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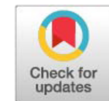
Impeding Effect of Polystyrene Microplastic Pollutants on Hg^{2+} Uptake Potential of *Aspergillus flavus*



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IG performed experiments and prepared the first draft of the article. AH supervised the work and finalized the article. AJ helped in data analysis. SM assisted in data compilation.

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Bioremediation system, Biosorption, Fungal remediation, Heavy metals, Microplastic pollutants

ABSTRACT

Microplastic pollution has aroused up to intimidating level around the globe and has become a focal point for researchers. However, data regarding the effects of microplastics on structure and function of filamentous fungal species is very scarce. Fungi have made a prominent mark in the area of heavy metals' bioremediation. This study attempts to check the influence of microplastic pollutants on Hg^{2+} uptake potential of metal-resistant *Aspergillus flavus* at laboratory scale under pre-optimized conditions. *A. flavus* showed a remarkable potential of remediating simulated wastewater, i.e., 100% Hg^{2+} reduction was achieved at 25 mg/L of the added metal in 15 days of incubation. On higher concentrations like 75 and 100 mg/L, *A. flavus* showed almost negligible reduction of Hg^{2+} but this strain was able to tolerate Hg^{2+} up to 200 mg/L. Polystyrene microbeads at a concentration of 100 mg/L reduced the metal uptake potential of *A. flavus* up to 21%. Polystyrene microparticles might have formed aggregates on fungal mycelia blocking the attachment sites for heavy metals. Our findings will be helpful in designing an efficient bioremedial system mediated by the pollution-resistant microflora. More research is required to check the possible effects of microplastic pollutants on the fungal mycelia to exploit their maximum potential.

INTRODUCTION

Rapid industrialization and development result in several environmental concerns. A variety of pollutants are damaging environment but heavy metal pollution is a serious distress due to its persistence and non-biodegradability (Ayele *et al*., 2021). Major industries that contribute in heavy metal pollution include metal-manufacturing plants, smelting and mining sites, tanneries and coating and painting industries. Other than anthropogenic activities some natural sources like volcanic eruptions, geysers, deep-sea vents, and forest fires also result in metal contamination of the environment (Tchounwou *et al*., 2012; Ghaffar *et al*., 2023). In addition to environmental pollution heavy metals are also a threat to human beings and all other living organisms. Some metals have a potential of chronic toxicity even at lower concentrations (Ayele *et al*., 2021; Ghaffar *et al*., 2023). To deal with heavy metal pollution bioremediation has highly been recommended by the researchers because of its sustainable and cost-effective nature (Ghaffar *et al*., 2023). Currently, biosorption nature of fungus has been explored to some extent (Ayele *et al*., 2021). Reports have suggested that fungi have successfully been exploited for the remediation of several heavy metals including Ni, Cr, Pb, Hg, Zn, and Cd (Chaurasia *et al*., 2023; Sharma *et al*., 2023).

Three groups (mushrooms, molds and yeast) of this eukaryotic microorganism are considered important in various applications (Mohmand *et al*., 2011; Carris *et al*., 2012). Fungal cell wall is of prime importance in bioremediation it has been revealed that its structure has high metal-binding properties than other biosorption agents. Fungal biomass shows extensive tolerance towards high metal concentrations and low pH (Ghaed *et al*., 2013). Dead and alive both types of fungal biomass exhibit sorption qualities (Ayele *et al*., 2021). Biosorption by live fungal biomass is an active process, in this process

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external and internal metabolism like volatilization, detoxification, bioaccumulation and chelation occur while inactivated (dead) biomass displays passive adsorption i.e., only cell surface binding occurs (Javanbakht *et al*., 2014; Cai *et al*., 2016). For the removal of metals from a liquid medium filamentous fungal species are more effective. The commonly used fungal species for the treatment of heavy metals include yeast (*Saccharomyces, Penicillium*), mushrooms and molds (*Rhizopus*, *Aspergillus*) (Pansuphaphol *et al*., 2016; Alothman *et al*., 2020; Gajewska *et al*., 2022).

Microplastics are omnipresent, i.e., they are literally found in every ecosystem (i.e., water, air, soil) and all living forms. Reports have suggested that synthetic microfibers are also present in air other than water and beaches (Sutton *et al*., 2016; Horn *et al*., 2019). Taking in to account the shape of microplastics they can be found in the form of fibers, pellets, foams, fragments, granules or films. Fibers originate from synthetic clothes; pellets are small spheres manufactured to prepare bigger plastic items and foam is coming in the environment from single-use styrofoam containers. Periodic break down of plastic made bottles and other objects made from plastic result in the formation of plastic fragments in the environment. Granules intrude in environment from the products used in personal care products e.g., toothpaste whereas plastic bags or food packaging materials are the main sources of plastic films. Pellets and granules are similar in shape (i.e., round), but pellets are much larger than granules (Lujan-Vega *et al*., 2021; Ghaffar *et al*., 2022a). Microplastics can handicap biosorption potential and growth of microbes by inducing structural and functional changes (Ghaffar *et al*., 2022a, b). Keeping in view the omnipresence and hazardous nature of microplastics, it is obvious that they can handicap the in-situ bioremediation. As fungi are one of the most potent microbes used for the removal of heavy metals so the present study is conducted to assess the impeding effect of microplastics on Hg^{2+} uptake potential of a metal-resistant fungal strain.

MATERIALS AND METHODS

Sample collection

To isolate a metal-resistant fungal strain sample was collected from a highly polluted drain known as Hudiara drain located in Lahore, Pakistan. This drain receives several types of anthropogenic and industrial discharge, providing an optimum environment to the pollution resistant microbes. Sampling of wastewater was performed using precise protocols of sterility and hygiene. While sampling some parameters of wastewater such as pH, humidity and temperature were measured as 7.4, 70% and 32 °C, respectively. The sample obtained from the wastewater drain was then carefully taken to PG (post-graduate) Laboratory, Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Lahore, Pakistan to perform analysis and further examination.

Isolation of pure culture of metal-resistant fungal strain

The collected sample was spread over MEA (malt extract agar) plates amended with various concentrations (up to 200 mg/L) of metal (Hg) and incubated in a dark incubator (OMEGA 1-52) for 3-5 days at 30 °C. Some fungal species appeared on plates after 5 days of incubation out of which the most resistant one was picked and pure cultured. Pure culturing was performed by inoculating MEA containing sterile petri plates with fungal spores in the center and incubating for 5 days in a dark incubator (OMEGA 1-52), the procedure was repeated if required. The strain was further proceeded for identification and experimentation.

Morphological identification of the fungal isolate

For morphological identification, color and texture of the fungal colony was checked as macroscopic features. For microscopic features fungal mycelia were stained with Lactophenol cotton blue and studied under microscope. Diba *et al*. (2007) was followed to identify the morphology of the isolated fungal strain.

Molecular level identification of the fungal isolate

Fungal strain was identified at molecular level by 18S rRNA gene sequencing. Dneasy® plant mini kit (Qiagen, Hilden, Germany) was used to isolate DNA of the freshly cultured fungal mycelia. Amplification of 18S rRNA was performed by using primer pairs for amplifying regions ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (Zhang *et al*., 2010). In a in a thermal cycler (Hamburg 22331, Germany) the initial denaturation was conducted at 95°C for 5 min followed by 35 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s), extension of primer (72°C for 2 min) and the final extension (72°C for 7 min). Electrophoresis was then performed by using stained ($C_{21}H_{20}BrN_3$) agarose gel to separate the PCR product. The PCR product was then purified with the help of Gene Purification Kit (Hunan Runmei Gene, CE, ISO 13485, China) and the amplicon obtained was then got sequenced and compared with the most alike sequences by using NCBI blast tool.

Optimization of growth parameters

Fungal strain was allowed to grow in different conditions to determine the optimum level of different

parameters. For this purpose, malt extract agar (MEA) was used and following parameters were optimized as:

Temperature

Fungal mycelia were incubated at 20 °C, 30 °C and 40 °C on petri plates in a dark incubator (OMEGA 1-52) and growth was observed. Maximum growth was observed at 30 °C. Temperature was further narrowed down at 28 °C, 29 °C, 31 °C, and 32 °C to achieve the accuracy. At every temperature colonies were counted on regular basis up to 5 days.

pH

pH for the fungal mycelia was set at 5, 7 and 9 and petri plated inoculated with fungal spores were incubated for 5 days in a dark incubator (OMEGA 1-52) and it was detected that number of colonies was the highest at pH 5. Then the growth was further checked at pH 4.8, 4.9, 5.1 and 5.2 by counting the number of colonies for 5 days.

Incubation period

Sterile petri plates containing growth medium of fungi along with fungal spores were incubated at 30 °C in a dark incubator (OMEGA 1-52) and the number of colonies was observed after every day for consecutive 5 days.

Experimental design

The fungal-microplastic interaction was checked on the basis of the concentration of Hg adsorbed by *Aspergillus flavus.* Experimentation was carried out in three groups to check the impact of polystyrene microbeads on the efficiency of *A. flavus* to adsorb Hg^{2+}. Microplastics (0.5µm) used for this purpose were purchased from Poly Sciences Europe GmbH (Eppelheim, Germany). The 1st group deals with the interaction of fungi with microplastics (i.e., experimental flasks with inclusion of microbeads 100 mg/L). While the 2nd group only contained microplastics (100 mg/L) and heavy metal to check the microplastic-metal interaction and the 3rd group was kept as control i.e., without inclusion of microbeads. All experimentation was conducted in triplicates. In all three sets Hg concentrations of 25, 50, 75 and 100 mg/L were added. Tolerance of the fungal isolate was pre-assessed and it was revealed that this strain was able to withstand Hg up to 200 mg/L.

The fungal spore suspension was prepared by adding a loop full of freshly cultured fungal mycelia and sporangia into a 250mL Erlenmeyer flask containing sterile Basal medium. The flask was placed in dark incubator for 3-5 days in a dark incubator (OMEGA 1-52). 10% (v/v) of fungal spore suspension was used as inoculum for group 1 and group 3 harboring about 10^9 mg/L spores. While group 2 remained un-inoculated. To prepare concentrations of Hg, a compound of $HgSO_4$ was used. Basal medium ((gL^{-1}) NaCl, 0.01; K_2HPO_4, 1.5; $MgCl_2.7H_2O$, 0.05) was used to prepare all the mentioned concentrations of the mercury (II) and microplastics and pH was maintained at 4.8.

The fungal mycelia were permitted to uptake Hg^{2+} from artificially prepared wastewaters with the inclusion and/or exclusion of polystyrene microbeads in a 15 days trial. All experimental and control flasks were placed in dark incubator at 29 °C. Our results only showed the final concentration of Hg^{2+} biosorbed by *A. flavus* with the inclusion and/or exclusion of polystyrene microparticles. The final concentration of Hg^{2+} was achieved by subtracting the Hg^{2+} concentration absorbed in group 2 from group 1.

Data analysis

10 mL of sample was removed periodically (i.e., after every 5 days) from all the experimental and control flasks, and filtered with a Whatman cellulose filter paper. Filtrates were analyzed by using Atomic Absorption Spectrophotometer (CE-2041, UK) to check the varying concentration of Hg.

Statistics of the analyzed data

Statistical analysis of Hg concentrations adsorbed by fungal hyphae in all groups were carried out by using R software. Means were considered significant at $p\text{-value}<0.05$. Differences between Hg concentration of both control and experimental groups were compared by using T-test. Results are graphically expressed with the help of origin software 6.0.

RESULTS AND DISCUSSION

The morphological characteristics have revealed that the isolated strain showed resemblance with *A. flavus*. The colony color of the isolated strain was yellowish-green with powdery texture. Microscopic study has revealed that its hyphae were non-septate, shape of vesicle was globose, and phialides were biseriate loosely present all over the vesicle, and philiades were radiating from metulae. Our findings are in accordance with Afzal *et al*. (2013) and Okayo *et al*. (2020). So, the morphological characterization and BLAST search of 18S rDNA nucleotide sequence revealed that the metal-resistant fungal isolate belonged to the genus *Aspergillus* and species was identified as *Aspergillus flavus*.

Optimized parameters of the fungal isolate

Growth of *A. flavus* was effected by varying the parameters like temperature, pH and incubation period. To obtain the optimum pH *A. flavus* was incubated with different pH values and optimum growth of *A. flavus* was

observed at pH 4.8 and optimum temperature was 29 °C among 28 °C, 29 °C, 31 °C and 32 °C. Our results are related to Kote *et al.* (2009) according to their report *A. flavus* showed optimum growth and activity at pH 5 and Samapundo *et al.* (2007) suggested that temperature range for *A. flavus* is 25 to 30 °C. Casquete *et al.* (2017) isolated different strains of *A. flavus* which showed maximum growth in range of pH from 5 to 5.5 and temperature from 25 to 30 °C. Gunasekaran (1981) reported that *A. flavus* showed optimum mycelial growth at pH 4.5 results of this study are almost similar to our findings while optimum temperature reported in this study was 37 °C. Gallo *et al.* (2016) reported that maximum expression of regulatory genes by *A. flavus* was at 28 °C while minimum was at 37 °C. The isolated fungal strain showed maximum number of colonies on 5th day of incubation period at optimized temperature and pH.

Effect of metal-concentrations on metal uptake potential of fungal isolate

A. flavus has a tremendous potential for the biosorption of heavy metals (Anupong *et al.*, 2022). Present study showcases the capability of a metal-resistant strain *A. flavus* to remediate artificially prepared mercury polluted wastewater and results have revealed that the biosorption potential of the isolated strain reduces with an increase in the concentration of mercury (II) i.e., Hg^{+2} uptake potential was maximum i.e., 100% at 25 mg/L on 15th day of incubation period (Fig. 1A). At higher concentrations (75 and 100 mg/L) reduction of Hg^{+2} was almost negligible (Fig. 1C, D). The isolated fungal strain was able to withstand Hg (II) up to 200 mg/L but only lower concentrations were degraded efficiently. The overall metal-uptake sequence of *Aspergillus flavus* was as follows: 25 > 50 > 75 > 100 mg/L (Figs. 1-2). *A. flavus* showing 100% and 96% reduction at 25 and 50 mg/L (Fig. 1A, B), respectively of a toxic metal like mercury (II) is remarkable. Kurniati *et al.* (2014) reported 98.73% reduction of Hg^{+2} by *A. flavus* at 10 mg/L which is lower than our findings. Several reports have shown that *A. flavus* has an amazing potential to remediate a large number of heavy metals. *A. flavus* is able to tolerate metals like Cr and Cu up to 1000 mg/L (Dusengemungu *et al.*, 2020; Palanivel *et al.*, 2023). Live and dead biomass of filamentous fungi have the ability to remove mercury through various mechanisms like reduction, biosorption and bioaccumulation (Arıca *et al.*, 2003). Villalba-Villalba *et al.* (2022) reported that *A. flavus* has high tolerance against higher concentration of Cu, Zn, and Pb and was able to withstand only lower concentrations of Hg, Cd, and Ag. Mart´ınez-Ju´arez *et al.* (2012) reported the removal of mercury from aqueous solution by 14 different fungal species and *Mucor* spp. showed the maximum reduction i.e., up to 95% at 100 mg/L and *Aspergillus flavus* showed comparatively lower Hg removal efficiency among all. Acosta-Rodríguez *et al.* (2018) reported that *Aspergillus niger* removed 83.2% of mercury at 100 mg/L and was able to tolerate mercury up to 2000 mg/L which shows *A. niger* has remarkably higher remedial potential for mercury (II) than the fungal species isolated in present study.

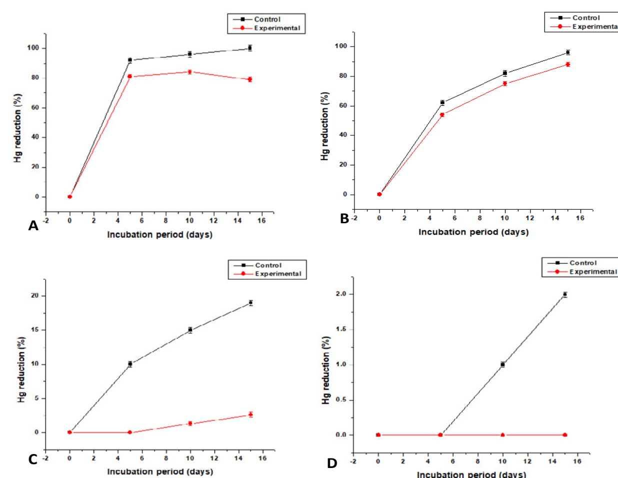

Fig. 1. Periodic removal of Hg by *A. flavus* at 25 ppm (A), 50 ppm (B), 75 ppm (C) and 100 ppm (D) of the added metal.

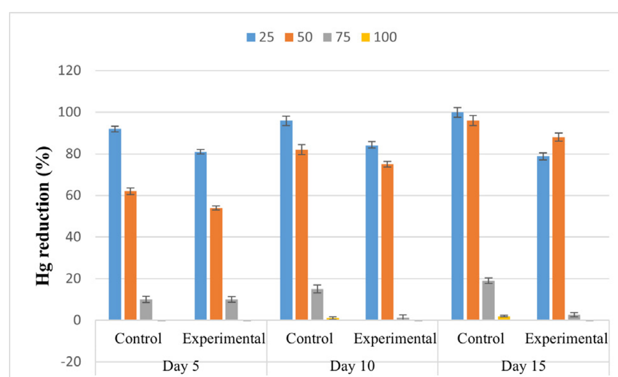

Fig. 2. Periodic removal of Hg by *A. flavus* in control (without microplastic) and experimental (with microplastics) samples.

Effect of microplastic bead size on remedial potential of fungi

The smaller polystyrene microparticles (0.5µm) influenced the remedial potential of *A. flavus*, i.e., in experimental sets slightly lesser Hg^{+2} uptake was observed (Fig. 2) as compared the control group (without inclusion of microplastics) which depicts that small sized microplastics have regressive effects on fungal mycelia

because they can block the attachment sites for metals and cause impairment of fungal cell wall. Studies about effects of microplastics on structure and bioremedial potential of filamentous fungi are scarce. Fan *et al.* (2022) reported that fungal communities are more sensitive towards microplastic pollutants than bacterial communities and microplastics declined the abundance of different fungal communities. Some studies report the effects of different size and charge of microplastics on microbes (Ghaffar *et al.*, 2022a). Microplastics significantly reduce the growth and biosorption potential of microalgae at various concentrations (Sjollema *et al*., 2016; Ghaffar *et al*., 2022b) which is in accordance to our findings. Concentration of microplastics is directly proportional to the severity of damage (Besseling *et al*., 2014; Sjollema *et al*., 2016). In the present study, 100 mg/L of PS microbeads reduced biosorption potential of *Aspergillus flavus* up to 21% which is not very remarkable but statistically significant while Ghaffar *et al*. (2022b) reported a remarkable decrease in the metal uptake potential of microalgae at the same concentration of microbeads. Lagarde *et al*. (2016) showed a decreased microbial growth at 400 mg/L of microbeads. At higher concentration of metals fungal mycelia exposed with PS microbeads showed 0% removal of Hg^{+2} (Fig. 1D). Microplastics may also form aggregate around the fungal hyphae, ultimately repressing their biosrption potential, as aggregation of microplastics has been reported by Lagarde *et al*. (2016) and Ghaffar *et al*. (2022b).

Effect of incubation period

After 5 days of incubation *Aspergillus flavus* showed a remarkable removal of Hg^{+2} i.e., 92% and on 15th day the metal-reduction potential of *Aspergillus flavus* was at its fullest. While the maximum removal of heavy metals by *Aspergillus* sp. was reported on the 7th day of incubation by Acosta-Rodríguez *et al.* (2018) and Kumar and Dwivedi (2020) reported the maximum reduction of heavy metals by *A. flavus* on 8th day of incubation. Sharma *et al*. (2022) reported 70-84% Hg removal by white rot fungi in 7 days of incubation. Ozsoy (2010) reported maximum biosorption of Hg by *Rhizopus oligosporus* after 6 hours of incubation and Dusengemungu *et al*. (2020) reported that maximum biosorption of heavy metals by filamentous fungi was observed after 24 h of incubation which indicates that lag phase of our isolate is longer than the other fungal species but *A. flavus* was able to easily acclimatized in growth medium and showed efficient metal reduction as compared to some bacterial and microalgal species (Hussain and Qazi, 2016; Muneeb *et al*., 2020; Ghaffar *et al*., 2022b). The log phase i.e., 10-15 days showed maximum reduction and then fungal mycelia entered in stationary phase and formed spores possibly due to the scarcity of nutrients and abundance of metabolites.

CONCLUSIONS

A. flavus has an efficient remedial potential for mercury which is toxic even in minute concentrations. To achieve the maximum metal reduction determination of the optimum conditions is important. Other than optimum conditions several other pollutants can also affect the remedial efficacy of the fungal species in in-situ wastewater treatment plants. So, in this study effect of microplastic pollutants on remedial potential of *A. flavus* was studies at laboratory scale and it was concluded that microplastics can handicap the heavy metal removal efficiency of the fungal mycelia by blocking the attachment sites available for mercury (II). Almost no data is available regarding the effects of microplastic pollutants on filamentous fungi and their capability to remove heavy metals. Investigations are needed to determine the extent up to which microplastics cause functional stress in filamentous fungi because in present age plastics are everywhere and damaging almost all living entities.

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IRB approval

The current research work was approved by Board of Studies of Department of Wildlife and Ecology, University of Vet-erinary and Animal Sciences, Lahore.

Ethical statement

The current research work didn't involve any animal model and thus ethical statement was not needed.

Statement of conflict of interest

The authors have declared no conflict of interest.

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