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Assessment Of Bioremediation Potential And Sequencing Of Fungi Isolated From Contaminated Soil

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Abstract

Fungi effectively eliminate heavy metals from wastewater through the processes of bioaccumulation and biosorption. Fungi were isolated from contaminated locations to enhance their capacity to endure and eliminate heavy metals from wastewater. Bacterial and fungal samples were extracted from Hisar, Haryana using an enrichment culture. We conducted experiments on fungal isolates that exhibited resistance to heavy metals (Pb, Cd, Cr, and As) at concentrations of up to 100 ppm. The objective was to determine their ability to eliminate these metals from liquid media containing 50 ppm of each metal. This study investigated the species Aspergillus nidulans, Rhizopus arrhizus, and Trichoderma hamatum. Aspegillus nidulans and Rhizopus arrhizus could absorb 31.04mg/g and 26.55mg/g of Lead, respectively. A. nidulans and R. arrhizus shown high efficiency in removing Cd from polluted samples, with uptake capacities of 25.01 mg/g and 23.71 mg/g, respectively. Trichoderma hamatum efficiently absorbed Cr (13.21mg/g) and As (7.17mg/g). The molecular sequencing analysis identified three distinct strains: Aspergillus nidulans strain PSGSS08, Trichoderma hamatum strain FBL587, and Rhizopus arrhizus strain FBL578. These fungi can effectively eliminate elevated levels of metals from wastewater and industrial effluents, making them a highly attractive option for biosorption.

Keywords: Aspergillus, Bioremediation, Heavy metals, Molecular sequencing, Rhizopus, Screening, Trichoderma

Introduction

The growing use of metals and chemicals in process industries has led to the production of substantial amounts of wastewater that include elevated concentrations of hazardous heavy metals (Shrestha et al., 2021; Barakat, 2011). Their existence, resulting from inadequate disposal methods, presents environmental challenges because of their non-biodegradable and long-lasting characteristics (Azimi et al., 2017; Leal et al., 2023). These metals are absorbed by humans and animals through the food chain, leading to various metabolic diseases (Fu and Xi, 2021; Angon et al., 2023). In contrast to organic substances, metals have a long-lasting presence in the environment, offering continuous hazards to all species that come into contact

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with them. Soil can have a multiple composition if it gets polluted (Rashid et al., 2023). In other cases, it can be a mixture of many dissolved metal ions at different pH levels, along with salts, colloidal, and particulate matter, coming from different industries (Rai et al., 2023). Utilising microorganisms as biosorbents for heavy metals presents an appealing alternative to current methods like chemical precipitation, chemical oxidation or reduction, electrochemical treatment, filtration, ion exchange, and membrane technologies (Ghosh et al., 2023). This approach offers effective toxicity reduction and recovery of valuable metals from industrial effluents, while also being cost-effective due to the excellent performance and low cost of biosorbent materials (Gupta et al., 2023). These procedures can be inefficient or costly, particularly when the concentration of dissolved heavy metals in the solution falls within the range of 1-100 mg/l (Singh et al., 2024). Microorganisms can be used to remediate heavy metals by many ways, including bio-accumulation, biosorption, bio-precipitation, and uptake by pure biopolymers from microbial cells (Cheema, 2023). Hence, it is preferable to eliminate the presence of heavy metals in wastewater using eco-friendly and cost-effective technology before its use in agriculture or release into water sources (Pratap et al., 2023). Heavy metal-tolerant microorganisms may be found in locations contaminated by heavy metals (Alvarado-Campo et al., 2023). The susceptibility and efficacy of microorganisms in eliminating heavy metals exhibit significant variation (Wang et al., 2023). Hence, it is necessary to isolate and screen fungi and bacteria that are tolerant to heavy metals from areas that are contaminated with heavy metals. The current investigation aims to identify and evaluate fungi and bacteria that are capable of tolerating heavy metals, specifically lead (Pb), cadmium (Cd), and chromium (Cr). Their efficacy in extracting heavy metals from liquid medium was also assessed in controlled laboratory circumstances and molecular sequencing was performed.

2. Materials and Methods

2.1 Soil sample collection

We have discussed the method of isolation of Fungi in our previous paper (Khicher et al., 2023). To isolate fungal organisms, a total of 20 soil samples were collected from different areas in Hisar district. These samples were taken at four different time intervals and were selected specifically from regions that had a previous record of being treated with pesticides many times. The specimens were gathered in aseptic polypropylene resealable pouches and subsequently kept at a temperature of 4℃ until further handling.

2.2 Preparation of a solution containing heavy metals

The 1000 ppm stock solutions of lead (Pb), cadmium (Cd), and chromium (Cr) were prepared by dissolving Pb $(NO_3)_2$, $CdCl_2$, and $K_2Cr_2O_7$ (obtained from SD Fine-Chem Ltd., Mumbai, India) in double distilled water. The 25, 50, 100, and 400 ppm solutions of these heavy metals were generated by diluting a 1000 ppm stock solution with double distilled water. The stock

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solution of heavy metals was sterilized and then combined with sterilised potato dextrose and nutritional broth to achieve concentrations of 25, 50, and 100 ppm.

2.3 Primary and Secondary Screening of Fungal Samples

Fungal strains were obtained from different location samples using the serial dilution technique. Potato dextrose agar (Hi-Media, Mumbai, India) supplemented with 25 ppm of lead (Pb), cadmium (Cd), and chromium (Cr) separately was used for the isolation. A serial dilution was performed on each sample, resulting in a dilution of 10^6. One millilitre of the dilution at 10^4 and 10^6 was then placed on sterilised petri plates in duplicate. A volume of 20 ml of PDA medium, which contained 25 ppm of one of these heavy metals, was added to the petri plates. The plates were then incubated at a temperature of 28°C for a duration of 96 hours. The colonies consisting of the most prevalent genera of fungi were collected and purified using the streak plate method. Fungal isolates that were resistant to heavy metals at a concentration of 25 ppm were subjected to additional screening to determine their tolerance to Pb, Cd, and Cr at concentrations of 50 and 100 ppm individually on PDA. The fungal isolates were streaked on PDA medium supplemented with 50 and 100 ppm of each of the three heavy metals individually. The streaking of fungal isolates on normal PDA medium was used as a reference to compare their development on PDA medium with varying concentrations of heavy metals. Fungal isolates were observed for growth after 96 hours of incubation. The fungal isolates' growth was assessed as either normal or nonexistent in contrast to the control. This study comprised three fungi: *Trichoderma hamatum*, *Rhizopus arrhizus* and *Aspergillus nidulans*.

2.4 Assessment of heavy metal absorption by fungal strains from liquid medium

The fungal isolates, which have a high tolerance for heavy metals, were tested for their ability to absorb heavy metals in a potato dextrose broth medium. The medium included a concentration of 50 ppm of several heavy metals, including Pb, Cd, and Cr, each tested in triplicate. A solution of potato dextrose broth, with a concentration of 50 parts per million of one specific heavy metal, was distributed into 100 ml portions and placed in 250 ml conical flasks. The flasks were then sterilised at a pressure of 15 pounds per square inch for a duration of 15 minutes. The flasks were infected with 1 ml of a recently generated spore suspension (containing 106 to 107 spores/ml) of each fungal isolate. The flasks were then placed on a shaker at 150 rpm and kept at a temperature of 28°C for a duration of 96 hours. Control samples consisted of un-inoculated flasks with PD broth with a concentration of 50 ppm of various heavy metals. The fungal growth was collected after 96 hours by filtering it using a Whatman filter No. 42. The collected fungal biomass was washed with double distilled water three to four times and then dried in a hot air oven at a temperature of 80°C for a duration of 18 hours. The desiccated fungal biomass was measured in weight, and the quantity of heavy metals inside it was determined through digestion using a mixture of nitric acid and perchloric acid in a ratio of 3:1. The fungal biomass that had undergone digestion

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was passed through a Whatman filter No. 42 and the resulting filtrate was then adjusted to a volume of 50 ml in a volumetric flask.

The quantification of heavy metal absorption by fungal and bacterial biomass was determined using the equation:

$$q_e\,(mg/kg) = (1000\ CV)/W$$

where q_e represents the concentration of heavy metal accumulated by fungal/bacterial biomass in milligrammes per gramme (mg/g), C represents the concentration of heavy metal in parts per million (ppm), V represents the volume of the aqueous medium in millilitres (ml), and W represents the dry weight of the fungal biomass in grammes (g).

2.5 Molecular Sequencing by 18sRNA method

Fungal species were identified based on their Internal Transcribed Spacer rDNA region (ITS) sequencing data. The genomic DNA was extracted using the ZR Fungal DNA MiniPrepTM kit, following the manufacturer's procedure. The amplification of ITS1-5.8S rDNA-ITS2 areas was performed using universal primers ITS5: 5′-GGAAGTAAAAGTCGTAACAAGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ (Rakotonirainy et al., 2007) by PCR (Polymerase chain reaction). The experiment was conducted using the methodology outlined by Tarr (2004). The PCR product underwent purification using a PCR purification kit (AXYGEN) following the instructions provided by the manufacturer. The ABI 377 DNA sequencer was employed to sequence the amplified ITS sections in both directions using the Big Dye Terminator v3.1 cycle sequencing kit. The acquired sequences were used as a query sequence to search for similarities. In addition, the BLAST algorithm was employed to search the database hosted at NCBI (http://www.ncbi.nlm.nih.gov). The contiguous rDNA sequences of the highly effective fungal strains were uploaded to the GenBank database using the SEQUIN programme.

Results and Discussions

3.1 Isolation and identification of fungus that can tolerate high levels of heavy metals

Fungal enzymes break down heavy metals by integrating them into their metabolic pathways and using them as a source of carbon and energy (Sharma and Kumar, 2021). A total of 54 fungal isolates were obtained from different samples as studied in our previous study (Rimple et al, 2023). The fungal isolates were obtained using established procedures (Obire, and Anyanwu, 2009). Out of the total isolates, there were 10 that showed tolerance to Pb, 12 that showed tolerance to both Cd/Cr, and 24 that were fungal isolates. Additionally, there were 3 specific fungi (Aspegillus nidulans, Rhizopus arrhizus, and Trichoderma hamatum) that showed tolerance to Pb. These 3 fungi were further tested for their tolerance to Pb at concentrations of 50 and 100 ppm. The data shows a decline in the number of isolates that are resistant to increased concentrations of Pb. Among the 10 fungal isolates tested, only 3 were

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able to withstand Pb at a concentration of 100 ppm, while all 10 isolates were tolerant to Pb at a concentration of 25 ppm. A similar pattern was noted when examining fungal isolates for their resistance to Cd and Cr. This demonstrates the suppression of fungal development when exposed to greater concentrations of heavy metals. Prior studies have also documented the detrimental impact of elevated levels of heavy metals on the growth of fungi (Ameen et al., 2021; Riaz et al., 2021; Gajewska et al., 2022; Chen et al., 2022; Zhang et al., 2022)

3.2 Measurement of the absorption of Lead by fungal isolates from liquid media

Over the past decade, biomaterials have been tested for water metal removal (Sheraz et al., 2024). *Ganoderma lucidum* fruiting body fragments and culture substrate provided the study biomass (Rozman et al., 2020) and Waste Fungal Biomass (WFB) was utilised as an adsorbent straight after drying and pre-treatment with three chemicals, increasing Pb(II) and Cd(II) removal from 87% and 84% to 93% and 97%. This indicated their potential as biosorbents for the removal of Pb from industrial effluent with high concentrations of Pb (Rozman et al.,2022; Robinson et al., 2021). Previous research by Sharma et al showed that P. brevispora can efficiently remove Pb, Cd, and Ni from metal-contaminated water, which can be used to bioremediate industrial effluent. On the same grounds in another study Aspergillus biomass was tested for lead metal ion biosorption. Pretreated A. niger biomass eliminated 31.25 and 48.44 mg/g of lead metal ion at the same base concentration, compared to 3.84 and 16.42 mg/g at 2 and 9 mM (Chauhan et al, 2020). Nevertheless, the elimination of Pb^{2+} in our study was in line with the results reported by previous researchers. Our research exhibited a maximum absorption of 31.04 mg/g of Pb by *Aspergillus nidulans*. The lowest recorded absorption of Pb was 10.86 mg/g in Trichoderma hamatum. (Table 1). The significant uptake of Pb by A. nidulans suggested that these microorganisms have a greater number of binding sites on their cell walls (Kumar and Dwivedi, 2021; Emri et al., 2021).

3.3 Measurement of the absorption of Cadmium by fungal isolates from liquid media

Study conducted by Rozzman et al have represented the WFB a promising alternative for the removal of lead and cadmium ions from aqueous solutions. Further, a study conducted by Sharma et al showcased the use of *P. brevispora* to effectively eliminate Pb, Cd, and Ni from water contaminated with metals. This method can be employed for the bioremediation of heavy metals found in industrial wastewater. In another research, Aspergillus fumigatus showed the highest tolerance to Cd (II) at higher concentrations, with a clearance rate of 74.76 ± 0.24 and absorption of 5.02 ± 1.21mg/g. This fungus strain's metal tolerance may make it a promising mediator for bio-remediation of heavy metal damaged environments (Talukdar et al., 2020). In or research work, Aspergillus nidulans exhibited the highest Cd absorption, reaching a maximum of 25.04 mg/g, followed by Rhizopus arrhizus and (26.71mg/g) the lowest amount of Cd absorption (10.86 mg/g) was recorded in Trichoderma hamatum, as shown in Table 2. Aspergillus nidulans demonstrated the maximum absorption of Cd, suggesting their potential as effective biosorbents for removing Cd from aqueous solutions.

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3.4 Measurement of the absorption of Chromium by fungal isolates from liquid media

The Agency for dangerous Compounds and Disease Registry ranks chromium among the top 20 dangerous substances. Especially for soil-plant systems, environmental monitoring and chromium knowledge are crucial. Bioremediation is becoming the standard method for restoring heavy-metal-contaminated soils because it is cheaper and more environmentally friendly than physical and chemical methods, which are ineffective and expensive, especially when metal concentrations are low and produce massive amounts of toxic sludge (Sriharsha et al., 2021). In a study conducted by Wang et al., 2023 fungal pellet and fungal biofilm were used as biosorbents to sequester Cr(VI). The fungal biofilm removed much more Cr(VI) (97.81 ± 2.5%) than the fungal pellet (79.14 ± 2.4%), according to the results ($P < 0.05$; two-way ANOVA). Furthermore, studies conducted by Modkovski et al, tested two filamentous fungi from chromium-contaminated soil (Aspergillus fumigatus and Cladosporium spp.) and three microorganism bank organisms (Penicillium commune, Paecilomyces lilacinus, and Fusarium equiseti) to develop sustainable, safe biotechnologies for heavy metal removal in industrial effluent These organisms were tested for effluent Cr(VI) tolerance and removal. Exposure to Cr(VI)-containing conditions altered fungus growth. At 20 mg/L, metal-removal assays revealed 99% to 35% effectiveness, with Cladosporium spp. reaching the highest values. on the similar lines fungal isolates from liquid medium absorb Chromium in our current study. *Trichoderma hamatum* had the highest Cr absorption (13.21mg/g) and *Rhizopus arrhizus* had the lowest Cr absorption (2.52 mg/g) in PD broth with 50 ppm Cr. *Trichoderma hamatum* removed Cr from aqueous solutions with greater Cr concentrations by uptaking the most Cr.

3.5 Measurement of the absorption of Arsenic by fungal isolates from liquid media

Arsenic pollution is commonly acknowledged as a significant environmental hazard caused by human activities (Gupta et al., 2022). The issue of arsenic toxicity and its cleanup has garnered significant attention from various institutions, such as industries, environmental organisations, and the general public (Kamal et al., 2023). Gao et al demonstrated that with an initial algal biomass of 4.8×10^7 cells mL^{-1}, the fungal-algal pellets show a considerable capacity for eliminating As (V) from water, and microalgae may be crucial for the absorption and conversion of inorganic arsenic. In another study *Aspergillus* spp APR-1 and APR-2 demonstrated biosorption rates of 53.94% and 52.54%, respectively, when exposed to a 250 mM arsenic solution, as determined by inductively coupled plasma-optical emission spectrometry analysis (Tanvi et al., 2020). similar studies were obtained in our current research work. Fungal strains obtained from a liquid medium could absorb Arsenic. *Trichoderma hamatum* exhibited the greatest Arsenic absorption rate (7.17 mg/g), whereas *Rhizopus arrhizus* demonstrated the lowest chromium absorption rate (0.41 mg/g) in PD broth containing 50 ppm of Arsenic. *Trichoderma hamatum* effectively eliminated Arsenic (As) from high-concentration aqueous solutions by absorbing the highest amount of As.

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3.6 Identification of Fungal Strains

The fungal strains were identified by examining the amplified gene sequence of ITS1-5.8S rDNAITS2. The sequences were obtained from the NCBI nucleotide database using the BLASTN programme. The sequence exhibiting a similarity exceeding 90% was successfully retrieved. the strains obtained are as follows.

3.6.1 Aspergillus nidulans strain PSGSS08

ORIGIN

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      1 ctacctaaca ctgttgcttc ggtggggagc cccccagggg cgagccgccg gggaccactg
     61 aacttcatgc ctgagagtga tgcagtctga gcctgaatac aaatcagtca aaactttcaa
    121 caatggatct cttggttccg gcatcgatga agaacgcagc gaactgcgat aagtaatgtg
    181 aattgcagaa ttcagtgaat catcgagtct ttgaacgcac attgcgcccc ctggcattcc
    241 ggggggcatg cctgtccgag cgtcattgct gccctcaagc ccggcttgtg tgttgggtcg
    301 tcgtcccccc cgggggacgg gcccgaaagg cagcggcggc accgtgtccg gtcctcgagc
    361 gtatggggct ttgtcacccg ctcgattagg gccggccggg cgccagccgg cgtctccaac
    421 cttatttttc tcatggttga cctcggacac gtagggatac ccgctgaact taagcatatc
    481 aataagcggc aggaaaagaa accaacaggg attgccccag gaacggcg
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3.6.2 Trichoderma hamatum strain FBL587

ORIGIN

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      1 attgtgccag acaattctgt tctcagtctt gtcaacattt tttcccacca agcatcgcac
     61 cccgctttgt ctgcctacct acccctcctt tggcacagca aaaattttct ggctgccttg
    121 gttggttttt agtggggtgc caaatttttg gcagtgaccc cgccatcgcc aatggtcctc
    181 atgcactacc caacacatgc tacatatcaa ctgcttgatt caatgtgcta atcatacttc
    241 aatcaatagg aagccgccga actcggcaag ggttcattca agtatgcgtg ggttcttgac
    301 aagctcaagg ccgagcgtga gcgtggtatc accatcgaca ttgccctgtg gaagttcgag
    361 actccaaagt actatgtcac cgtcattggt atgttttcag tccgactggt cactatccca
    421 acatcatcat gctaacgtgc gactccacag acgctcccgg tcaccgtgat ttcatcaaga
    481 acatgatcac tggtacctcc caggccgatt gcgctatcct cattatcgct gccggtactg
    541 gtgagttcga ggctggtatc tccaaggacg gccagacccg tgagcacgct ctgctcgcct
    601 acaccctggg tgtcaagcag ctcattgttg ccatcaacaa gatggacact gccaactggg
    661 ccgaggctcg ttaccttgag atcatcaagg agacctccaa cttcatcaag aaagtcggct
    721 tcaaccccaa gaccgttgcc tttgtcccca tctctggctt caacggtgac aacatgctcc
    781 aggcctccac caactgcccc tggtacaagg gttgggagaa ggagaccaag gctggcaagt
    841 ccaccggtaa gaccctcctc gaggccattg acgccatcga gccccccaag cgtcccacag
    901 acaagcccct ccgtctgccc cttcaagatg tctacaagat cggtggtatc ggaacagtcc
    961 ctgtcggccg tatcgagact gatgtcctca agcccggtat ggtcgttacc tacgctccat
   1021 ccaacgtcac cactgaagtc aagtccgtag agatgcacca cgagcagctc gttgaggatg
   1081 tccccggtga caacgttgga ttcaacgtca agaacgtctc cgtcaaggat atccgccgtg
   1141 gtaacgttgc cggtgactcc aagaacgacc cccccatggg tgccgcttct ttcaacgccc
   1201 aggtcatcgt catgaaccac cctggccagg tcggtgccg
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3.6.3 Rhizopus arrhizus strain FBL578

ORIGIN

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   1 tgatttcatt cagaacatga ttactggtac ttctcaagcc gattgtgcta ttcttatcat
  61 tgctggtggt actggtgaat tcgaagctgg tatctccaag gatggtcaaa cccgtgaaca
 121 cgccctttg gctttcaccc tcggtgtccg tcaattgatt gttgctgtta acaagatgga
 181 taccaccaag tggtccgaag ctcgtttcaa cgaaatcgtc aaggaagttt cctccttcat
 241 caagaagatt ggttacaacc ccaagtctgt tcccttcgtc cccatctctg gttggcacgg
 301 tgacaacatg ttggaagaat ctaccaacat gccctggtac aagggatgga acaaggaaac
 361 caaggctggt gccaagtctg gtaagactct cttggatgcc attgacaaca ttgaccctcc
 421 tacccgtcct gttgacaagc ctctccgtct tcctcttcaa gatgtttaca agatcggtgg
 481 tatcggtact gtccccgtcg gtcgtgtcga aactggtgtc atcaaggctg gtatggttgt
 541 caccttcgct cctgctgctg tcaccactga agttaagtcc gtcgaaatgc accacgaaac
 601 cctcactgaa ggtcttcctg gtgacaacgt cgttttcaac gtcaa
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4. Conclusion

To summarise, we have collected samples of contaminated soil from various sites of Hisar, Haryana, India. These places are notorious for their high concentration of heavy metals, particularly lead (Pb, Cr, Cd and As), in the waste materials. The current study aimed to investigate a cost-effective and practical bioremediation approach for the elimination of Pb, Cr, Cd and As metals. Three fungal strains, *Trichoderma hamatum*, *Rhizopus arrhizus* and *Aspergillus nidulans*, have been obtained from the effluents of the batch cultures. All three fungi have been selected for optimisation studies to remove Pb, Cr, Cd, and As and have demonstrated efficient removal of these metal ions. According to the data, *Rhizopus arrhizus* and *Aspergillus nidulans* are suitable options for removing Pb and Cd from contaminated soil. Whereas, *Trichoderma hamatum* could be selected for removal of Cr and As from contaminated soil on a commercial scale.

Table 1: Table depicting Lead uptake from a 50 ppm Pb liquid medium by fungi

S.NO.	Fungal Strain	Uptake (mg/g)
1.	Aspergillus nidulans	31.04
2.	Rhizopus arrhizus	26.55
3.	Trichoderma hamatum	10.86

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Table 2: Table depicting Cadmium uptake from a 50 ppm Cd liquid medium by fungi

S.NO.	Fungal Strain	Uptake (mg/g)
1.	Aspergillus nidulans	25.01
2.	Rhizopus arrhizus	23.71
3.	Trichoderma hamatum	8.13

Table 3: Table depicting Chromium uptake from a 50 ppm Cr liquid medium by fungi

S.NO.	Fungal Strain	Uptake (mg/g)
1.	Aspergillus nidulans	12.4
2.	Rhizopus arrhizus	2.52
3.	Trichoderma hamatum	13.21

Table 4: Table depicting Arsenic uptake from a 50 ppm As liquid medium by fungi

S.NO.	Fungal Strain	Uptake (mg/g)
1.	Aspergillus nidulans	6.30
2.	Rhizopus arrhizus	0.41
3.	Trichoderma hamatum	7.17

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