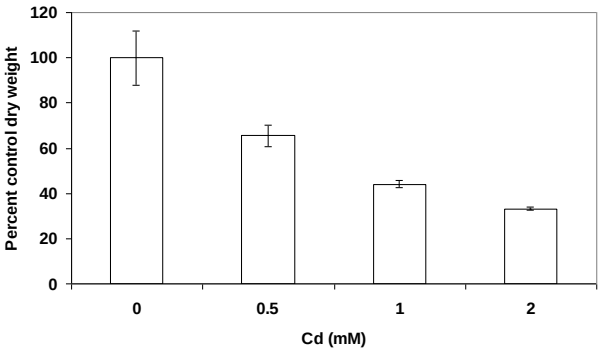

Figure 1a: Cadmium tolerance in Phanerochaete chrysosporium , the fungal cultures were grown at 37°C in an environmental shaker (180 rpm) for 72 hours; Cd was added at variable concentrations at the 24 hour time-point. Fungal E L R P D V V Z D V K D U Y H V W H G E \ Y D F X X P ¿ O W U D W L R Q D Q G G U \ L Q J D W 80°C. Values plotted represent average of triplicates ± standard deviation.

inoculating the culture medium (malt extract broth, pH 4.5) at 10% v/v. e cultures were grown for 24 hours by shaking (180 rpm) at 37°C. For assessing the e ect of metal and/or PAH on the growth, calculated amount of the test xenobiotic were spiked and the growth continued until 72 hours under the same incubation conditions. Mycelial biomass was harvested by vacuum ltration using Whatman No.1 lter paper (Fisher Scienti c, USA) and dried at 80°C for 24 hours or until a constant weight was achieved.

Fluorescence microscopy

Superoxide radicals were detected in the fungal mycelium by uorescence microscopy based on dihydroethedium (DE) staining. Fluorescence microscopy technique was also employed for the live/dead mycelia staining. While staining with 4',6-diamidino-2-phenylindole (DAPI) at 1% revealed all cells (live and dead), the dead cells were selectively detected by propidium iodide (PI) stain (0.5% concentration).

Results and Discussion

Cadmium tolerance of *P. chrysosporium*

Atrend of decreasing fungal biomass (in comparison to the control culture) with an increasing concentration of Cd was observed (Figure 1A). e mycelial mass (dry weight-basis) was decreased by 35% and 67% at 0.5 mM and 2 mM concentrations of Cd, respectively. e decrease in biomass could be due to the toxic e ect of Cd which is known to generate reactive oxygen species (ROS). In this context, uorescence staining-based microscopy employed fordetection of super oxide radicals showed increasing number of fungal laments with a higher red uorescence (Figure 1B, i-viii) upon metal accumulation [2,6,7]. is implied that with an increasing concentration of Cd, the fungus could experience elevated levels of ROS resulting in oxidative stress-induced toxicity leading to a reduced growth.

PAH tolerance of *P. chrysosporium*

In order to assess the tolerance to PAHs, three di erent compounds (containing 3-5 aromatic rings), namely phenanthrene (Phe), pyrene (Pyr) and benzo(a)pyrene (BaP) were tested at 10 and 25 ppm concentrations. Phenanthrene at a 10 ppm concentration had no e ect

on fungal growth as the mycelia dry weight in the treated cultures was similar to that of the control untreated cultures (Figure 2A). On the other hand, at a 25 ppm concentration, the dry weight reduced by more than 50% indicating toxicity. In contrast, pyrene showed a 20% reduction in mycelia mass even at 10 ppm concentration (Figure 2B), indicating greater sensitivity of the fungus to pyrenein comparison to phenanthrene. A further increase in pyrene concentration to 25 ppm led to a two-fold decrease in biomass level (40% decrease,); this suggestedthat a greater sensitivity to pyrene than phenanthrene at a common low concentration (10 ppm) does not necessarily result in a proportionatelyhigher toxicity at elevated concentrations (such as 25 ppm). A completely di erent pattern was observed in case of benzo(a) pyrene (Figure 2C) in comparison to phenanthrene and pyrene. At 10 ppm concentration, there was a signi cant increase (20%) in biomass productionin comparison to the control probably because of the recognized cell proliferation e ect of this PAH toward eukaryotic cells [8,9]. At 25 ppm concentration, there was a growth inhibition but the mycelial mass was reduced by only 30%, a gure signi cantlylower than those observed for phenanthrene (50%) and pyrene (40%). Taken together (10 and 25 ppm), fungal growth inhibition e ect exhibited by the PAHs followed the trend pyrene>phenanthrene>benzo(a)pyrene, suggesting that there was no uniform pattern of tolerance across the PAH types. Since pyrene showed the lowest threshold limit of tolerance (10 ppm), it was used as a model PAH for further investigationsin mixture studies with Cd.

E ect of cadmium and pyrene, singly and in mixture on mycelial pellet morphology

Visual di erences: Morphological changes in the mycelial mass in P. chrysosporiumcultures grown in presence of Cd, pyrene, or Cd+pyrene were monitored a er 72 hours of growth; equal aliquots from the individual cultures (freshly stirred to generate a uniform suspension) were transferred to the respective petriplates and visual di erences in mycelia morphology were recorded using photography. e control (untreated) culture was lled with 'loose ball-shaped pellets' (mycelia pellets) in addition to the free suspended laments in a light clear liquid phase of the medium (Figure 3A-Le panel). In contrast, the Cd-containing cultures showed light yellow colored, 'tightly-knit spherical ball-shaped mycelia pellets' in a colored liquid phase containing only a few suspended laments (Figure 3A-Right panel). e pyrene culture was similar to the control culture in that the mycelial pellets covered the entire liquid phase (light, clear) albeitata lower density (Figure 3B). In the mixed treatment (Cd+pyrene), the mycelia pellets turned out to be more like those in the pyrene culture (loose ball-shaped pellets surrounded by free suspended laments) albeit with a darker appearance (Figure 3B). is indicates the overarching in uence of pyrene on pellet morphology in the co-contaminated medium.

Microscopic di erences: A closer look at the culture suspensionusing microscopy provided further insights into the mycelial growth patterns. e culture suspensions were loaded onto the glass slides (without any treatment) and examined under a light microscope (10×magni cation). Micrographs of the control (untreated) culture showed loose mycelial masses (pellets) with a fuzzy boundary (Figure 4A), whereas the mycelia growing in presence of Cd alone (Figure 4B) showed tightly packed ball-shaped structures (pellets) with a distinct boundary and hairy small laments protruding at the surface. On the other hand, the pellet morphology in pyrene cultures were similar to that in the control culture except that it had a more distinct appearance of the otherwise loosely bound smaller mycelia mass (Figure 4C). In theco-treated (Cd+pyrene) cultures (Figure 4D), the mycelial mass appeared as a tightly knit ball-shaped structure with a distinct boundary albeit with a lower density than that inthe Cd-onlycultures.

Toxicity of Cd and Pyrene based on live/dead staining: e results on toxicity of Cd (0.5 mM) and pyrene (10 ppm) measured in terms of cell death based on live/dead staining are presented in (Figure 5A-5H).

mM Cd and 10 ppm pyrene, considering the preceding observations on the independent toxicity of these test concentrations (as evident in terms of their e ect on both fungal biomass yield and morphology). Interestingly, the overall toxicity outcome in the co-exposure treatment did not increase beyond what was seen with Cd alone indicating some kind of protective e ect of pyrene. To further prove this point, the experiments were carried out at a much safer nontoxic concentration (1 ppm) of pyrene. Interestingly, it improved the biomass growth, meaning that presence of even low amount of pyrene in the medium containing Cd interacts in a manner that protects the fungus from metal toxicity.

In speci c terms, when Cd (0.5 mM) and pyrene (10 ppm) were added alone at their respective toxic concentrations, the fungal growth was reduced by 35% and 20%,respectively (Figures 1 and 2A) Interestingly, in a co-exposure at these toxic concentrations, the growth was reduced by 35% (Figure 6A), same as that for the Cd only cultures is means, the two toxicants do not exert an additive e ect. Instead, pyrene seems to exert a protective e ect. We then asked the question, if this assumption was true even co-exposure to a lower non-toxic amount of pyrene should protect the fungus from the toxic e ect of

and 5H) showed the dead lament population, indicating that Cd was toxic but presence of Cd along with pyrene (Figure 5G and 5H) increased the toxicity more than Cd alone (Figure 5C and 5D). is lament-speci c observation contrasted with the overall protective in uence of pyrene on the fungal pellet morphology and biomass yield in the co-contaminated medium.

Role of pyrene in alleviation of Cd toxicity

ese co-exposure experiments were carried out in presence of 0.5

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