**BIODIVERSITAS**
Volume 25, Number 8, August 2024
Pages: 2504-2511

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250822

# Bioremoval of $Pb^{2+}$ by *Aspergillus niger* D1RA, A heavy metal-resistant fungus isolated from an illegal gold mining site

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Manuscript received: 30 May 2024. Revision accepted: 12 August 2024.

**Abstract.** *Nofiani R, Mu'in R, Hafizah, Ardiningsih P. 2024. Bioremoval of $Pb^{2+}$ by* Aspergillus niger *D1RA, A heavy metal-resistant fungus isolated from an illegal gold mining site. Biodiversitas 25: 2504-2511.* Heavy metal pollution can cause serious problems for the environment and human health. One of the methods to eliminate this pollution is to use heavy metal-resistant fungi isolated from heavy metal-polluted environments. This study aimed to investigate the ability of heavy metal-resistant fungi to remove $Pb^{2+}$ in liquid media, Potato Dextrose Broth (PDB). Samples were collected from two locations, namely an abandoned illegal gold mining site and illegal gold mine, Samalantan, Bengkayang District, West Kalimantan, Indonesia. Each sample was inoculated on two different agar media (PDA= Potato Dextrose Agar and MEA= Malt Extract Agar) supplemented with 7.5 ppm $HgCl_2$. All fungal species that grew on the surface media were isolated, identified (based on spore morphology and Internal Transcribed Spacer [ITS]), evaluated (tolerance index [TI] against $Hg^{2+}$, $Pb^{2+}$, and $Zn^{2+}$), and assessed for their bioaccumulation capacity and $Pb^{2+}$removal efficiency. Four isolates (*Aspergillus* sp. OK2A, *Aspergillus* sp. OEA, *Aspergillus* sp. OEB and OEC) were successfully isolated from the abandoned illegal gold mine, while only one isolate (*Aspergillus niger* D1RA) was isolated from the illegal gold mining site. On the eighth day of incubation, the high tolerance level of each fungus to various selected metal concentrations was *Aspergillus* sp. OK2A in 40 ppm $HgCl_2$ and 300 ppm $ZnCl_2$; *Aspergillus* sp. OEA in 40 ppm $HgCl_2$ and 1,200 ppm $ZnCl_2$; *Aspergillus* sp. OEB in 800 ppm $Pb(NO_3)_2$; OEC in 20 ppm $HgCl_2$; *A. niger* D1RA in 40 ppm $HgCl_2$, 1,200 ppm $Pb(NO_3)_2$, 300 ppm $ZnCl_2$. Only *A. niger* D1RA showed a high tolerance for three metals and was further analyzed to determine the bioaccumulation capacity and removal efficiency of $Pb^{2+}$. The best bioaccumulation capacity and removal efficiency of $Pb^{2+}$ in PDB medium supplemented with 100 ppm $Pb(NO_3)_2$ at pH 4 were 237.776 mg/g dried biomass and 93.266 %, respectively. In conclusion, *A. niger* D1RA has the potential as a bioremediation agent to remediate $Pb^{2+}$ environments.

**Keywords:** *Aspergillus niger* D1RA, bioaccumulation, heavy metal-resistant fungi, tolerance index

## INTRODUCTION

Heavy metals (Cd, Cu, Cr, Hg, Mn, Ni, Pb and Pb) are categorized as toxic metals that can harm ecosystems and human health (Li et al. 2019). They are released into the environment by anthropogenic activities such as preservatives, fertilizers, pesticides, gold mining, metallurgical activities, etc. As a result, they accumulated and exceeded the normal average in the soil of mine drainage and surrounding agricultural fields throughout the world (Li et al. 2019; Zhang et al. 2024). Afterwards, they can enter the food chain and finally accumulate in biological tissues (Collin et al. 2022). Therefore, heavy metal pollution in water and soil is difficult to remove (Jing et al. 2021).

Heavy metals can be removed from the environment by physical and chemical techniques, such as coagulation, flocculation, membrane filtration, chemical precipitation, flotation, photocatalysis, ion exchange, electrochemical treatments, adsorption, etc. These techniques have many limitations such as failures which include insufficient metal sequestration, high costs, high reagents and/or energy requirements, and generation of toxic sludge or other waste products that require disposal (Liaquat et al. 2020). These techniques also become ineffective in removing heavy metal concentrations ≤100 ppm (Priyanka and Dwivedi 2023). The modern biological technique employs plants, microbes, or fungi to effectively eliminate low levels of heavy metals, such as heavy metal-resistant fungi (Liaquat et al. 2020).

Heavy metal-resistant fungi are defined as fungi that are able to survive in media containing toxic metals. Their ability to develop heavy-metal tolerance is either due to mutation or adaptation (Mohammadian et al. 2017). The adaption can occur through various mechanisms of detoxification or bioaccumulation either extracellular or intracellular (cell metabolisms). In the extracellular detoxification mechanism, most heavy-heavy metal fungi can excrete multiple organic acids, polymers, and anions (such as sulfides and phosphates), which can react with heavy-metal ions to obtain slightly soluble or insoluble compounds or salts (Calderón et al. 2020; Priyanka and Dwivedi 2023). For example, *Pleurotus ostreatus* ISS-1 excretes oxalate that can precipitate $Pb^{2+}$ by chelating $Pb^{2+}$ with oxalate to limit $Pb^{2+}$ entry into the cell (Xu et al. 2020).

In the intracellular mechanism, heavy metals enter inside a cell by active transport then accumulate by entrapping in the cell wall through biosorption and then undergo processes such as chelation, ion exchange, reduction, complexation, precipitation, and surface adsorption in the fungal cells (Priyanka and Dwivedi

2023). The heavy-metals with the active groups of the cell wall can form a polyatomic complex molecule, namely association of metal cations with a surrounding array of bound ligands (Escudero et al. 2019). In addition, $Pb^{2+}$ can be inhibited from entering cells by binding $Pb^{2+}$ to various functional groups in the fungal cell wall structure, such as hydroxyl, amides, carboxyl, and sulfhydryl groups. Cytochrome P450 and calcium signaling also play a role in $Pb^{2+}$ detoxification under $Pb^{2+}$ stress (Wang et al. 2019).

Heavy metal-resistant fungi are usually isolated from heavy metal-polluted environments such as an abandoned- or illegal gold mining site (Širić et al. 2016; Sanjaya et al. 2021; Văcar et al. 2021; Nofiani et al. 2022). Fungal species isolated from heavy metal-polluted environments can improve or increase their resistance or tolerance level (Raftos and Radford 2015; Firincă et al. 2024). However, their ability to remove heavy metals depends on their species and habitats (Fazli et al. 2015; Iram et al. 2015; Navnage et al. 2020; Galgowska and Pietrzak-Fiecko 2021). For example, *Aspergillus fumigatus* isolated from the Chemu Lagoon, Ghana can accumulate $Ar^{3+}$, $Pb^{2+}$ and $Zn^{2+}$ higher than *Aspergillus niger* D1RA *niger* (Doku and Belford 2015). In this study, we explored heavy metal-resistant fungi from two locations, the abandoned illegal gold mining site and illegal gold mine, Samalantan, Bengkayang District, West Kalimantan, Indonesia. This study aimed to investigate the ability of the best heavy metal-resistant fungus for $Hg^{2+}$, $Pb^{2+}$, and $Zn^{2+}$ based on its tolerance index (TI) and its bioaccumulation capacity and percentage removal in PDB media supplemented with 100 ppm $Pb(NO_3)_2$ at certain pH and time.

## MATERIALS AND METHODS

### Soil sampling

Sampling was performed on November 27th 2020, at two locations Samalantan, Bengkayang District, West Kalimantan, Indonesia: an abandoned illegal gold mining site and illegal gold mine (Table 1). Each sample was placed in sterile polyethylene plastic, tightly tied, labelled, and transferred to the laboratory for further analysis.

### Isolation of heavy metal-resistant fungi

Each soil sample (1-3 grams) was resuspended in 10 mL of distilled water, shaken at 120 rpm for an hour, and left until it separated into precipitation and liquid. The liquid was inoculated in three different media, malt extract agar (MEA), potato dextrose agar (PDA), and Czapek dox agar (CDA) supplemented with 7.5 ppm of $HgCl_2$ and incubated at 30°C and observed every day. $HgCl_2$ was a representative metal used to screen heavy metal-resistant fungi. Each appeared fungal colony was transferred to a new media until obtaining a homogenous colony.

### Identification of heavy metal-resistant fungi

The fungal isolates were identified using morphological and molecular approaches. For the morphological approach, each fungus was inoculated on a PDA medium and incubated at 30°C for five days, and then, the naked eye observed spore colour. The morphological identifications were performed by taking the spores from an MEA plate using a sterilized inoculating loop, then mixed with one drop of lactophenol cotton blue stain on a glass slide, then covered in a cover glass and observed under light microscopes with 100× magnification. The results were then matched with fungal spore standards or references.

The molecular approach was carried out using the ITS (internal transcribed spacer) region. The fungal DNA was extracted using a Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). The ITS region was amplified using MyTaq HS Red Mix (Bioline, BIO-25048) with ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') as primers, and the PCR reaction and program were carried out following the manufacturer's guidelines. The PCR product was sequenced by Sanger DNA sequencing and the sequence could be accessed on GenBank with accession number PP837326. The partial ITS sequences were used as a query sequence against the NCBI ITS sequences database using BLAST search. All the ITS sequences were aligned using the ClustalW method and then constructed a phylogenetic tree using the Neighbour-Joining method with 1,000 bootstrap replications using Mega 11 software (Tamura et al. 2021).

**Table 1**. Location for sampling in Samalantan, Bengkayang District, West Kalimantan, Indonesia

| Location coordinate | Sample code | Sample collection point |
|---|---|---|
| The abandoned illegal gold mining site | | |
| N 0°49'24.0492" E 109°09'05.9652" (abandoned for six months) | D1K | Mine excavation pond |
| | D1L | Mine waste disposal site |
| | D1R | Rhizosphere around the mine |
| N 0°49'11.7012" E 109°09'09.4932 (abandoned for two years) | D2K | Mine excavation pond |
| | D2L | Mine waste disposal site |
| | D2R | Rhizosphere around the mine |
| N 0°49'14.3112" E 109°09'03.3552" (abandoned for three years) | D3K | Mine excavation pond |
| | D3L | Mine waste disposal site |
| | D3R | Rhizosphere around the mine |
| The illegal gold mining site | | |
| N 0°48'59.3028" E 109°09'11.4588" | OK1 | Mine excavation ponds at a depth of 3 meters |
| | OK2 | Mine excavation ponds at 10-15 meters depth |
| | OE | Gold extraction ponds |
| | OL | Mine waste disposal sites |

**Determination of tolerance index**

The tolerance index (TI) value was determined by inoculating a 5 mm agar plug of each isolate on a PDA medium (pH 5.5) as a control and PDA medium (pH 5.5) with different heavy metal concentrations ($Hg^{2+}$, $Pb^{2+}$, and $Zn^{2+}$) in six replicates. The different heavy metal concentration was 5; 7.5; 10; 15; 20; 40; 80 and 160 ppm for $HgCl_2$; 400; 800; 1,200 and 1,600 ppm for $Pb(NO_3)_2$; 300; 600; 1,200 and 2,400 ppm for $ZnCl_2$. After incubation at 30°C, the diameter of fungal mycelia was measured on days 2 and 8 using a vernier calliper. The TI was calculated as follows:

$$TI = \frac{\text{Diameter of colony grown in media supplemented with heavy metal}}{\text{Diameter of colony grown in media without heavy metal}}$$

**Pb uptake of heavy metal-resistant fungi for different pH and time**

The starter was prepared by collecting fungal spores from PDA media using a sterilized inoculating loop and subsequently resuspended with 5-7 mL of sterilized distilled water to obtain a spore suspension. The concentration of the spore suspension was measured using a hemocytometer. A $1\times10^6$ spores/mL was inoculated on 40 mL of PDB with different pH (3, 4, 5, 6, and 7) and supplemented with 100 ppm of heavy metal ($Pb(NO_3)_2$), then shaken at 200 rpm, 30°C for five days. For non-spore fungus, a 10 mm agar plug was inoculated on 50 mL of PDB medium and shaken at 200 rpm and 30°C. The 100 mL Erlenmeyer flask with a spring was used for all this test. Bioaccumulation capacity or heavy metal uptake was calculated as follows:

$$Q = \frac{V(Co - Cf)}{m}$$

Removal efficiency was calculated as:

$$Y = \frac{Co - Cf}{Cf} x100\%$$

Where:

Q : bioaccumulation capacity or heavy metal uptake (mg/g)
Y : removal efficiency (%)
V : the initial sample volume (L)
Co, Cf : the initial and final of heavy metal in the liquid medium respectively (mg/L)
m : the dried fungal biomass (g)

## RESULTS AND DISCUSSION

**Sampling and isolation of heavy metal-resistant fungi**

Fungi resistant to one metal typically also exhibit resistance to other metals. Therefore, screening of heavy metal-resistant fungi, specifically $Hg^{2+}$, $Pb^{2+}$, and $Zn^{2+}$ was conducted using $HgCl_2$ 7.5 ppm added in a growth media. $HgCl_2$ played a role as a representative metal to screen heavy metal-resistant fungi.

Thirteen samples collected from the abandoned illegal gold mining site and illegal gold mine, Samalantan, Bengkayang District, West Kalimantan, Indonesia, were inoculated in three different media (CDA, MEA and PDA) supplemented with 7.5 ppm of $HgCl_2$. The results showed four fungi were isolated from 2 sample location points (OE and OK2), the illegal gold mining samples, which three fungi (OEA, OEB, and OEC) were isolated from the OE using MEA medium, while one fungus (OK2A) was isolated from the OK2 using PDA medium (Figure 1.A). Only one fungus (D1RA) was isolated from the D1R sample location point, the abandoned illegal gold mining (D1R), using MEA medium (Figure 1.B). Most mercury-resistant fungi grew on MEA compared to PDA, while none on CDA. MEA is a rich media for fungal growth due to its high protein and essential amino acid content, such as cysteine, whose thiol can form a complex with $Hg^{2+}$, probably reducing $Hg^{2+}$ intake to fungi (Ajsuvakova et al. 2020). The Hg content in the media becomes lower, causing more fungi to grow compared to the other media. PDA is also a rich media with low protein content, while CDA is a poor media that does not contain protein or amino acids due to its use of $NaNO_3$ as an N source.

Preliminary identification of each fungus was carried out by morphological observation using macroscopic and microscopic approaches. One of five fungi (OEC) did not produce spores on MEA and PDA media. Therefore, microscopic identification using morphological spores could not be used. The other fungi that produced spores: yellow (OK2A and OEA) with concentric rings and black (OEB and D1RA) on MEA for 5 days of observation (Figure 1.C), while all the reverse colonies were pale yellow. The morphological spores of OEB, and D1RA exhibited a swollen vesicle entirely or partially covered with flask-shaped phialides, which might develop into a uniseriate form for OK2A and OEA or biseriate form for OEB and D1RA and produced chains of mostly round shapes (Figure 2). *Aspergillus oryzae* is identified as a uniseriate form and produces concentric rings with yellow-green spores on MEA (Saikia et al. 2022) while *A. niger* has a biseriate form and dark brown or black spores (Šimonovičová et al. 2021). OK2A and OEA were probably the same species while OEB with D1RA based on their spore color and morphological spores. From spore color and morphological spores, OK2A and OEA were probably close to *A. oryzae* while D1RA and OEB were perhaps close to *A. niger*. However, toxic compound content in contaminated sites may affect microscopic fungi and cause genome alteration, which may change their physiology (Šimonovičová et al. 2021). *Sarocladium* sp. M2 and *Sarocladium* sp. M6 grown in the presence of different concentrations of Cd(II) changed the morphology and size of spores compared with the control (Zhang et al. 2024). Therefore, their species were confirmed using a molecular approach.

D1RA, one of five heavy metal-resistant fungi, was re-confirmed using the ITS region in its species. A PCR product of approximately 700 bp was obtained by amplifying the ITS region using ITS1 and ITS4 primers (Figure 3.A). The PCR product was subsequently sequenced and phylogenetic analysis. The result indicated D1RA was in one clade with *A. niger* (Figure 3.B), which also was consistent with its morphological characteristics. Therefore, D1RA was identified as *A. niger* and named *A. niger* D1RA.

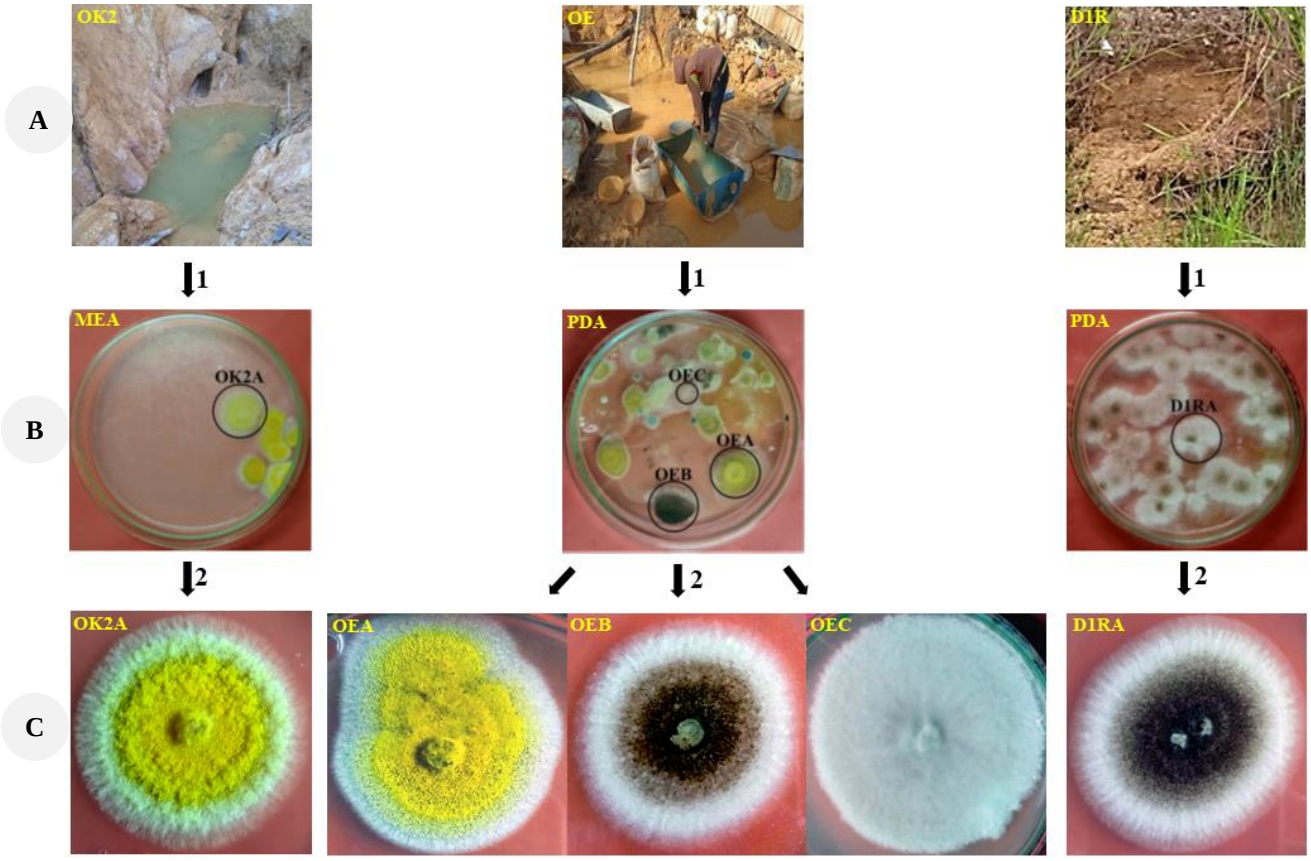


**Figure 1**. A. Selected sampling locations; B. Colonies obtained from inoculation of the samples on MEA media supplemented with 7.5 ppm of $HgCl_2$ for D1R sample and PDA supplemented with 7.5 ppm of $HgCl_2$ for OK and OE samples; C. A pure fungal colony was incubated for 5 days in MEA. 1. Inoculation of the samples 2. Purification of colonies

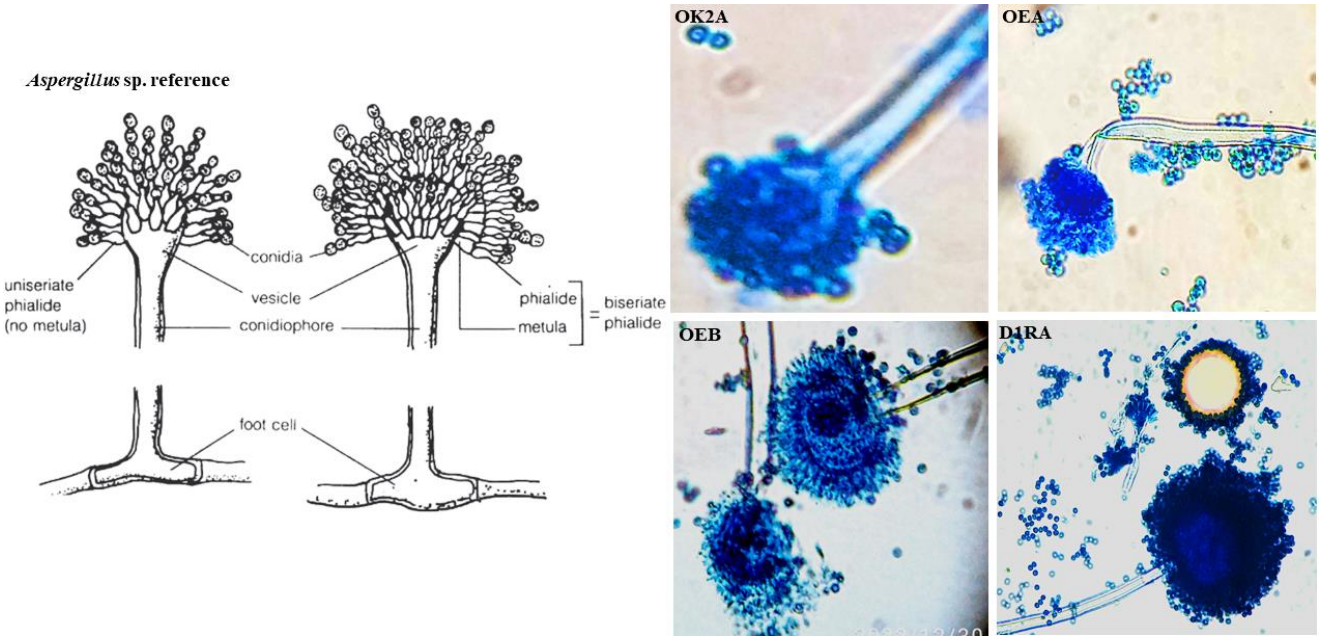


**Figure 2.** Morphological spores of mercury-resistant fungi under the light microscope with 100× magnification. *Aspergillus* sp. Reference (Larone et al. 2023). OK2A, OEA, OEB, D1RA. Mercury-resistant fungi

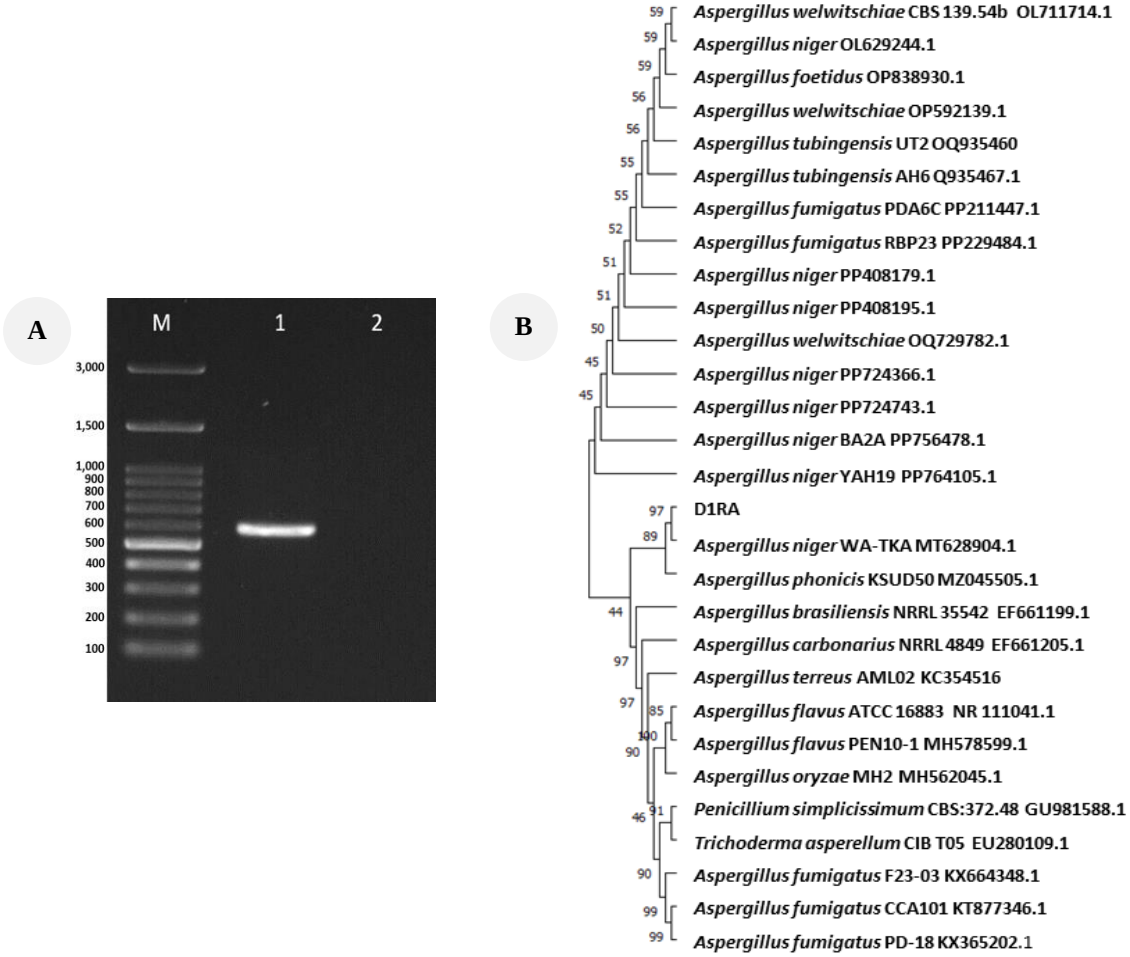


**Figure 3**. A. Electrophoregram of the ITS fragment amplified PCR product; B. Neighbour-joining phylogenetic of strain D1RA and the other 28 fungi using 1,000 replicates of the bootstrap consensus tree built Mega 11 software. M. 100bp DNA ladder. 1. ITS fragment amplified PCR product; 2. PCR negative control

**TI of heavy metal-resistant fungi**

A fungus resistant to metal usually develops resistance to other heavy metals such as $Pb^{2+}$ or $Zn^{2+}$ $Cd^{2+}$ and $Cu^{2+}$ (Văcar et al. 2021).Therefore, each fungus in this study was tested for its resistance to $Pb^{2+}$ and $Zn^{2+}$. The results showed that they also developed resistance to $Pb^{2+}$ and $Zn^{2+}$. Therefore, all fungi were categorized as heavy metal-resistant fungi.

The tolerance level for each heavy metal-resistant fungus can be determined by TI. The TI also can describe the effect of heavy metal on fungal growth. Based on the TI value, fungi can be classified as follows complete fungal intolerance towards metal (TI=0); fungi suppressed by metal (TI<1); fungi show similar absolute growth with or without the presence of metal (TI=1); while TI>1 showed that absolute growth of fungus in heavy metal exceeded that of control (TI>1) (Valix et al. 2001). The other classification according to Oladipo et al. 2018 is very low tolerance (TI=0.00-0.39), low tolerance (TI=0.40-0.59), moderate tolerance (TI: 0.60-0.79), high tolerance (TI=0.80-0.99) and very high tolerance (TI=≥1.00) (Oladipo et al. 2018). Each heavy metal-resistant fungus in this study showed a different TI level for each heavy metal concentration (Table 2). OEC TI value >1 fell for concentration $HgCl_2$ 5-15 ppm observed on days 2 and 8, and $Pb(NO_3)_2$ 400 ppm on day 8 because it outgrew the petri dish on PDA medium supplemented with $HgCl_2$ 5-15 ppm observed on days 2 and 8; and $Pb(NO_3)_2$ 400 ppm on day 8 (Table 2). Meanwhile, OEC TI was less than 1 for OEC grew slowly on PDA supplemented with $Pb(NO_3)_2$ 800-1,600 ppm and $ZnCl_2$ 300-2,400 ppm on day 8 compared to PDA (control, Table 2). Based on these results, OEC was only resistant to $Hg^{2+}$ and $Pb^{2+}$ but not $Zn^{2+}$. OEC TI level for $Hg^{2+}$ was lower than *Aspergillus* sp. OK2A, *Aspergillus* sp. OEA and *A. niger* D1RA. *Aspergillus* sp. OK2A and *Aspergillus* sp. OEA was probably a similar species but with different tolerance levels of heavy metals. For example, *Aspergillus* sp. OK2A and *Aspergillus* sp. OEA exhibited the same high tolerance level to 40 ppm $HgCl_2$ but *Aspergillus* sp. OK2A only showed a high tolerance to 300 ppm $ZnCl_2$ and a very low tolerance to 400 ppm Pb(NO3)$_2$, while *Aspergillus* sp. OEA exhibited a high tolerance to 1,200 ppm $ZnCl_2$ and a moderate tolerance to 400 ppm $Pb(NO_3)_2$ for observation

on day 8 (Table 2). *Aspergillus* sp. OEB had only a high tolerance for 800 ppm $Pb(NO_3)_2$. Only *A. niger* D1RA showed a high tolerance for three different heavy metals; 1,200 ppm $Pb(NO_3)_2$, 40 ppm $HgCl_2$, and 300 ppm $ZnCl_2$ (Table 2). Among all heavy metal fungi, *A. niger* D1RA showed the best TI for $Pb^{2+}$ 1,200 ppm. According to Iskandar et al. (2011) and Oladipo and Salam (2024), *Aspergillus niger* had a tolerance limit of 1.07 and 1.01 at 200 ppm of $Pb(NO_3)_2$ and $PbSO_4$, respectively. Therefore, *A. niger* D1RA was selected as a model to study the bioaccumulation capacity of $Pb^{2+}$.

**$Pb^{2+}$ uptake of *A. niger* D1RA in PDB medium with different pH**

*Aspergillus niger* D1RA were grown in the aqueous growth medium, PDB supplemented with and without 100 ppm of $Pb(NO_3)_2$ and adjusted to a different pH (3,4,5,6, and 7). After incubation at 5 days, *A. niger* D1RA biomass was collected and dried to determine its growth pattern, while the supernatants were used to assess its bioaccumulation capacity and removal efficiency. The amount of *A. niger* D1RA dry biomass prepared from PDB supplemented with 100 ppm of $Pb(NO_3)_2$ was higher than that of *A. niger* D1RA dry biomass prepared from PDB for all pH. These results exhibited that *A. niger* D1RA had a better growth rate on PDB supplemented with 100 ppm of $Pb(NO_3)_2$ compared to the PDB medium, particularly for pH 6-7 (Figure 4). *Trichoderma* spp. had also reported having grown faster when the media was supplemented up to 100 ppm of $Pb(NO_3)_2$ (Prakash et al. 2023). *Corollospora lacera* mycelia also increases its growth rate with increasing $Pb^{2+}$ concentration (Taboski et al. 2005). However, *A. niger* D1RA cultivated on PDB supplemented with 100 ppm of $Pb(NO_3)_2$ was suppressed on pH its growth at pH 3 and 4. Different pH causes differences in $Pb^{2+}$ behavior that probably affect fungal growth. For example, below pH 5 dominated $Pb^{2+}$, at pH 6 approximately 50 % exists as $Pb^{2+}$, and 50 % as $PbOH^+$; at pH 7 approximately 70 % occurs as $PbOH^+$ and 30 % as $Pb^{2+}$; and at pH 8-9, most of the Pb exists as $PbOH^+$ and above pH 9 formed $Pb(OH)_2$ (Hahne and Kroontje 1973; Bagy et al. 1991).

The effect of pH on the bioaccumulation capacity of $Pb^{2+}$ by *A. niger* D1RA was evaluated. The results showed that the bioaccumulation capacity of $Pb^{2+}$ was different for each pH value. The highest bioaccumulation capacity was at pH 4, which was able to accumulate 78.75 mg/g dried biomass even though the dry biomass mass was the lowest than the other due to its growth suppression (Figure 4.A). This value was higher than the results of *A. niger* which is reported by Dursun et al. (2003), namely 16.8 mg/g dry biomass at pH 4.5.

The removal efficiency of $Pb^{2+}$ by *A. niger* D1RA fluctuated depending on the pH value. Unlike pH for the bioaccumulation capacity, pH 4 is not the best removal efficiency of $Pb^{2+}$ for *A. niger* D1RA (Figure 4.B). The best pH for the removal efficiency of $Pb^{2+}$ was pH 3 and 7, reaching 97 and 95%, respectively, even though the amount of biomass pH 3 was less than pH 7 (Figure 4). According to these results, the pH was a more important factor compared to the amount of biomass to remove $Pb^{2+}$ by *A. niger* D1RA.

**$Pb^{2+}$ uptake of *A. niger* D1RA in PDB medium at different incubation times**

Different times of $Pb^{2+}$ uptake by *A. niger* D1RA cultivated on PDB medium supplemented with 100 ppm $Pb(NO_3)_2$ at pH 4 were observed in its growth curve, bioaccumulation capacity and removal efficiency every day for 5 days (Figure 5.A). *A. niger* D1RA growth curve was determined by its dry biomass mass. The exponent phase was reached between days 2 and 3. The maximal dry biomass was on day 3.

**Table 2**. TI of each heavy metal-resistant fungi in PDA media with different heavy metal concentrations

| Heavy metal | Conc, ppm | *Aspergillus* sp. OK2A | | *Aspergillus* sp. OEA | | *Aspergillus* sp. OEB | | *A. niger* D1RA | | OEC | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Day 2 | Day 8 | Day 2 | Day 8 | Day 2 | Day 8 | Day 2 | Day 8 | Day 2 | Day 8 |
| $HgCl_2$ | 5 | 0.94 | 0.89 | 0.88 | 0.90 | 0.94 | U | 0.91 | U | >1 | >1 |
| | 10 | 0.87 | 0.90 | 0.85 | 0.93 | 0.79 | U | 0.93 | U | >1 | >1 |
| | 15 | 0.70 | 0.85 | 0.75 | 0.88 | 0.69 | U | 0.82 | U | >1 | >1 |
| | 20 | 0.62 | 0.84 | 0.64 | 0.78 | 0.72 | U | 0.79 | U | 0.64 | 0.82 |
| | 40 | 0.20 | 0.94 | 0.73 | 0.96 | 0.00 | 0.00 | 0.40 | 0.86 | 0.15 | 0.56 |
| | 80 | 0.00 | 0.00 | 0.41 | 0.78 | 0.00 | 0.00 | 0.44 | 0.75 | 0.00 | 0.00 |
| | 160 | 0.00 | 0.00 | 0.06 | 0.54 | 0.00 | 0.00 | 0.00 | 0.58 | 0.00 | 0.00 |
| $Pb(NO_3)_2$ | 400 | 0.20 | 0.21 | 0.76 | 0.65 | 0.84 | 0.82 | 1.03 | 0.87 | 0.83 | >1 |
| | 800 | 0.12 | 0.14 | 0.45 | 0.55 | 0.76 | 0.80 | 0.76 | 0.76 | 0.29 | <1 |
| | 1,200 | 0.00 | 0.10 | 0.27 | 0.35 | 0.35 | 0.77 | 0.54 | 0.80 | 0.00 | <1 |
| | 1,600 | 0.00 | 0.03 | 0.25 | 0.27 | 0.14 | 0.43. | 0.39 | 0.63 | 0.00 | 0,00 |
| $ZnCl_2$ | 300 | 0.77 | 0.90 | 0.87 | 0.57 | 0.20 | 0.66 | 0.46 | 0.90 | 0.48 | <1 |
| | 600 | 0.55 | 0.71 | 0.26 | 0.51 | 0.18 | 0.17 | 0.00 | 0.13 | 0.06 | <1 |
| | 1,200 | 0.40 | 0.65 | 0.64 | 0.93 | 0.00 | 0.10 | 0.00 | 0.00 | 0.03 | <1 |
| | 2,400 | 0.24 | 0.56 | 0.72 | 0.73 | 0.00 | 0.90 | 0,00 | 0,00 | 0.00 | <1 |

Note: Unmeasured colony growth diameter in either media supplemented with heavy metal or without heavy metal; Red. Very high tolerance (≥1); Green. High tolerance (0.80-0.99); Yellow. Moderate tolerance (0.60-0.79); Blue. Low tolerance (0.40-0.59); Grey. Very low tolerance (≤0.39)

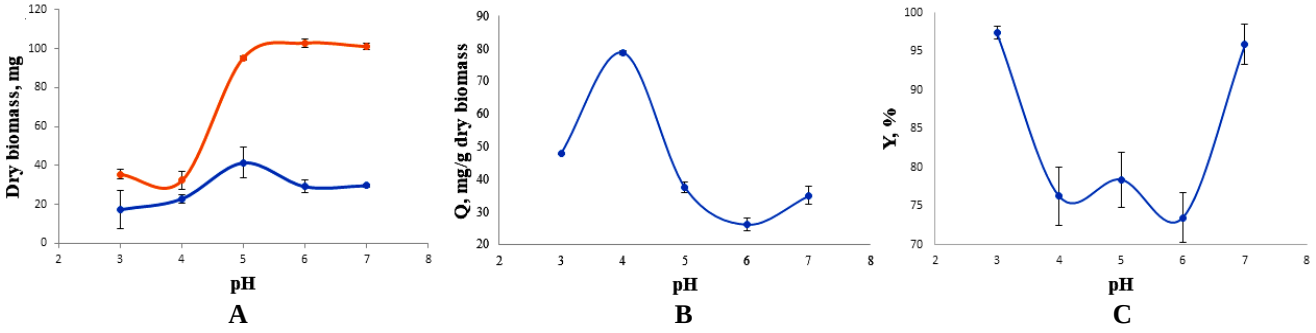


**Figure 4.** Effect of different pH on *A. niger* D1RA growth on PDB (blue line) and PDB supplemented with A. 100 ppm $Pb(NO_3)_2$ [orange line]); B. Bioaccumulation capacity; C. Removal efficiency (C). Bars indicated the standard error of the mean (±SE)

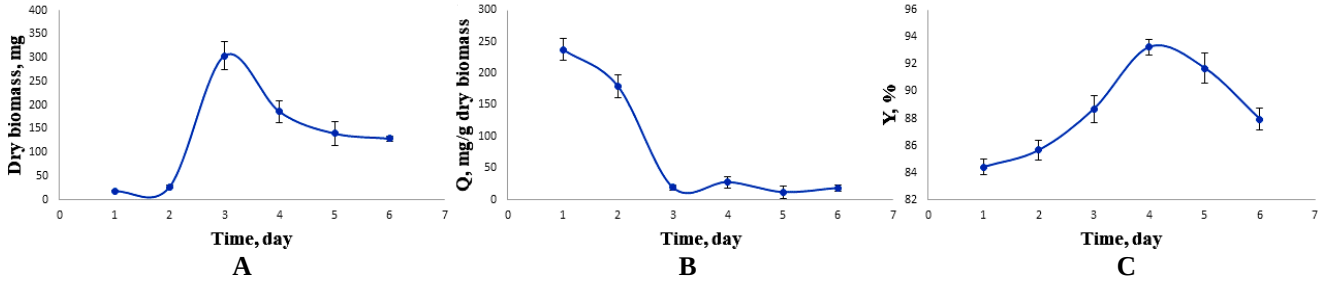


**Figure 5**. Effect of different incubation times on A. *A. niger* D1RA growth; B. Bioaccumulation capacity; and C. Removal efficiency on PDB medium supplemented with 100 ppm $Pb(NO_3)_2$. The bars indicated a standard error of the mean (±SE)

The bioaccumulation capacity of *A. niger* D1RA reached the highest value on day 1 (237.776 mg/g dry biomass). It decreased from day 2 to 3, while the maximum removal efficiency reached 93.266% on day 4 (Figures 5.B and 5.C). The other study reports that the bioaccumulation capacity of *A. niger* at 100 ppm $Pb(NO_3)_2$ is 34.4 mg/g dry biomass (Dursun et al. 2003) and 24.701 mg/g dry biomass (Iskandar et al. 2011) which was lower *A. niger* D1RA. *C. lacera* can accumulate levels up to 250 mg/g while *Monodictys pelagica* can accumulate 6 mg/g (Taboski et al. 2005). *Penicillium simplicissimum* and *Trichoderma asperellum* at 100 ppm $Pb(NO_3)_2$ can remove 4.309 mg/g and 20.831 mg/g dry biomass (Iskandar et al. 2011). *Penicillium* sp. EN1 only can accumulate 85.7% of $Pb^{2+}$ at a concentration 3 ppm (Navnage et al. 2020).

In conclusion, four heavy metal-resistant fungi were isolated from the illegal gold mining samples (*Aspergillus* sp. OK2A and *Aspergillus* sp. OEA, *Aspergillus* sp. OEB, OEC) and one heavy metal-resistant fungus was isolated from the abandoned illegal gold mine sample (*A. niger* D1RA). All of them also showed the ability to resistance to the other heavy metals, such as $Pb^{2+}$ and $Zn^{2+}$, but only *A. niger* D1RA isolated from the abandoned illegal gold mining site showed the best tolerance to three different heavy metals, namely 40 ppm $HgCl_2$, 400 ppm $Pb(NO_3)_2$ and 600 ppm $ZnCl_2$. The best bioaccumulation capacity and removal efficiency of $Pb^{2+}$ by *A. niger* D1RA were 237.776 mg/g dry biomass and 93.266%, respectively. *A. niger* D1RA has significant potential to be developed as bioremediation agents in wastewater exposed to lead.

## ACKNOWLEDGEMENTS

This research was funded by the Budget Implementation Checklist (DIPA) of Universitas Tanjungpura, Pontianak, Indonesia, with the contact number SP. DIPA-023.17.2.677517/2022, dated 30 November 2022, fiscal year 2023.

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