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Comparative assessment of free, immobilized, and nanoconjugate form of fungal biomass for biosorption of heavy metal

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Abstract
Heavy metal contamination poses a major environmental threat due to the toxic and persistent nature of metals such as chromium (Cr), lead (Pb), aluminum (Al), and mercury (Hg). The present study aimed to evaluate the biosorption efficiency of three fungal isolates (RF1, RF7 and RF16) obtained from contaminated sites, and to compare their metal removal potential in free, immobilized, and nanoconjugate forms. Three isolates (RF1, RF7, and RF16) showing promising results were further processed for immobilization in calcium alginate beads and for ZnO nanoparticle conjugation. Biosorption experiments were conducted using metal solutions (80 ppm) at pH 5.0 and 25 ± 2 °C, and residual metal concentrations were quantified by UV–Visible spectrophotometry. Statistical analysis (t-test, $p < 0.05$) confirmed significant differences among fungal forms. Results revealed that immobilized and nanoconjugate forms showed markedly higher removal efficiencies compared to free forms. Immobilized fungi exhibited the best biosorption for Cr, Pb, and Al (up to ~70%), while nanoconjugate forms were most effective for Hg removal (~22%). Microscopic identification confirmed the isolates as *Aspergillus flavus* (RF1), *Penicillium* sp. (RF7), and *Talaromyces* sp. (RF16). Overall, the study highlights that immobilization and nanoconjugation enhance fungal biosorption potential, making these modified biomasses promising candidates for eco-friendly heavy metal remediation.

Keywords: Biomass, Biosorption, Fungi, heavy metal, Immobilized, Nanoconjugate

Introduction

Heavy metal contamination in soil and water has become a major environmental concern due to the toxic, persistent, and bioaccumulative nature of metals such as chromium (Cr), lead (Pb), aluminum (Al), and mercury (Hg). These metals are released into the environment from industrial effluents, mining operations, and agricultural activities, causing serious ecological and health hazards (Nayak et al., 2020; Singh et al., 2022). Conventional physicochemical techniques for metal removal—such as chemical precipitation, ion exchange, and membrane filtration—are often expensive, energy-intensive, and generate toxic sludge as a secondary pollutant (Fu & Wang, 2011; Barakat, 2011). Consequently, biological methods such as biosorption have emerged as sustainable, cost-effective, and eco-friendly alternatives for the remediation of heavy metals from contaminated environments (Ayele et al., 2021).

Fungi are recognized as potent biosorbents due to their high tolerance to metals, large surface area, and cell wall composition rich in functional groups such as carboxyl, hydroxyl, phosphate, and amino groups that facilitate metal binding (Verma et al., 2014; Gadd, 2009). The versatility of fungal metabolism and their ability to thrive in metal-polluted environments make them ideal candidates for biosorption-based remediation (Volesky, 2007). Additionally, modifying fungal biomass through immobilization in polymer matrices or nanoconjugation with metal oxide nanoparticles further enhances their biosorption potential by increasing surface stability, accessibility of active sites, and reusability (Bouabidi et al., 2019; Abbas et al., 2023).

The present study focuses on the screening and comparative evaluation of fungal isolates (RF1, RF7, and RF16) in free, immobilized, and ZnO-nanoconjugate forms for them biosorption efficiency against Cr, Pb, Al, and Hg. The goal is to determine the most effective fungal form for metal removal and to statistically validate biosorption performance. This research contributes to advancing eco-friendly fungal-based bioremediation approaches for the sustainable management of heavy metal pollution.

Materials and methods

Biosorption Experiments to Evaluate the Removal of Heavy Metals with Immobilized fungi

Immobilization of fungal cells Instead of employing free cells as catalysts, cell immobilization allows for the effective limitation of cell movement through the use of artificial or natural polymers (Bouabidi et al., 2019).

Materials required: Broth culture of fungal sample, 2% sodium alginate solution, 2% chilled CaCl2 solution, Sterile syringe, Glassware (Beaker, storage bottle), Equipment (Deep freezer)

Procedure

50 ml of fungal mycelium was mixed with 2% sodium alginate solution. This mixture was taken in a sterile syringe and poured drop by drop into pre-chilled CaCl2 solution. The solution was stirred to prevent aggregation of fungus-entrapped Ca alginate beads. After 1h, the beads were washed twice with 200 mL sterile distilled water. Ca-alginate beads with entrapped mycelia were then transferred to PDA media and allowed to incubate on a shaker incubator for 3 days (at 150 rpm, 30°C temperature). These beads were filtered from the medium and then washed twice with distilled water (Arica et al., 2001).

Biosorption of heavy metal with immobilized fungal strain-Chromium (Cr) from potassium dichromate ($K_2Cr_2O_7$), mercury (Hg) from mercury sulfate ($HgSO_4$), lead (Pb) from lead acetate, and aluminum (Al) from aluminum chloride ($AlCl_3$) - each at a concentration of 80 ppm using six immobilized fungal strains (RF1, RF12, RF7, RF11, RF16, and RF1G) individually. For each experiment, 80 ppm of the metal solution was transferred into 250 mL Erlenmeyer flasks having PDB (Potato dextrose broth) media, and the alginate beads entrapped live fungus biomass equivalent to 1 g L^{-1} were added. The pH of each solution was adjusted to 5.0 ± 0.1 using 0.1 M HCl or NaOH. The flasks were incubated on an orbital shaker at 120 rpm and 25 ± 2 °C for a specific contact time to ensure effective interaction between the metal ions and the immobilized fungal biomass. After incubation, the samples were centrifuged or filtered through a 0.45 µm filter paper to separate the immobilized fungal biomass. The residual concentration of metal ions in the supernatant was measured using a UV–Visible spectrophotometer at their respective λmax values specific to each metal complex for blank, 5 days, 10 days and 15 days.

Control experiments containing only the metal solution without biomass were also maintained to account for any abiotic changes (Oyewole et al. 2019).

Synthesis of ZnO Nanoparticles and Preparation of ZnO–Fungal Nanoconjugates (RF1G, RF7, RF16)

Pre–formed ZnO–fungal nanoconjugates (From Siddhacham laboratory, Raipur, Chhattisgarh), of three fungal strains—RF1G, RF7, and RF16—were used individually.

Chemical synthesis method was applied for nanoparticle formation - For each strain, 0.5 g of dried fungal biomass powder was dispersed in 50 mL of distilled water and sonicated for uniform suspension. A pre-synthesized ZnO nanoparticle suspension (containing 0.5 g ZnO NPs in 50 mL distilled water) was then added slowly to the fungal suspension under magnetic stirring. The mixture was stirred continuously for 3–4 hours at room temperature to facilitate surface adsorption and binding interactions between ZnO nanoparticles and fungal biomolecules, forming ZnO–fungal composites. The resulting mixture was centrifuged and washed with distilled water to remove unboundnanoparticles. The obtained ZnO–RF1G, ZnO– RF7, and ZnO–RF16 composites were dried at 60 °C for 12 hours and stored in airtight containers for further characterization and adsorption studies.

Biosorption of heavy metal using ZnO–fungal nanoconjugate – Biosorption experiments were carried out to investigate the removal of heavy metals—chromium (Cr) from potassium dichromate ($K_2Cr_2O_7$), mercury (Hg) from mercury sulfate ($HgSO_4$), lead (Pb) from lead acetate, and aluminum (Al) from aluminum chloride ($AlCl_3$)—each at a concentration of 80 ppm using four nanoparticle adsorbents: ZnO nanoparticles (ZnO NPs), ZnO-RF1G, ZnO-RF7, and RF16. For each experiment, 50 mL of the respective metal solution was placed in 250 mL Erlenmeyer flasks, and a known amount of nanoparticle adsorbent equivalent to 1 g L^{-1} was added. The pH of each solution was adjusted to 5.0 ± 0.1 using dilute HCl or NaOH. The flasks were shaken at 120 rpm on an orbital shaker and maintained at 25 ± 2 °C for different contact times ranging from 5 to 240 minutes to ensure efficient interaction between metal ions and the adsorbents. After incubation, the mixtures were centrifuged or filtered through 0.45 µm filter paper to remove the nanoparticles. The residual concentrations of Cr, Hg, Pb, and Al in the supernatant were analyzed using a UV–Visible spectrophotometer at their respective λmax values specific to each metal ion. Control samples containing metal solutions without nanoparticles were also run simultaneously to correct any non-adsorptive losses. The adsorption efficiency and percentage removal of metals were calculated from the difference between the initial and final absorbance values obtained from the spectrophotometric analysis (Oyewole et al. 2019).

Comparative Analysis of Free-Form, Immobilized and Nanoconjugate Form for Biosorption Efficiency

The comparative analysis of free form, immobilized and nanoconjugate form was done by comparing the residual concentration of Cr, Pb, Al and Hg in the solution against control after fixed period of time. The results were expressed as percentage of removal efficiency of heavy metals from all three forms (Oyewole et al. 2019). Finally, metal removal efficiency of biosorption was calculated by following formula given by Sahin and Keskin (2013) **% Removal Efficiency = C0 – C/ C0 * 100** Where, C0 = Initial metal concentration before biosorption, C = Final metal concentration after biosorption.

Statistical Analysis

Microsoft Excel 2021 version was used for plotting Error bar graphs for studying removal efficiency and for comparison of removal efficiency between all fungal forms t-test was calculated (at p<0.05% significant level).

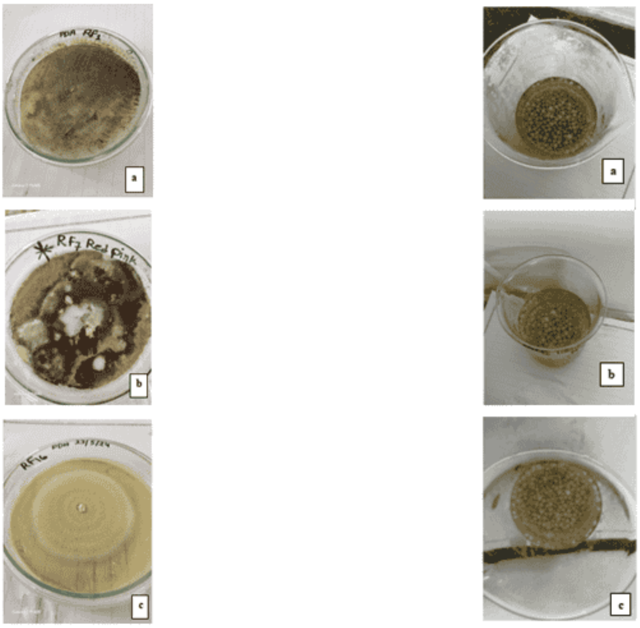
Phenotypical Identification of Fungal Strain by Microscopic Examination

Fungal strains were sent to the Agharkar Research Institute, Pune, Maharashtra), India, for authentication by microscopic examination of morphological characters using microscope [Labomed, Digital Digi 21500, Merck, USA].

Result

Comparative Analysis of Free, Immobilized and Nanoconjugate Forms of Fungal Isolates for Biosorption Efficiency

The removal efficiency of RF 1, RF 7, RF 9, RF 11, RF 12 and RF 16 for Cr, Pb, Al and Hg was reported to be in the approximately in similar percentage range without any sharp significant difference. Thus, it was reported that mostly all the isolates could be used for further investigations. Hence, out of 6 isolates, randomly 3 fungal isolates named as RF 1, RF 7 and RF 16 were further processed for immobilization in calcium alginate beads (Photo 1a & 1b) and conjugation with Zinc oxide (ZnO) nanoparticle. The comparative analysis of the biosorption potential between the free form, immobilized form and nanoconjuagte form for all the four metals (Cr, Pb, Al and Hg) were performed. The statistical t-test was also performed to compare the significant/ or non-significant removal efficiency of all the four metals by free, immobilized and nanoconjugate forms of 6 fungal isolates.



Photograph 1a & 1b: Free and Immobilized beads of three fungal isolates named RF 1(a), RF 7(b) and RF 16(c).

Biosorption Efficiency of Free, Immobilized and Nanoconjugate Forms *Vs* Chromium

The bar graph represented the percentage of Cr removal efficiency by free, immobilized and nanoconjugate form of RF 1, RF 7 and RF 16 isolates **(Fig. 1).** The results indicated that the free form showed very low removal efficiency (close to 0–5%) for all isolates (RF 1, RF 7, and RF 16) whereas a significant increase in removal efficiency was reported ranging from ~40–70% for immobilized form. This indicated poor chromium uptake capacity when the cells are used directly in suspension as compared to immobilized one. The RF 7 had the highest removal efficiency at approximately 70% among immobilized forms of all 3 isolates, followed by RF 16 ~50% and RF 1 ~40%. The result suggested that immobilization enhances stability and reusability, improving chromium biosorption. On the other hand, the nanoconjugate form also showed high removal efficiency (≈55–65%), but comparable to or slightly lower than the immobilized form. The nanoconjugate of RF 1 and RF 16 showed similar performance (~60%), while RF 7 showed slightly lower (~60%) efficiency than its immobilized counterpart **(Table 1).**

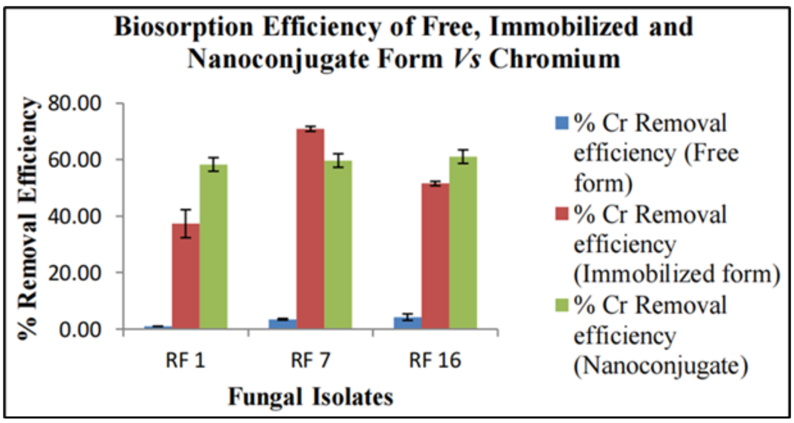


Fig. 1: The comparison between free, immobilized and nanoconjugate form of RF 1, RF 7 and RF 16 for removal efficiency of chromium (in percentage). Each bar represents Mean ± SE of three experiments.

Table 1: The table represents the chromium removal efficiency (in percentage) of free, immobilized and nanoconjugate form of RF 1, RF 7 and RF 16.

S.No.	Fungal Isolate	% Cr Removal Efficiency (Free form)	% Cr Removal Efficiency (Immobilized form)	% Cr Removal Efficiency (Nanoconjugate)
1	RF 1	1±0.02	37.34±4.96	58.33±2.40
2	RF 7	3.5±0.28	70.93±0.88	59.72±2.41
3	RF 16	4.25±1.15	51.58±0.77	61.10±2.40

The percentage of chromium removal efficiency is expressed as Mean ± SE of three replicates. The free, immobilized and nanoconjugate forms of fungal isolates were compared statistically by t-test for Cr removal efficiency. It was seen that immobilized and nanoconjuagte forms were almost significantly showing high removal efficiency as compared to free from. The t-stat values were greater than the t-critical value which proved the significant removal efficiency at $p<0.05$ **(Table 1).**

Table 2: The Statistical t-test comparison of all 3 fungal isolates (RF 1, RF 7 and RF 16) in their free, immobilized and nanoconjugate forms for chromium removal efficiency at $p<0.05$.

Metal	Fungal Isolates	Comparison	t-stat (two tail)	t-critical (two tail)	p-value	Significance	Null hypothesis
Chromium (Cr)	RF 1	Free *Vs* Immobilized	-7.33	4.30	0.02	P<0.05 (Significant)	Reject
		Free *Vs* Nanoconjugate	-23.86	4.30	0.00	P<0.05 (Significant)	Reject
		Immobilized Vs Nanoconjugate	-7.09	4.30	0.02	P<0.05 (Significant)	Reject
	RF 7	Free *Vs* Immobilized	-61.20	12.71	0.01	P<0.05 (Significant)	Reject
		Free *Vs* Nanoconjugate	-25.05	12.71	0.03	P<0.05 (Significant)	Reject
		Immobilized *Vs* Nanoconjugate	2.76	12.71	0.22	p>0.05 (Non-significant)	Accept
	RF 16	Free *Vs* Immobilized	-26.57	4.30	0.00	P<0.05 (Significant)	Reject
		Free *Vs* Nanoconjugate	-27.34	4.30	0.00	P<0.05 (Significant)	Reject
		Immobilized *Vs* Nanoconjugate	-3.02	4.30	0.09	p>0.05 (Non-significant)	Accept

This research indicates that using immobilized and nanoconjugated fungal biomass was more efficient for Cr removal compared to free fungal cells. Among the three tested isolates, RF7 showed the best overall performance. This finding highlighted that modifying fungal biomass can significantly enhance its ability to remove metals. Other studies have reported similar outcomes. For instance, Verma, Sati, and Rai. (2014) found that immobilized cells of *Ganoderma lucidum* and *Mucor hiemalis* were more effective at removing Cr (VI) than their free counterparts. Additionally, Chhikara and Dhankhar (2008) demonstrated that *Aspergillus niger* immobilized in alginate beads had a greater biosorption capacity and could be reused multiple times. Similarly, Oliveira et al. (2021) discovered that immobilized fungal biomass was more effective in removing Cd (II) due to its increased surface area, more binding sites, and enhanced stability. The superior performance of the modified RF7 in this study can be attributed to its larger surface area and stronger structure, which provides more contact points for metal ions and helps retain active sites. Overall findings suggest that immobilization and nanoconjugation are effective strategies to improve the biosorption efficiency and reusability of fungi. The RF7 isolate shows significant potential for treating industrial wastewater with heavy metals. Future research should utilize techniques like FTIR and XPS to identify the active binding groups and refine the process for larger-scale applications.

Biosorption Efficiency of Free, Immobilized and Nanoconjugate Forms *Vs* Lead

The bar graph in the figure represented the percentage removal efficiency of Pb by all three forms of fungal isolates (RF1, RF 7 and RF 16) (Fig. 2). The results indicated that the immobilized form has high removal efficiency of around 60-65% as compared to nanoconjugate form and free form (approximately 1-3%). Among all the isolates, RF 1 has moderate removal efficiency against RF 7 and RF 16 form (Table 3)

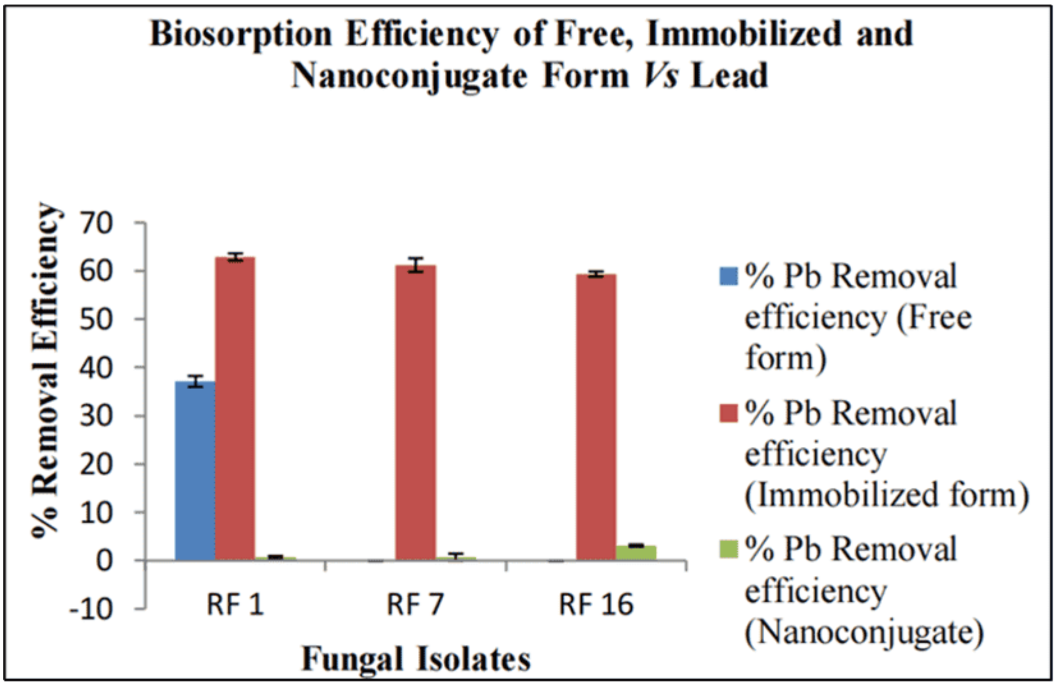


Fig. 2: The difference between free, immobilized and nanoconjugate form of RF 1, RF 7 and RF 16 for removal efficiency of lead (in percentage). Each bar represents Mean ± SE of three experiments.

Table 3: The table represents the lead removal efficiency (in percentage) of free, immobilized and nanoconjugate form of RF 1, RF 7 and RF 16.

S.No.	Fungal Isolate	% Pb Removal efficiency (Free form)	% Pb Removal efficiency (Immobilized form)	% Pb Removal efficiency (Nanoconjugate)
1	RF 1	37.1±1.12	62.85±0.75	0.68±0.30
2	RF 7	0	61.2±1.40	0.71±0.7
3	RF 16	0	59.37±0.52	3.09±0.23

The percentage of lead removal efficiency is expressed as Mean ± SE of three replicates. The percentage of Pb removal efficiency by free, immobilized and nanoconjugate forms of fungal isolates were compared statistically by t-test. It was seen that immobilized and nanoconjuagte forms were almost significantly showing high removal efficiency as compared to free from. The t-stat values were greater than the t-critical value which proved the significant removal efficiency at $p<0.05$ (Table 3)

Table 4: The Statistical t-test comparison of all 3 fungal isolates (RF 1, RF 7 and RF 16) in their free, immobilized and nanoconjugate forms for lead removal efficiency at $p<0.05$.

Metal	Fungal Isolate	Comparison	t-stat (two tail)	t-critical (two tail)	p-value	Significance	Null hypothesis
Lead (Pb)	RF 1	Free Vs Immobilized	-9.87	12.71	0.06	p>0.05 (Non-significant)	Accept
		Free Vs Nanoconjugate	25.01	12.71	0.03	P<0.05 (Significant)	Reject
		Immobilized Vs Nanoconjugate	56.74	12.71	0.01	P<0.05 (Significant)	Reject
	RF 7	Free Vs Immobilized	-54.89	12.71	0.01	P<0.05 (Significant)	Reject
		Free Vs Nanoconjugate	0.00	0.00	0.00	-	-
		Immobilized Vs Nanoconjugate	54.89	12.71	0.01	P<0.05 (Significant)	Reject
	RF 16	Free Vs Immobilized	-114.24	4.30	0.00	P<0.05 (Significant)	Reject
		Free Vs Nanoconjugate	75.42	4.30	0.00	P<0.05 (Significant)	Reject
		Immobilized Vs Nanoconjugate	-13.55	4.30	0.01	P<0.05 (Significant)	Reject

In this study, immobilized fungal forms were found to be the most effective at removing lead Pb ions from solution, outperforming both free and nanoconjugate forms. This enhanced performance was attributed to greater surface stability and more accessible binding sites in the immobilized forms. Verma, Sati, and Rai 2014 reported similar findings, noting that immobilized fungal biomass was more efficient in removing heavy metals compared to free cells, as immobilization increases surface area and protects cells during absorption/ adsorption. Conversely, the free fungal forms in this study exhibited lower Pb removal, likely due to a reduced number of active binding sites available for adsorption. This aligns with the observations of Ayele et al. 2021, who noted limited metal uptake in unmodified fungal biomass. The nanoconjugate forms showed very little Pb uptake, possibly because the nanoparticle coating limited the availability of active functional groups. Abbas et al. 2023 also noted that certain surface modifications could obstruct metal binding by blocking adsorption sites. Overall, these results indicate that immobilization improves metal removal efficiency, while excessive surface modification may sometimes diminish biosorption capacity.

Biosorption Efficiency of Free, Immobilized and Nanoconjugate Forms *Vs* Aluminum

The variation between free, immobilized and nanoconjugate forms of 3 isolates namely RF 1, RF 7 and RF 16 for Al removal capacity was represented by **Fig. 3, Table 5.** The results of the comparison indicated that all forms of fungal isolates showed good removal property. But, still free form of RF 1 had high removal efficiency of ~61% followed by immobilized form (~57%) and nanoconjugate form around 55–56%. In case of isolate RF 7 immobilized form had high removal efficiency (~60%) against the nanoconjugate (~58%) and free form (~38%). Similar results were reported for RF 16 isolate too, Al removal efficiency was in decreasing order as immobilized (~61%) > nanoconjugate (~59%) > free form (~6%). The overall results indicated that immobilization and nano conjugation enhance the removal efficiency as compared to free form.

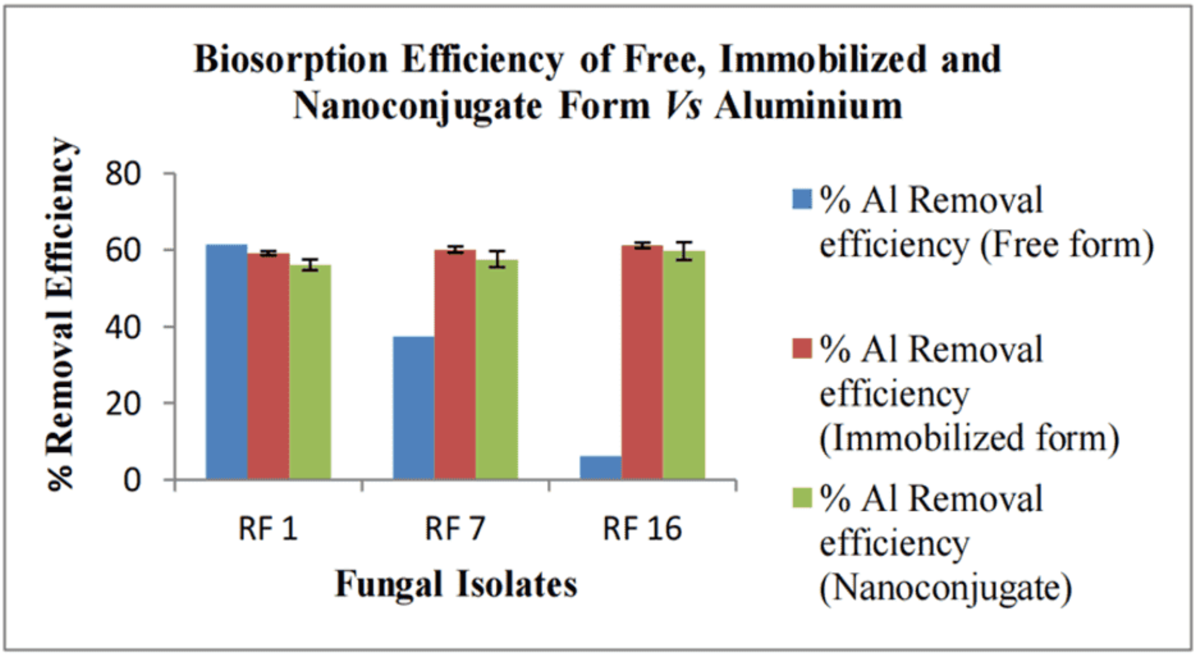


Fig. 3: Variation in aluminum removal efficiency (in percentage) of free, immobilized and nanoconjugate form of RF 1, RF 7 and RF 16. Each bar represents Mean ± SE of three experiments.

Table 5: The table represents the aluminum removal efficiency (in percentage) of free, immobilized and nanoconjugate form of RF 1, RF 7 and RF 16.

S.No.	Fungus Isolate	% Al Removal efficiency (Free form)	% Al Removal efficiency (Immobilized form)	% Al Removal efficiency (Nanoconjugate)
1	RF 1	61.32±1.29	59.16±0.54	56.12±1.41
2	RF 7	37.45±1.75	60.12±0.80	57.61±2.11
3	RF 16	6.17±1.16	61.20±0.71	59.7±2.32

The percentage of aluminum removal efficiency is expressed as Mean ± SE of three replicates. The free, immobilized and nanoconjugate forms of fungal isolates were compared statistically by t-test for Al removal efficiency. It was witnessed that almost all forms were non-significant except free ***Vs*** immobilized or free ***Vs*** nanoconjugate forms of RF 7, RF 16 showing high removal efficiency. The t-stat values was greater than the t-critical value which proved the significant removal efficiency at $p<0.05$ (Table 5).

Table 6: The comparison of all 3 fungal isolates (RF 1, RF 7 and RF 16) in their free, immobilized and nanoconjugate forms for aluminum removal efficiency by statistical t-test at p<0.05.

Metal	Fungal Isolate	Comparison	t-stat (two tail)	t-critical (two tail)	p-value	Significance	Null hypothesis
Aluminum (Al)	RF 1	Free Vs Immobilized	1.60	12.71	0.35	p>0.05 (Non-significant)	Accept
		Free Vs Nanoconjugate	2.45	12.71	0.25	p>0.05 (Non-significant)	Accept
		Immobilized Vs Nanoconjugate	6.55	12.71	0.10	p>0.05 (Non-significant)	Accept
	RF 7	Free Vs Immobilized	-5.18	12.71	0.12	p>0.05 (Non-significant)	Accept
		Free Vs Nanoconjugate	-32.98	12.71	0.02	P<0.05 (Significant)	Reject
		Immobilized Vs Nanoconjugate	0.48	12.71	0.72	p>0.05 (Non-significant)	Accept
	RF 16	Free Vs Immobilized	-36.39	4.30	0.00	P<0.05 (Significant)	Reject
		Free Vs Nanoconjugate	-15.37	4.30	0.00	P<0.05 (Significant)	Reject
		Immobilized Vs Nanoconjugate	0.70	4.30	0.56	p>0.05 (Non-significant)	Accept

The study revealed that both immobilized and nanoconjugate forms were more effective in removing aluminum than the free form, particularly for RF7 and RF16. This was consistent with the findings of Verma, Sati, and Rai (2014), which showed that immobilized fungal biomass was more effective at removing heavy metals due to its enhanced surface stability and greater availability of binding sites. Likewise, Ayele et al. (2021) found that altering the structure of fungal biomass improved its metal adsorption capabilities by increasing the number of active binding sites. The strong performance of the free form of RF1 indicates it has a natural capacity to bind metals, which align with Ayele et al. (2021), where certain native fungal isolates exhibited high tolerance and absorption/ adsorption abilities without any modifications. Overall, these results reinforce the notion that immobilization and nanoconjugation enhance the efficiency of fungal biosorption, supporting earlier research findings.

Biosorption Efficiency of Free, Immobilized and Nanoconjugate Forms *Vs* Mercury

The percentage of Hg removal efficiency of 3 fungal isolates in their free, immobilized and nanoconjugate form was represented by the graph given in **Fig. 4 and Table 7.** The results depicted that among all 3 isolates in their free form, RF 7 has moderate Hg removal of approximately 10% followed by RF 1 (~6%) and RF 16 (~2%). It indicated that free form had little potential for Hg biosorption due to presence of surface functional groups. The overall percentage of Pb removal was low in immobilized form also ranging from ~7–12% which was highest for RF1 followed by RF 7 and RF 16. Alternatively, the nanoconjugate forms of all 3 fungal isolates showed high Hg removal efficiency ranging from (≈20–22%).

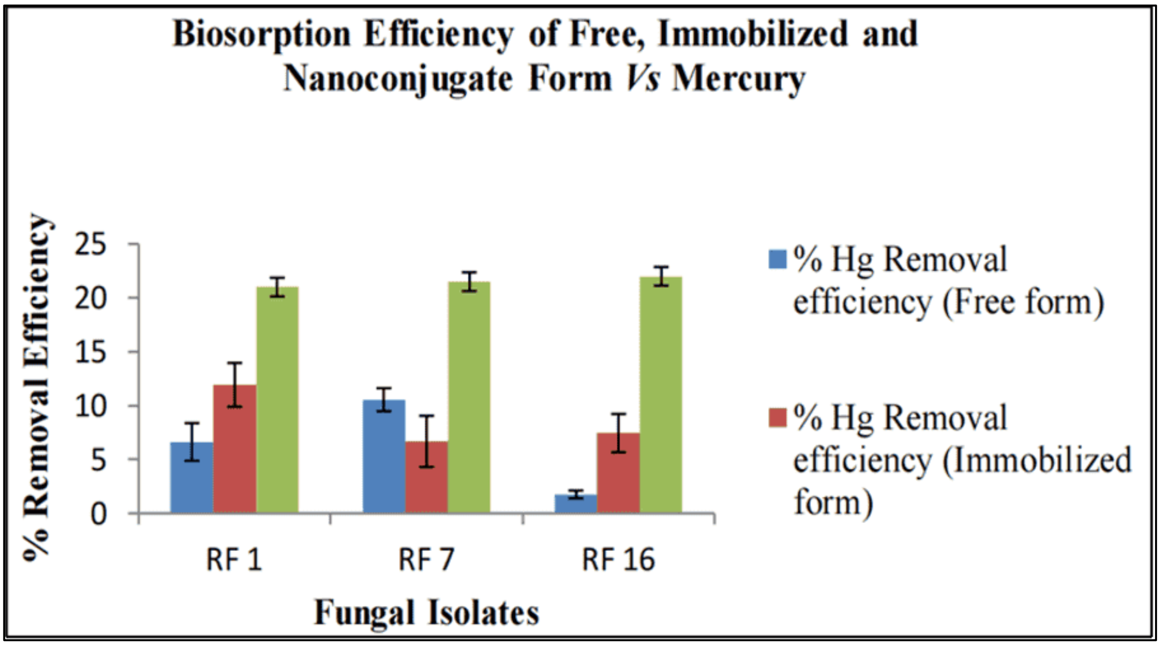


Figure 4: Variation in mercury removal efficiency (in percentage) of free, immobilized and nanoconjugate form of RF 1, RF 7 and RF 16. Each bar represents Mean ± SE of three experiments.

Table 7: The table represents the mercury removal efficiency (in percentage) of free, immobilized and nanoconjugate form of RF 1, RF 7 and RF 16.

S.No.	Fungal Isolate	% Hg Removal efficiency (Free form)	% Hg Removal efficiency (Immobilized form)	% Hg Removal efficiency (Nanoconjugate)
1	RF 1	6.625±1.75	11.925±2.03	21±0.87
2	RF 7	10.55±1.07	6.68±2.27	21.5±0.87
3	RF 16	1.75±0.36	7.44±1.78	22±0.87

The percentage of mercury removal efficiency is expressed as Mean ± SE of three replicates. The free, immobilized and nanoconjugate forms of fungal isolates were compared statistically by t-test for Hg removal efficiency. It was found that RF 1 fungus in their free, immobilized and nanoconjuagte forms was non-significant but while considering RF 7 and RF 16, free Vs immobilized and nanoconjugate were significant showing high removal efficiency as compared to other froms. The t-stat values greater than the t-critical value proved the significant removal efficiency at $p<0.05$ and t-stat smaller than t-critical value was non-significant at $p<0.05$ (Table 7).

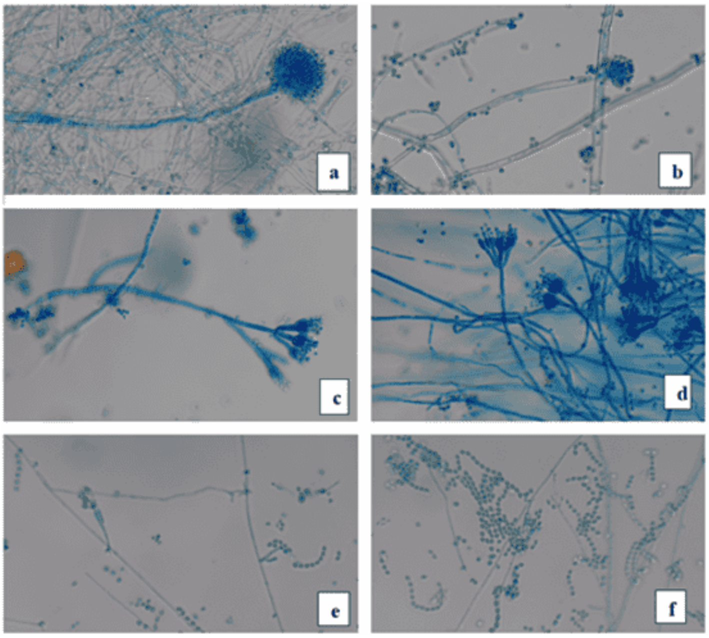
Table 8: The comparison of all 3 fungal isolates (RF 1, RF 7 and RF 16) in their free, immobilized and nanoconjugate forms for mercury removal efficiency by statistical t-test at p<0.05.

Metal	Fungal Isolate	Comparison	t-stat (two tail)	t-critical (two tail)	p-value	Significance	Null hypothesis
Mercury (Hg)	RF 1	Free Vs Immobilized	-1.12	12.71	0.46	p>0.05 (Non-significant)	Accept
		Free Vs Nanoconjugate	-12.15	12.71	0.05	p>0.05 (Non-significant)	Accept
		Immobilized Vs Nanoconjugate	-2.24	12.71	0.27	p>0.05 (Non-significant)	Accept
	RF 7	Free Vs Immobilized	52.11	12.71	0.01	P<0.05 (Significant)	Reject
		Free Vs Nanoconjugate	-4.56	12.71	0.14	p>0.05 (Non-significant)	Accept
		Immobilized Vs Nanoconjugate	-7.12	12.71	0.09	p>0.05 (Non-significant)	Accept
	RF 16	Free Vs Immobilized	-3.34	4.30	0.08	p>0.05 (Non-significant)	Accept
		Free Vs Nanoconjugate	-25.58	4.30	0.00	P<0.05 (Significant)	Reject
		Immobilized Vs Nanoconjugate	-5.77	4.30	0.03	P<0.05 (Significant)	Reject

This research indicated that nanoconjugate forms of fungi were significantly more effective at removing Hg compared to both free and immobilized forms. This suggests that combining fungal biomass with nanomaterials enhances the binding of metal ions by boosting surface activity, adsorption potential, and the availability of functional groups. For example, a review by Abate Ayele et al. 2021 noted that nanostructured fungal and other microbial composites have a higher capacity for heavy metal biosorption due to their improved surface reactivity and stability. In this study, free fungal forms achieved moderate Hg removal, which aligns with findings from A. Bahobil et al. 2017 that showed unmodified fungal biomass can remove metals like Hg but is not very efficient due to having fewer active sites. Conversely, the immobilized forms showed relatively low Hg biosorption, likely because diffusion limitations hinder metals from reaching active binding sites. This issue has been discussed in broader reviews on fungal biosorption, such as by Duraisamy et al. 2021, who pointed out that excessive immobilization can limit mass transfer and decrease metal uptake efficiency. Overall,these results suggest that nano conjugation enhances biosorption performance for Hg remediation, making it a promising approach.

Microscopic Identification of RF1, RF 7 and RF 16

Microscopic examination of three fungal isolates (RF1, RF7 and RF16) was carried out from Agharkar Research Institute, Pune, which was solely based on morphological characters. RF1 was identified as *Aspergillus flavus* Link (Photo. 2a & b), RF7 was identified as *Penicillium* sp. (Photo. 2c & d) and RF16 was identified as *Talaromyces* sp. (Photo. 2e & f).



Photograph 2: Microscopic images of 3 important fungal isolates (a & b) RF1- Aspergillus flavus Link, (c & d) RF7-Penicillium sp., (e & f) RF16- Talaromyces sp.

Conclusion

The comparative evaluation of the free, immobilized, and nanoconjugate forms of fungal isolates RF1, RF7, and RF16 revealed that biomass modification significantly enhanced biosorption efficiency for all tested metals. Immobilized forms showed the highest removal of chromium, lead, and aluminum, while nanoconjugates exhibited superior mercury uptake (~22%). Among the isolates, Penicillium sp. (RF7) proved most effective for Cr, Pb, and Al removal, whereas Talaromyces sp. (RF16) demonstrated the best performance for Hg. The enhanced metal uptake in modified forms can be attributed to increased surface area, structural stability, and availability of active binding sites.

Microscopic identification confirmed the isolates as Aspergillus flavus (RF1), Penicillium sp. (RF7), and *Talaromyces* sp. (RF16). Overall, the study establishes that immobilized and nanoconjugated fungal systems offer efficient, eco-friendly, and sustainable approaches for the bioremediation of heavy metal–contaminated environments.

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