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TRANSFORMING WASTEWATER: THE POWER OF NON-LIVING FUNGAL BIOMASS TO REMOVE HEAVY METALS

Mohammed Kadhem Abid[1*], Ahmed S. Abd Askar[2], Mahmood Jamal Abdulhasan[2], Fatma M. Mostafa[3]

[1]*Environmental Research Center, Al-Ayen Iraqi University, Dhi-Qar, 64001, Iraq*
[2]*Marshes Research Center, University of Thi-Qar, Thi-Qar, 64001 Iraq*
[3]*Microbiology Department, College of Pharmacy, Al-Ayen Iraqi University, Dhi-Qar, 64001, Iraq*

The occurrence of heavy metals in the ecosystem, particularly in water resources, poses a significant threat to human health. Our research examines the efficiency of fungal non-living biomass prepared from Aspergillus terreus, Aspergillus niger, Rhizopus arrhizus, and Rhizopus oligosporus in reducing heavy metal concentrations—cadmium (Cd), copper (Cu), and lead (Pb)—in wastewater. Wastewater samples were collected from the Nasiriyah station in Dhi Qar, and initial concentrations of (0.96 ppm) for lead, (0.122 ppm) for cadmium, and (0.67 ppm) foor copper were recorded. Within 24 hours, fungal biomass achieved statistically significant reductions ($p < 0.01$), with removal efficiencies ranging from 16.4% to 100% for lead, 19.7% to 100% for cadmium, and 18.9% to 100% for copper. Among the tested fungi, Aspergillus niger demonstrated the highest removal efficiency, while Rhizopus arrhizus exhibited the lowest. These findings highlight the aptitude of fungal non-living biomass, particularly Aspergillus niger, as a sustainable, eco-friendly, and affordable solution for the remediation of heavy metal contaminants in industrial wastewater.

Keywords: *Industrial wastewater treatment, heavy metal remediation, non-living fungus masses, Aspergillus niger*

INTRODUCTION

The industrial sector's quick development has significantly shared in global economic growth and human welfare. However, it has also led to increased waste generation, resource depletion, and energy consumption. These challenges necessitate the development of innovative strategies and effective methods to limit their environmental and societal effects[1-4]. The production of large volumes of wastewater, containing a diverse array of contaminants, represents one of the most significant environmental impacts of human activities[5,6]. Depending on the industry, different types of pollutants are created and released into the environment. Textile dyes, heavy metals, organic compounds, and any more persistent substances that pose a risk to the environment could be considered pollutants[7]. The pollution of water, which eventually causes major threats to aquatic life and public health, is the result of these various contaminants being released into the environment, such as in natural water bodies[8,9].

Traditional wastewater treatment techniques like coagulation, flocculation, adsorption, and the process of activated sludge are still in use today and are frequently employed to solve problems related to industrial wastewater contamination[10,11]. These traditional techniques have repeatedly shown themselves to be highly successful in treating common environmental contaminants, but they are ineffective at breaking down complex and resistant industrial pollutants such as heavy metals and aromatic dyes. In addition to this

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*Corresponding author: Mohammed Kadhem Abid, E-mail: Mohammedk.A.K@alayen.edu.iq

bottleneck, the degrading process of these traditional technologies results in the production of numerous secondary pollutants that are detrimental to the environment[12].

Heavy metal elements in soil and aquatic environments can be effectively reduced and removed by filamentous fungi via its adsorption ability on the external mycelia walls. Adsorption is the process of heavy precipitated complexes formation or the process of biosorption and element storage inside their cells[13,14]. Bennet et al.[15] showed that fungi are highly effective in bioaccumulation heavy metals; they also have the ability to absorb iron, cadmium, zinc and mercury in the aquatic environment, efficiently. Mycelium's effective absorption of heavy metals is due to the fungal hyphae's cell wall structure which comprises a set of compounds entailing protein and polysaccharides; the latter is further made up of secondary elements like hydroxyl and carboxyl on top of phosphate and various amino acids, all of which work together to bind heavy metal molecules[16,17.]

Aquatic plants, fish, and other environmental species are poisoned when heavy metals enter water bodies, disrupting the ecosystem's equilibrium. When these minerals get into the food chain, they become extremely harmful to people. Because they have several different mechanisms that make them effective in lowering the heavy metals' concentrations, such as adsorption on the walls of external fungal adsorption or the formation of heavy complexes that sedate and store elements within their cells, fungi play a significant task in lowering and the concentrations of many heavy metals' concentrations in the soil and aquatic environment[18].

The mycelium capability to effectively absorb iron, cadmium, mercury, and zinc in the aqueous environment, as well as the bioaccumulation of heavy metals, were observed. A collection of substances, including proteins and polysaccharides, make up the fungal mycelium. The latter contains amino acids and phosphates and is made up of secondary groups like carboxyl and hydroxyl. Each of these material classes affects how heavy element molecules bond. In order to lower concentrations of heavy metals like lead and cadmium as well as various physical and chemical contaminants, the current study compares the effectiveness of the Mycelium fungus to fungal biomass, *Aspergillus* niger, and Rhizopus oligosporium[19].

MATERIALS AND METHODS

Sample Collection

Samples of final wastewater were collected from the Nasiriyah station in Dhi Qar using clean, sterile 2-liter polyethylene bottles. The bottles were thoroughly rinsed with sterile distilled water before sample collection to ensure cleanliness. The collected samples were then transported to the laboratory in an ice box for subsequent fungal isolation[20].

Isolation of Fungi

The morphological characteristics of the isolates of fungi were examined and identified based on both macroscopic and microscopic features. Macroscopic features included colonial morphology, color, shape, diameter, and overall colony appearance. Microscopic examination revealed structures such as septation in mycelium, conidiophores, vesicles, conidia, and hyphae[21]. Visual investigations of the mycelia were supported by microscopic observations, and the results were further confirmed using PCR for accuracy.

After removing the effluent samples from the ice box,they were processed in sterile lab workstation. To concentrate the samples, 10 mL aliquots were aseptically distributed into centrifuge tubes and then centrifuged for 10 minutes at 250 rpm. The resulting residue (0.1 mL) from each sample was spread-plated using a sterile bent glass rod on duplicate plates of potato dextrose agar (PDA) and malt extract agar (MEA) (Oxoid Ltd., Basingstoke, UK), both prcessed with 50 µg/L of chloramphenicol to inhibit bacterial growth. The plates were incubated at room temperature (30°C) for seven days to allow fungal growth[22].

Selection of Primers

Based on the particular identification panel, **Table 2** displays the primer sequences that were used.

Table 2: Primer sequence used for identification of fungi[23]

Filamentous fungi panel	Species Primer sequence (5′ to 3′)
Aspergillus niger *Aspergillus terreus* *Rhizopus arrhizus*	**F-**ccctccttccaaacaaacaa **R-**tccagatcggctacacagaa **F-**gcggatgcaaggtgtaattt **R-**tactgcgcgttagttgaagc **F-**agaagcaaaatcatcgtcgaaag **R-**cgtaggtccagcgtaaacttg

Genomic DNA Extraction from Filamentous Fungi

Filamentous fungal strains were cultured on agar medium for four days at 30 °C prior to the genomic DNA extraction. DNA was isolated from filamentous fungus using the method outlined by Carvalho-Pereira et al [23].

In order to encourage a mechanical rupture of cell walls, A fungal biomass volum of500 µL was frozen inby liquid nitrogen and subsequently macerated. DNA was isolated in the aqueous phase using chloro-form: isoamyl alcohol (24: 1), and DNA precipitation was encouraged using sodium acetate (3 M). About 50 µL of warm, sterile water was used to resuspend the pellet after it had been dried overnight. After 45 minutes of incubation at 65 °C in a thermoblock, the samples were posteriorly homogenized. As directed by the manufacturer, Genomic DNA Purification Kits of JetQuick® (Invitrogen, Carlsbad, CA, USA) were used to purify the genomic DNA. To avoid contamination, all procedures were carried out in a category 2 laminar flow cabinet[24].

PCR Amplification

The CFX 96 TM Real-time PCR Detection Systems (Bio-Rad) was used to perform real-time PCR reactions. The PCR program included a pre-incubation step of three minutes at 95 °C, followed by 40 cycles of the following steps: 15 seconds at 95 °C, 30 seconds of annealing at 60 °C (for singleplex reactions) and 61 °C (for multifocal reactions), and 30 seconds of extension at 72 °C. A heat preservative step at 65 °C for 5 seconds and a step that matched the temperature ramp from 65 to 95 °C , the heating rate was 0.05 °C/s comprised the melting curve analysis[24-27].

Preparation of Non-Living Fungal Biomass

Fungal cells were cultivated for 36 hours, allowing complete utilization of the available sugar. The cells were then gathered by centrifugation at 5000 rpm for 5 minutes, then washed thoroughly by distilled water for impurities removal, and then dried at 80°C for 24 hours. The resulting dried biomass was utilized in subsequent experiments[28].

For the experiment, 50 ml of final discharge water from a sterile sewage treatment plant was combined with the prepared fungal biomass in 100 ml sterile glass beakers. Each fungal isolate was tested in triplicate, with an average biomass weight of 795 mg per replicate. Then we incubated beakers at 25°C for 24 hours, maintaining a pH range of 6.5 to 7.6. The concentrations of lead, cadmium, and copper were measured both pre and post treatment of by using an atomic absorption spectrophotometer. The APHA method[29] was employed for laboratory measurements, including the use of a pH meter to determine the pH value of the samples. The percentage reduction in heavy metal concentrations was calculated using the following formula:

% reduction in concentration = initial concentration - final concentration / initial concentration x 100%.

After the purification process, the used fungal biomass was safely removed and disposed of following the procedures outlined by Eckenfelder and O'Connor[30].

Statistical Analysis

Statistical analysis was accomplished by using the Analysis of Variance (ANOVA) test with the assistance of the SPSS software package.

RESULTS AND DISCUSSION

Results

The morphological and cultural characteristics of the isolated fungal strains were examined and are presented in **Fig. 1**. *Aspergillus terreus* colonies **(Fig. 1A)** displayed a distinct texture and pigmentation, while microscopic analysis **(Fig. 1B)** revealed its characteristic conidiophores and vesicles. *Aspergillus niger* **(Fig. 1C)** formed dense black spores on culture media, with microscopic imaging **(Fig. 1D)** highlighting its vesicles and radiating phialides. *Rhizopus arrhizus* colonies **(Fig. 1E)** exhibited a light brown growth pattern, with sporangiophores and sporangia visible under the microscope **(Fig. 1F).** Similarly, *Rhizopus oligosporus* colonies **(Fig. 1G)** demonstrated greenish-yellow pigmentation on culture media, and microscopic analysis **(Fig. 1H)** revealed well-defined sporangia and hyphal structures. These findings confirm the unique morphologic and microscopic characteristics of each fungal isolate.

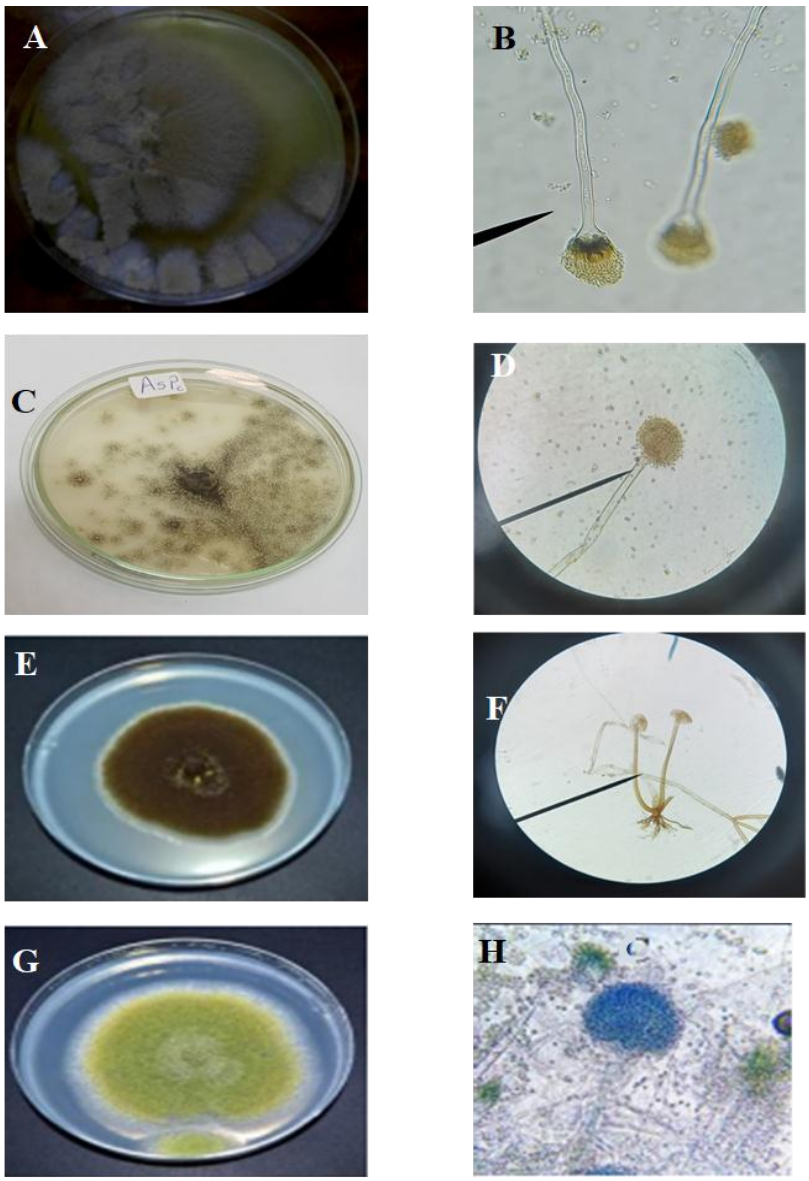

Fig. 1: Morphology and culture characteristics of isolated fungal strains. (A) Culture of *Aspergillus terreus*. (B) Microscopic images of *Aspergillus terreus*. (C) Culture of *Aspergillus niger*. (D) Microscopic images of *Aspergillus niger*. (E) Culture of *Rhizopus arrhizus*. (F) Microscopic images of *Rhizopus arrhizus*. (G) Culture of *Rhizopus oligosporus*. (H) Microscopic images f *Rhizopus oligosporus*.

The initial analysis of the wastewater samples revealed that copper had the highest concentration among the tested heavy metals, followed by lead and cadmium. This pattern underscores the prominence of copper contamination in the samples. The distribution of these metal concentrations is shown in **Fig. 2**.

The reduction in heavy metal concentrations (lead, cadmium, and copper) in wastewater samples before and after treatment with non-living fungal biomass demonstrates significant effectiveness. The results indicate a substantial decrease in metal concentrations after a 24-hour treatment period. Among the fungal isolates, *Aspergillus niger* achieved complete removal of all tested heavy metals, showcasing its superior efficiency. *Aspergillus terreus*, *Rhizopus arrhizus*, and *Rhizopus oligosporus* also contributed to significant reductions, albeit with varying degrees of efficiency, as detailed in **Table 1**.

Our findings indicate that *Aspergillus niger* demonstrated exceptional efficiency in removing heavy metals from wastewater within 24 hours of treatment, achieving highly significant reductions ($p < 0.01$). Similarly, *Aspergillus terreus* effectively reduced lead concentrations to 0.04 ± 0.02 mg/L, cadmium to 0.03 ± 0.02 mg/L, and copper to 0.08 ± 0.02 mg/L, with high significance ($p < 0.01$). Furthermore, *Rhizopus oligosporus* exhibited notable efficacy in reducing lead concentrations to 0.059 ± 0.001 mg/L ($p < 0.05$), cadmium to 0.029 ± 0.003 mg/L ($p < 0.01$), and copper to 0.076 ± 0.002 mg/L ($p < 0.05$) following a 24-hour treatment period. These results highlight the effectiveness of non-living fungal biomass in heavy metal remediation.

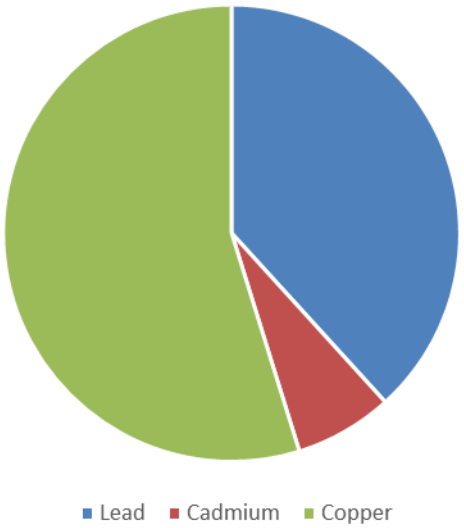

Fig. 2: Initial concentrations of heavy metals (lead, copper and cadmium) in wastewater samples before treatment.

Table 1: Reduction in lead, cadmium, and copper concentrations with treatment of indicated non-living fungal masses.

	Element	**Concentration before treatment (mg/L)**	**Concentration after 24 hours treatment (mg/L)**			
			Aspergillus terries	***Aspergillus niger***	***Rhizopus arrhizus***	***Rhizopus oligosporium***
1	**Lead**	0.67	*0.02 ±0.04	0	**0.001 ± 0.56	**0.001±0.059
2	**Cadmium**	0.122	*0.02 ± 0.03	0	*0.002 ± 0.098	*0.003 ± 0.056
3	**Copper**	0.96	*0.02 ± 0.08	0	**0.002 ± 0.78	**0.002 ± 0.076

Results as shown as mean ± standard deviation p value was calculated using ANOVA test. * Denotes significant differences ($P<0.05$) in comparison to post-treatment. ** Denotes highly significant differences ($P<0.01$) in comparison to pre-treatment.

In contrast, *Rhizopus arrhizus* demonstrated lower efficiency in reducing heavy metal concentrations following 24 hours of treatment. Lead concentrations were reduced to only 0.56 ± 0.001 mg/L ($p < 0.05$), cadmium to 0.098 ± 0.002 mg/L ($p < 0.01$), and copper to 0.78 ± 0.002 mg/L ($p < 0.05$). These findings align with the study by Jassim and Al-Shammari[31], which reported that *Aspergillus niger*, *Mucor arcindloiddes*, *Saccharomyces cerevisiae*, and *Penicillium austurianum* effectively reduced heavy metal contamination in soil, with iron concentrations significantly reduced by 60–75%. The study also highlighted the role of fungal hyphae in facilitating the adsorption process of heavy metals onto their cell walls, emphasizing the potential of fungi as efficient biosorption agents.

The current findings are also in line with that of and Ateshan et al.[31] which showed that the *Aspergillus* and *Rhizopus* fungi can effectively remove heavy metals following an 18-hour liquid culturation. Also supportive of the current findings are the studies of Teskova and Petrov,[32] which showed that the mass of *Rhizopus delemar* can efficiently lower heavy metal concentrations following liquid culturation.

The reduction percentages of lead, cadmium, and copper concentrations in wastewater after treatment with non-living fungal biomass are presented in **Fig. 3**. The results reveal significant differences in the effectiveness of the fungal isolates. *Aspergillus niger* demonstrated the highest efficiency in diminishing of lead, copper and cadmium, concentrations in the wastewater, achieving complete removal (100%) within 24 hours of treatment. In comparison, *Aspergillus terreus* and *Rhizopus oligosporium* also showed significant removal efficiencies. *Aspergillus terreus* reduced lead, cadmium, and copper concentrations by 94.03%, 75.41%, and 91.67%, respectively, while *Rhizopus oligosporium* achieved reductions of 91.19%, 76.22%, and 92.08%, respectively. Conversely, *Rhizopus arrhizus* exhibited much lower removal efficiencies, reducing lead, cadmium, and copper concentrations by 16.4%, 19.67%, and 18.75%, respectively. These results underscore the superior biosorption capabilities of *Aspergillus niger* and highlight the variability in performance among the tested fungal species.

The excellent capability of *Aspergillus niger* in reducing the aforementioned heavy metals' concentrations is due to the cohesiveness and large mass (973.4 mg) of its mycelium, whereby the direct wastewater contact caused the surface area to expand. Brunner and Frey,[33] and Ha et al.[34] demonstrated the ability of the *mycorrhizal* fungus in absorbing aluminum from soil via the wall surface of its mycelium. Abraham,[35] and Azemi et al. [36] proved the capacity of the *Rhizopus nigricans* fungus in removing chromium from liquid culture as well as the capacity of its mycelium in substantially spreading all over the model and increasing the surface area for adsorption.

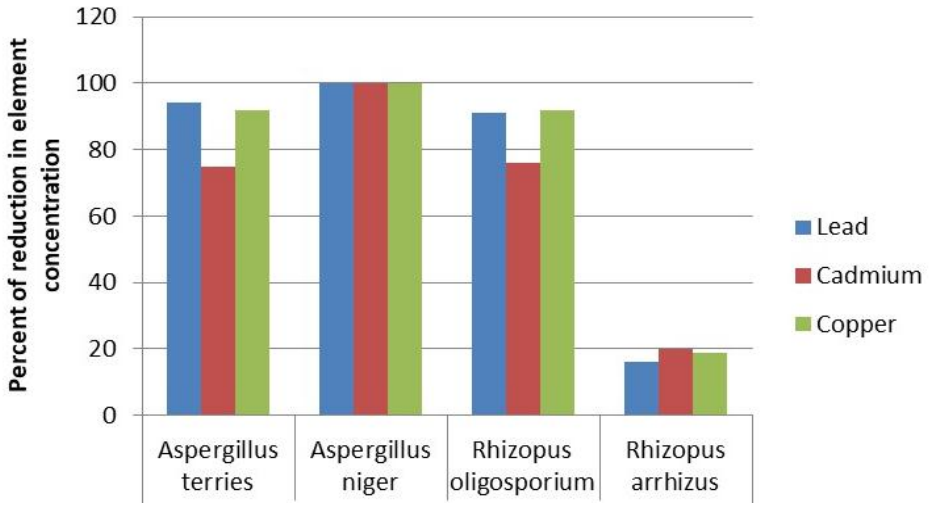

Fig. 3: Percentage reduction of heavy metal concentrations (lead, cadmium, and copper) in wastewater after 24-hour treatment with non-living fungal biomass. The bars represent the removal efficiency for each metal: blue for lead, red for cadmium, and green for copper**.**

Meanwhile, Rigling et al,[37] and Azemi et al.[36] demonstrated the excellent capacity of the *Armillaria* fungus in reducing zinc, lead, and cobalt concentrations from soil via bioaccumulation. Along the same line, Preetha and Viruthagiri,[38]and Ateshan et al.[31] proved the high efficiency of the *Rhizopus* fungus in reducing zinc concentrations from liquid culture. The current findings show that *Rhizopus arrhizus*, at 543.2 mg, is incapable of effectively lowering the lead, cadmium, and copper concentrations as a result of the non-cohesiveness of its mycelium and the build-up of light thickness mass, which ultimately weaken the absorption of the heavy metals. Fourest et al.[39] and Al Sailawi et al.[40] corroborated this finding by comparing the effectiveness of *Mucor miechei*, *Penicillium chrysogenum* and *Rhizopus arrhizus*, in lowering heavy metal concentrations from soil.

The stoichiometric interaction between the metal and the reactive chemical groups in the cell wall is the first step in what appears to be a two-part process of metal binding, followed by the inorganic deposition of larger amounts of metals. All of the metal ions pass through the cell wall before reaching the plasma membrane and cytoplasm of the cell. Therefore, the first element to come into contact with the metal ions is the cell wall. Because the cell wall functional groups provide multiple active sites that can bind metal ions, the process is thought of as a complicated ion exchanger that is comparable to a commercial resin. Variations in the composition of cell walls among various biosorbents, as well as variations within groups, might result in notable differences in the kind and quantity of metal ions that bind to them. Several distinct sequestering mechanisms have been proposed as the cause of metal binding in biosorption. They can be categorized based on a number of factors. The biosorptive mechanism can be classified as either metabolism-dependent or metabolism-independent processes[41].

Based on its reliance on the cell's metabolism. The interaction between metal nanoparticles and chemical or pharmaceutical active ingredients has been widely documented, with adsorption being the primary mechanism. For instance, the adsorption and subsequent removal of cefoperazone by CeO_2 nanoparticles have been reported[42].

Conclusions

Aspergillus terries, *Aspergillus niger*, and *Rhizopus oligosporium* have demonstrated excellent abilities to reduce heavy metal concentrations (such as lead, cadmium, and copper) in wastewater, achieving remarkable removal rates between 75% and 100%. Among these, *Aspergillus niger* stands out as particularly efficient, completely eliminating the studied heavy metals (100% reduction) after just 24 hours of treatment. In contrast, *Rhizopus arrhizus*, despite its thin and cohesive mass, proves to be less effective, with a modest reduction of only 16%-19% in heavy metal concentrations over the same 24-hour period.

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تحويل مياه الصرف الصحي: قدرة الكتلة الحيوية الفطرية غير الحية على إزالة المعادن الثقيلة

محمد كاظم عبيد[1*] – أحمد س. عبد عسكر[2] – محمود جمال عبد الحسن[2] – فاطمة مصطفى كمال[3]

[1]مركز البحث البيئي، جامعة العين العراقية، ذي قار، ٦٤٠٠١، العراق

[2]مركز أبحاث الأهوار، جامعة ذي قار، ذي قار، ٦٤٠٠١ ، العراق

[3]قسم الأحياء الدقيقة، كلية الصيدلة، جامعة العين العراقية، ذي قار، ٦٤٠٠١، العراق

نظرا لخطورتها علي صحة الانسان، نولي اهتمام خاص لوجود المعادن الثقيلة في البيئة خاصه في مصادر المياه. تم التحقيق في الكتلة الحيوية الفطرية غير الحية لـ Aspergillus niger و Aspergillus terries و Rhizopus arrhizus و Rhizopus oligosporium لإمكاناتها في تقليل تركيزات المعادن الثقيلة - الرصاص (٠.٩٦ جزء في المليون) والكادميوم (٠.١٢٢ جزء في المليون) والنحاس (٠.٦٧ جزء في المليون) - في مياه الصرف الصحي المجمعة من محطة الناصرية في ذي قار. أظهرت النتائج انخفاضًا مهمًا إحصائيًا ($P < 0.01$) في تركيزات المعادن خلال فترة ٢٤ ساعة. حققت الكتلة الحيوية الفطرية انخفاضات تتراوح من ١٦.٤٪ إلى ١٠٠٪ للرصاص، و ١٩.٧٪ إلى ١٠٠٪ للكادميوم، و ١٨.٩٪ إلى ١٠٠٪ للنحاس. أظهر Aspergillus niger أعلى كفاءة، حيث حقق إزالة كاملة (١٠٠٪) لجميع المعادن الثقيلة الثلاثة، مما يسلط الضوء على قدرته الفائقة على المعالجة البيولوجية. وعلى النقيض من ذلك، أظهرت Rhizopus arrhizus أدنى كفاءة، حيث تراوحت معدلات الإزالة بين ١٦.٥% و١٩.٦٨%. وتؤكد هذه النتائج على إمكانات الكتلة الحيوية الفطرية غير الحية، وخاصةAspergillus niger ، كطريقة مستدامة وصديقة للبيئة وفعالة من حيث التكلفة لإزالة الملوثات المعدنية الثقيلة من مياه الصرف الصناعي. وينبغي أن تركز الأبحاث المستقبلية على تحسين ظروف الكتلة الحيوية، وتوسيع نطاقها للتطبيقات الصناعية، والتحقيق في إمكانية إعادة استخدام الكتلة الحيوية الفطرية لتحسين الكفاءة.