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Heavy Metal Biosorption by Endophytic Fungi Isolated from *Azadirachta Indica*

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Abstract
Heavy metal contamination in industrial effluents, particularly in paper pulp industries, is a significant environmental concern due to the detrimental effects on living organisms and ecosystems. The use of biological materials, a process known as biosorption, has emerged as a promising eco-friendly and cost-effective approach for the remediation of these pollutants. This study investigates the potential of endophytic fungi isolated from the medicinal plant *Azadirachta indica* (neem) to remove heavy metals from paper pulp industry wastewater. Endophytic fungi were isolated from the leaves of *Azadirachta indica*, a plant commonly found in the vicinity of paper pulp industries, and their heavy metal biosorption capabilities were evaluated. The findings demonstrate the efficacy of these endophytic fungi in sequestering heavy metals, suggesting their potential application in the bioremediation of paper pulp industry effluents. Total 26 fungi were isolated and estimate the efficiency of biosorption.

Keywords: Contaminated soil, Environmental pollution, Fungal biodegradation, Industrial waste, Mycoremediation, Plant-fungi interaction

Introduction

Endophytic fungi, the microorganisms that reside within plant tissues without causing any visible harm, have garnered significant attention in recent years due to their immense potential in bioremediation (Dhankhar & Hooda, 2011). These fungi have the ability to produce a wide range of bioactive metabolites, which can be leveraged for various applications, including environmental remediation (Gupta, 2018).

Fungal endophytes establish a symbiotic relationship with their host plants, providing numerous benefits to the latter (Gupta et al., 2015). They can enhance plant growth, increase tolerance to biotic and abiotic stresses, and produce secondary metabolites that act as defensive compounds Interestingly, these endophytic fungi can also play a crucial role in the biodegradation and detoxification of various environmental pollutants, making them a promising solution for bioremediation efforts (Gupta et al., 2005).

The scientific literature is replete with studies that have explored the remarkable bioremediation potential of endophytic fungi. For instance, a study by (Kaur, 2020) demonstrated the ability of endophytic fungi to enhance the growth and fitness of their host plants, thereby improving their tolerance to various abiotic stresses, including heavy metal contamination. Similarly, research has shown that endophytic fungi can produce a variety of antimicrobial compounds, which can be leveraged for the treatment of various bacterial, fungal, and viral infections(Aamir et al., 2020) (Gautam & Avasthi, 2019).

One of the key advantages of utilizing endophytic fungi for bioremediation is their ability to adapt to diverse environmental conditions. These fungi can thrive in contaminated soil, water, and even air, allowing them to effectively target a wide range of pollutants. Furthermore, their metabolic versatility enables them to break down complex organic compounds, such as pesticides, heavy metals, and hydrocarbons, into less toxic or even harmless products (Nassos&Avlonas, 2020; Digra&Sumbali, 2023).

The field of bioremediation has seen a significant surge of interest in recent years, particularly with the exploration of the potential of endophytic fungi to serve as effective bioadsorbents for the removal of heavy metals from contaminated environments. Endophytic fungi, which are known to establish symbiotic relationships with their host plants, have demonstrated a remarkable ability to accumulate and sequester a wide range of heavy metals, including cadmium, lead, copper,and chromium (Pieper & Reineke, 2000).

The underlying mechanisms behind the biosorption capabilities of endophytic fungi are multifaceted and complex. These microorganisms have evolved various strategies to cope with the toxicity of heavy metals, such as the production of metal-binding proteins and the formation of intracellular complexes (Volesky & Holan, 1995). Thecell walls of endophytic fungi also play a crucial role, as they possess a variety of functional groups that can effectively chelate and immobilize heavy metal ions (Sharma et al., 2019)(Selvasembian et al., 2018). Moreover, thethe extracellular polymeric substances secreted by these fungi can further enhance the biosorption process by creating a favorable environment for the adsorption of metal ions (Volesky & Holan, 1995)(Sharma et al., 2019).

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Material and Methods
Survey and Sample:
All chemicals, materials, media, and solvents used in this study were obtained from Sigma-Aldrich (UK).

Collection of Plant Samples
Healthy fresh and green leaves of Neem (*Azadirachta indica*) in old age were obtained from near the Paper and Pulp industry, Bhanpuri, Raipur, Chhatisgarh.

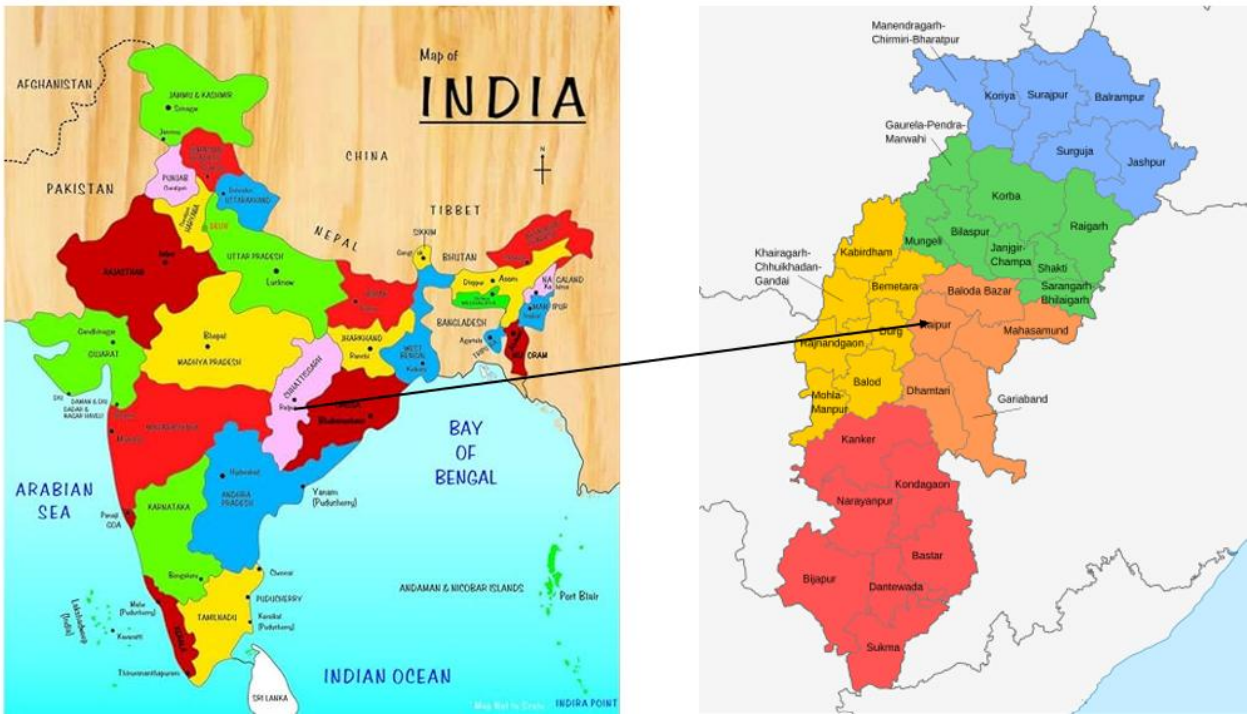

Fig 1: Site of Sample Collection

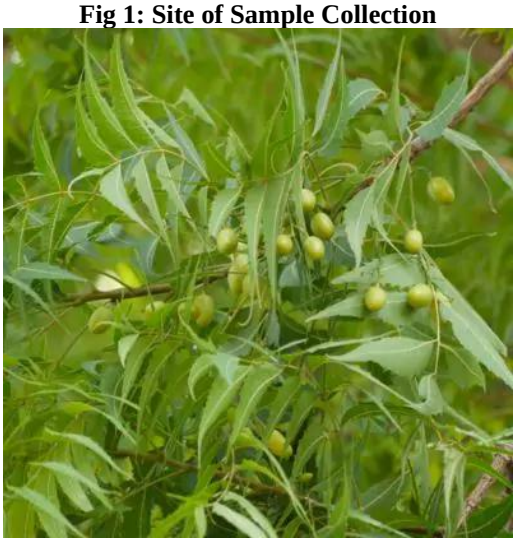
Fig 2:*Azadirachta indica*

Isolation and identification of endophytic fungi from *Azadirachta indica.* The plant material samples from *Azadirachta indica* (leaf) were thoroughly rinsed twice with running tap water and surface-sterilized by sequential immersion in 70% ethanol for 1 minute, followed by 5% (v/v) sodium hypochlorite for 5 minutes. The sterilized roots, stems, and leaves were then longitudinally sliced into segments of approximately 5 mm under sterile conditions. These segments were placed directly onto sterilized Petri dishes containing potato dextrose agar (PDA) supplemented with 100 U/mL chloramphenicol to inhibit bacterial growth. The plates were incubated at 28°C for 2-3 weeks to allow colony formation. The tips of the growing mycelia were transferred to fresh PDA plates for obtaining pure cultures, which were maintained through repeated sub-culturing (Sandhu et al., 20). Fungal colonies were identified based on microscopic

examination of mycelia, spores (asexual/sexual), colony morphology, and cultural characteristics (such as color, texture, and pigmentation), as well as spore-bearing structures, using standard identification manuals (Moharbi et al., 2019).

Heavy metal tolerance test

Potato Dextrose Agar (PDA) medium was prepared and supplemented with different concentrations of heavy metals, including Cu (CuSO4), Mn (MnCl2), and Co (CoCl2), to achieve target levels of 200, 400, 600, 800, and 1000 ppm. The pH was adjusted to 5.6 using NaOH or HCl solutions. After autoclaving, the medium was poured into Petri dishes. Fungal isolates, cultured for eight days, were cut into 4 mm discs using a cork borer. The fungal inoculum was placed at the center of both metal-treated and control plates (without metals). Plates were incubated at 27±2°C for 10 days, during which the radial colony growth was measured. Fungal growth was observed from the inoculation point, and the impact of heavy metals on growth was determined by comparing the colony radius (mm) with that of the control (Iram et al., 2012; Kapoor et al., 1999).

Result and Discussion

Diversity of cultural endophytic fungi from *Azadirachta indica*

Endophytic microorganisms play a vital role in promoting plant growth and restoring plant health through various mechanisms, such as managing phytopathogens, and producing phytohormones like indole-3-acetic acid (IAA), cytokinins, and gibberellic acids. Moreover, endophytes enhance plant development by nitrogen fixation, phosphate solubilization, nutrient cycling, and secreting unique bioactive compounds. Endophytic fungi also contribute to bioremediation efforts. In this research, 26 strains of endophytic fungi were isolated from the leaves of *Azadirachta indica*. Based on their morphological characteristics, the fungal isolates were identified as *Penicillium crustosum*, *Penicillium chrysogenum*, *Penicillium commune*, *Penicillium corylophilum*, *Alternaria infectoria*, *Penicillium caseifulvum*, *Alternaria alternata*, *Alternaria tenuissima*, *Aspergillus flavus*, *Aspergillus niger*, *Bionectriaochroleuca*, *Phomopsis mali*, *Fusarium oxysporum*, *Colletotrichum acutatum*, *Cladosporium* sp., *Tricharinagilva*, *Nigrospora oryzae*, *Cladosporium bruhnei*, *Phomopsis* sp., *Guignardiamangiferae*, *Alternaria* sp., *Cochliobolus sativus*, *Phyllostictagardeniicola*, *Aspergillus* sp., *Diaportheamygdali*, and *Trichoderma harzianum*. Ibrahim et al. (2021) investigated the antioxidant activity of endophytic fungi isolated from eight Nigerian medicinal plants, including *Acalypha ornata*, *Albizia zygia*, *Alchornea cordifolia*, *Chrysophyllum albidum*, *Ficus exasperata*, *Gomphrena celosioides*, *Millettiathonningii*, and *Newbouldia laevis*.

The effects of varying concentrations of cobalt (Co) on fungal isolates

The table presents the effects of varying concentrations of cobalt (Co) on the growth of 26 fungal species. The fungi were exposed to cobalt concentrations ranging from 0 to 1000 ppm, and their colony growth was measured after 10 days. Increasing cobalt concentrations generally inhibited fungal growth, with most species showing reduced colony growth at higher concentrations. Fungal species exhibited varying levels of sensitivity to cobalt. Some species, such as *Tricharinagilva* and *Guignardiamangiferae*, showed increased growth at lower cobalt concentrations, while others, like *Penicillium chrysogenum* and *Fusarium oxysporum*, were strongly inhibited.The inhibitory effect of cobalt was concentration-dependent. At lower concentrations (200-400 ppm), some fungi showed minimal growth reduction, while higher concentrations (600-1000 ppm) significantly inhibited growth. The mean colony growth decreased from 6.71 mm (control) to 3.29 mm (1000 ppm), indicating a 51% reduction in growth.

Table 1. Effect of Co heavy metals on growth of 26 isolates

Name of fungi		**Colony growth (mm) after 10 days**					
		Concentration of heavy metals, Cobalt (ppm)					
		0	200	400	600	800	1000
1	*Penicillium crustosum,*	7.33	6.67	5.00	3.60	5.67	3.80
2	*Penicillium chrysogenum,*	6.67	3.37	3.43	2.83	2.97	2.57
3	*Penicillium commune,*	5.47	4.10	5.23	4.13	4.37	3.63
4	*Penicillium corylophilum,*	6.79	6.28	4.52	3.54	3.23	3.01
5	*Alternaria infectoria,*	7.67	8.00	5.67	6.33	5.63	3.13
6	*Penicillium caseifulvum,*	6.01	5.94	4.76	4.22	3.58	2.79
7	*Alternaria alternata,*	6.35	5.76	5.32	3.98	3.33	2.96
8	*Alternaria tenuissima,*	7.23	7.67	6.50	5.67	5.83	3.53
9	*Aspergillus flavus,*	6.44	5.14	4.89	4.06	3.93	3.21
10	*Aspergillus niger,*	7.00	5.67	5.47	3.87	5.03	3.10
11	*Bionectriaochroleuca,*	8.67	3.77	2.83	2.17	2.67	3.00
12	*Phomopsis mali,*	6.99	6.01	5.76	5.01	4.98	3.21
13	*Fusarium oxysporum,*	7.33	8.50	3.50	2.00	2.90	2.07
14	*Colletotrichum acutatum,*	5.48	5.24	4.14	4.05	3.32	2.01

15	*Cladosporium sp.,*	5.29	5.09	4.90	4.34	3.87	3.54
16	*Tricharinagilva,*	7.33	8.00	6.00	7.13	8.27	3.37
17	*Nigrospora oryzae,*	5.37	4.65	5.68	5.26	4.69	4.16
18	*Cladosporium bruhnei,*	5.17	3.57	5.37	4.50	4.43	3.27
19	*Phomopsis sp.,*	6.15	5.90	5.84	5.04	4.42	3.92
20	*Guignardiamangiferae,*	8.50	8.50	7.50	5.17	7.00	3.60
21	*Alternaria sp.,*	6.29	5.33	4.49	4.23	3.78	3.39
22	*Cochliobolus sativus,*	6.16	6.06	5.65	5.30	4.07	3.71
23	*Phyllostictagardeniicola,*	7.13	7.17	6.77	7.33	5.20	3.67
24	*Aspergillus sp.,*	8.00	7.83	3.77	7.33	7.67	3.60
25	*Diaportheamygdali*	7.14	6.24	5.18	4.76	5.19	3.31
26	*Trichoderma harzianum*	6.50	4.50	5.50	4.57	5.00	4.00
Mean		**6.71**	**5.96**	**5.14**	**4.63**	**4.66**	**3.290**
S.E.		**0.95**	**1.51**	**1.05**	**1.37**	**1.43**	**0.53**

Effect of Cu heavy metals on growth of 26 fungal isolates

The table presents the effects of varying concentrations of copper (Cu) on the growth of 26 fungal species. The fungi were exposed to copper concentrations ranging from 0 to 1000 ppm, and their colony growth was measured after 10 days.Copper inhibits fungal growth in a concentration-dependent manner, with higher concentrations (600-1000 ppm) significantly reducing growth.Fungal species exhibit varying levels of sensitivity to copper, with some species (e.g., *Cladosporium sp.*, *Aspergillus sp*.) showing relatively higher tolerance.At lower concentrations (200-400 ppm), some fungi (e.g., *Penicillium commune*, *Phomopsis mali*) showed minimal growth reduction.The mean colony growth decreased from 6.89 mm (control) to 3.19 mm (1000 ppm), indicating a 53.5% reduction in growth.Standard error values indicate variability in growth responses among fungal species.*Cladosporium* sp. and *Aspergillus* sp. showed remarkable tolerance to copper, with minimal growth reduction even at 1000 ppm.*Fusarium oxysporum* and *Colletotrichum acutatum* were highly sensitive to copper, with significant growth reduction at concentrations as low as 200 ppm.*Trichoderma harzianum* exhibited unusual growth patterns, with increased growth at lower copper concentrations.

Table 2. Effect of Cuheavy metals, on growth of 26 fungal isolates

Name of fungi		**Colony growth (mm) after 10 days**					
		Concentration of heavy metals, Copper Cu(ppm)					
		0	200	400	600	800	1000
1	*Penicillium crustosum,*	7.43	7.30	5.85	5.30	5.33	3.79
2	*Penicillium chrysogenum,*	7.77	5.54	5.16	4.20	4.04	2.55
3	*Penicillium commune,*	5.73	6.29	6.06	5.50	4.18	3.14
4	*Penicillium corylophilum,*	7.29	7.04	6.28	4.08	4.30	3.72
5	*Alternaria infectoria,*	7.43	6.73	6.01	3.44	3.39	2.22
6	*Penicillium caseifulvum,*	6.28	5.69	4.83	4.95	3.12	2.83
7	*Alternaria alternata,*	6.35	5.87	5.61	3.13	3.10	2.90
8	*Alternaria tenuissima,*	6.51	5.03	4.01	3.42	2.94	2.71
9	*Aspergillus flavus,*	5.89	4.51	4.87	2.30	2.13	2.04
10	*Aspergillus niger,*	6.45	5.85	4.48	4.99	3.44	3.14
11	*Bionectriaochroleuca,*	7.81	6.99	6.98	6.41	5.40	3.32
12	*Phomopsis mali,*	6.86	6.13	5.66	5.07	4.28	2.83
13	*Fusarium oxysporum,*	7.50	6.47	6.27	5.65	4.20	2.66
14	*Colletotrichum acutatum,*	6.51	5.64	5.68	5.56	3.63	3.01
15	*Cladosporium sp.,*	7.44	6.97	6.95	6.8	6.52	5.82
16	*Tricharinagilva,*	7.09	6.45	5.62	3.7	3.25	2.57
17	*Nigrospora oryzae,*	7.09	5.92	5.21	4.72	3.78	3.97
18	*Cladosporium bruhnei,*	6.18	5.98	5.27	4.54	2.74	2.65
19	*Phomopsis sp.,*	6.13	4.49	4.0	3.67	3.33	2.77
20	*Guignardiamangiferae,*	6.99	6.45	5.21	4.73	3.03	2.82
21	*Alternaria sp.,*	7.42	6.38	4.19	3.8	3.38	2.95
22	*Cochliobolus sativus,*	7.44	5.4	3.72	3.51	3.44	3.02
23	*Phyllostictagardeniicola,*	7.28	6.98	6.31	6.07	5.16	3.39
24	*Aspergillus sp.,*	7.3	6.2	6.01	5.9	5.63	5.07
25	*Diaportheamygdali*	5.9	5.2	5.07	4.9	3.4	3.26

26	*Trichoderma harzianum*	6.99	6.97	4.66	4.1	3.99	3.79
Mean		**6.89**	**6.1**	**5.38**	**4.63**	**3.89**	**3.19**
S.E.		**0.62**	**0.77**	**0.88**	**1.10**	**1.01**	**0.82**

Effect of Mn heavy metalson growth of 26 fungal isolates

The table presents the effects of varying concentrations of manganese (Mn) on the growth of 26 fungal species. The fungi were exposed to manganese concentrations ranging from 0 to 1000 ppm, and their colony growth was measured after 10 days.Manganese generally inhibited fungal growth in a concentration-dependent manner.Fungal species exhibited varying levels of sensitivity to manganese.Lower concentrations (200-400 ppm) had minimal effects on growth, while higher concentrations (600-1000 ppm) significantly reduced growth.The mean colony growth decreased from 6.34 mm (control) to 3.54 mm (1000 ppm), indicating a 44.1% reduction in growth.*Cladosporium* sp. and *Trichoderma harzianum* showed relatively higher tolerance to manganese.*Phomopsis* sp. was highly sensitive to manganese, with significant growth reduction at concentrations as low as 200 ppm.*Alternaria infectoria*, *Alternaria alternata*, and *Aspergillus niger* exhibited moderate sensitivity to manganese.Manganese can be used as a fungicide to control fungal growth, but its effectiveness depends on the specific fungal species and concentration.Standard error (S.E.) values indicate variability in growth responses among fungal species.

Table 3. Effect of heavy metals, Mn on growth of 26 isolates

Name of fungi		**Colony growth (mm) after 10 days**					
		Concentration of heavy metals, Mn (ppm)					
		0	200	400	600	800	1000
1	*Penicillium crustosum,*	6.57	5.72	4.84	3.30	3.38	3.36
2	*Penicillium chrysogenum,*	6.02	5.95	5.03	4.11	3.77	3.60
3	*Penicillium commune,*	6.10	6.39	5.55	5.01	4.67	3.88
4	*Penicillium corylophilum,*	6.02	5.48	5.24	4.14	4.05	3.32
5	*Alternaria infectoria,*	6.46	6.06	5.65	5.30	4.07	3.71
6	*Penicillium caseifulvum,*	6.49	6.16	5.37	4.65	4.23	3.78
7	*Alternaria alternata,*	6.71	6.29	5.90	5.33	4.49	3.39
8	*Alternaria tenuissima,*	6.37	6.15	5.84	5.04	4.42	3.92
9	*Aspergillus flavus,*	6.58	5.68	5.26	4.69	4.16	3.54
10	*Aspergillus niger,*	6.62	5.29	5.09	4.90	4.34	3.87
11	*Bionectriaochroleuca,*	6.43	6.33	5.13	4.99	4.56	3.84
12	*Phomopsis mali,*	6.23	5.71	5.96	4.36	4.03	3.49
13	*Fusarium oxysporum,*	6.48	6.01	5.82	4.59	4.74	3.68
14	*Colletotrichum acutatum,*	6.53	6.31	5.45	5.17	4.08	3.94
15	*Cladosporium sp.,*	6.68	6.47	5.87	5.08	4.21	4.12
16	*Tricharinagilva,*	6.41	5.76	5.53	4.66	3.62	3.40
17	*Nigrospora oryzae,*	6.12	5.83	5.60	4.95	4.27	3.35
18	*Cladosporium bruhnei,*	6.05	5.63	5.22	4.53	4.18	3.29
19	*Phomopsis sp.,*	6.61	4.28	4.01	2.97	2.83	2.57
20	*Guignardiamangiferae,*	5.47	5.23	4.37	4.13	4.10	3.63
21	*Alternaria sp.,*	6.09	5.19	4.38	4.00	3.50	2.87
22	*Cochliobolus sativus,*	6.22	5.88	5.58	4.44	4.15	3.95
23	*Phyllostictagardeniicola,*	6.18	5.31	4.92	4.33	3.48	3.79
24	*Aspergillus sp.,*	6.04	5.40	4.82	3.85	3.55	2.68
25	*Diaportheamygdali*	6.73	5.18	4.97	4.25	4.03	3.45
26	*Trichoderma harzianum*	6.50	6.14	5.81	4.70	4.61	3.65
Mean		**6.335**	**5.763**	**5.277**	**4.518**	**4.058**	**3.541**
S.E.		**0.293547**	**0.501**	**0.513**	**0.579**	**0.451**	**0.384**

Conclusion

The study demonstrated that endophytic fungi isolated from *Azadirachta indica* possess significant potential for heavy metal biosorption, particularly for metals such as copper (Cu), manganese (Mn), and cobalt (Co). The fungi showed varying degrees of tolerance and biosorption efficiency across different metal concentrations, suggesting their adaptability and ability to survive in heavy metal-contaminated environments. This biosorption capability can be attributed to the unique metabolic properties of endophytic fungi, which allow them to interact with and immobilize metal ions. These findings highlight the potential of endophytic fungi as natural bioremediators for the detoxification of

heavy metal-polluted ecosystems. Further exploration of these fungal isolates could provide sustainable solutions for environmental management and pollution control.

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