RESEARCH ARTICLE

Lead (II) Tolerance and Uptake Capacities of Fungi Isolated from a Polluted Tributary in the Philippines

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Abstract: The Lead [Pb(II)] tolerance and uptake ability of four fungal species, two from the genus *Penicillium* and two from the genus *Talaromyces* were investigated in this study. The species were isolated from a polluted tributary and identified to be closest to *P. canescens*, *P. simplicissimum*, *T. macrosporus* and another *Talaromyces sp.* via PCR targeting their internal transcribed spacer 1 and 4 sequences. All isolates have tolerances for up to 2000 μg/mL and 3000 μg/mL Pb(II) on solid and liquid medium, respectively. Both *Penicillium* isolates showed increasing removal rates dependent on initial Pb(II) concentration at 500 to 2000 μg/mL, while removal rates of both *Talaromyces* isolates were not significantly influenced by initial Pb(II) concentrations. The Pb(II) uptake of all isolates increased with increasing Pb(II) concentration but was depressed at 3000 μg/mL, with the exception of *T. macrosporus.* The recorded total uptake capacities for both *Penicillium* isolates in this study were higher than in most literature, at 7.0 – 407.4 mg/g and 50.8 – 412.6 mg/g for *P. canescens* and *P. simplicissimum*, respectively. The study also reported the exemplary Pb(II) uptake capacities of both *Talaromyces* isolates at 58.9 – 601.0 mg/g and 60.9 – 402.3 mg/g for *T. macrosporus* and *Talaromyces sp.*, respectively. These results signify the excellent Pb(II) removal capabilities of all isolates which may further be developed for use as mycoremediation tools to remove Pb(II) from heavy metal contaminated environments.

Keywords: mycoremediation, tolerance index, biosorption, bioaccumulation, heavy metals

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1 Introduction

The exacerbation of heavy metal (HM) concentrations in various substrates is a global ecological and health concern. The direct or indirect release of Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pb), Mercury (Hg), Nickel (Ni), Uranium (U), and Zinc (Zn) whether from natural or anthropic origin, increases their environmental levels beyond acceptable thresholds (Chen et al., 2017; He and Chen, 2014; Say et al., 2003a). These HMs become pollutants that are non-biodegradable and are bioaccumulated and biomagnified (Elia et al., 2006) reaching human populations (Wong et al., 2017). Since these HMs are unnecessary for physiological function, minute quantities are enough to cause toxicity to humans and other forms of life as they leach from contaminated sources and enter soil, air, and water (He et al., 2005; Singh et al., 2011).

Heavy metal poisoning due to Lead [Pb(II)] is one of the most historically well documented causes of disease and disability (Tong et al., 2000). The impact of Pb(II) on human health affects all ages within a population, ranging from mild discomfort, persistent disability, or severe, multi-systemic dysfunction leading to death (Kim et al., 2015). In the Philippines, Pb(II) prevalence has been reported in urban centers and rural areas and waterways, making its management and damage mitigation a matter of significance (Navarrete et al., 2017; Ona et al., 2006; Riddell, 2007; Solon et al., 2008). While there are numerous methods available for rehabilitation of Pb-polluted environments, most of these are costly and inefficient, opening avenues for alternative methods of handling Pb(II) pollution (Ayangbenro and Babalola, 2017).

Mycoremediation has increasingly gained attention due to its cost-effectivity, remarkable success outcomes,

and fungal structural advantages over other rehabilitative agents (Dixit et al., 2015; Kapahi and Sachdeva, 2017). Filamentous fungi can be used in various environments because of their innate competence and responsiveness to fluctuations in temperature, pH, nutrient availability, and HM concentrations (Oladipo et al., 2018). Fungal species can also accumulate higher amounts of HMs compared to plants (Gube, 2016; Kalac and Svoboda, 2000) and bacteria (Ayangbenro and Babalola, 2017; Rhodes, 2014) This ability is based on several fungal constitutive and adaptive physiological and genetic mechanisms that have been summarized extensively in literature (Bellion, 2006; Gadd, 2007; Gube, 2016).

The total tolerance and metal uptake ability of a fungus is reliant on both bioaccumulation and biosorption (Iskandar et al., 2011; Joshi et al., 2011). The abilities of fungi to intracellularly accumulate (bioaccumulation) and extracellularly adsorb (biosorption) HMs may be dependent on one or more combinations of various biological strategies (Abd El Hameed et al., 2015; Machido et al., 2011; Romero et al., 2006; Zhang et al., 2016). These strategies can be harnessed to serve as means to remove HM toxicants from various substrates. Utilization of fungi that are part of the natural microbiota of an ecosystem exposed to HM damage may become a viable strategy in response to high costs and low effectivity of traditional HM treatment strategies.

2 Materials and Methods

2.1 Sample Collection and Fungal Isolation and Screening

Soil and water samples were collected at a depth of 5-10 cm from a small tributary creek (14°46'21"N, 121°1'37"E) of the Marilao River within the Meycauayan-Marilao-Obando River system (MMORS). This area was within range of a health status report that indicated Pb(II) exposure to the residential areas nearby (Ostrea et al., 2015). Each of the soil samples was diluted with sterile distilled water (sdH_2O) in a 1 g: 9 mL ratio. Both the diluted soil samples and water samples were diluted with sdH_2O to produce $1{\times}10^{-1}$ until $1{\times}10^{-5}$ dilutions within 6 h of obtaining the samples. One mL of each of these dilutions was placed in sterile disposable petri-plates and pour-plated with 20 mL potato dextrose agar (PDA, pH 5.5) and incubated at 30°C for 5-7 days.

Morphologically distinct mycelia grown on the dilution plates were sub-cultured via point inoculation on PDA supplemented with 1, 10, and 100 μg/mL Pb(II) and tetracycline (tet, 50 μg final concentration/plate). This served to generate isolated cultures and initially screen all isolates for tolerance against Pb(II). Fungi that survived at 100 μg/mL were then sub-cultured on both PDA/tet and PDA/tet/Pb(II) plates and were incubated for 7-14 days at 30°C. PDA/tet plates were used for both morphological and molecular identification. The same isolates were cultured on PDA/tet/Pb(II) and were used for tolerance index (TI) measurements and liquid culture uptake tests. All experiments were carried out in triplicate.

2.2 Morphological Identification

Standard protocols detailing the macroscopic and microscopic examination of fungal features such as reproductive structures, mycelium, spore shape, size, and color were used to differentiate fungi isolated from the environmental samples for Pb(II) screening and to corroborate the PCR-based identification (Ellis and Ellis, 1997; Gilman, 2001; Nagamani et al., 2006). Isolates were sub-cultured on PDA/tet every 15 days (5-7 days growth, 30°C) to maintain a working stock that was kept at 20°C.

2.3 DNA Extraction and PCR Identification

Genomic DNA was isolated using Vivantis GF-1 DNA isolation kit following the instructions of the manufacturer. Twenty-five grams of fungal tissue from each was used per isolation either being ground to fine powder using liquid nitrogen or through sonication (50 mHz, 5 reps, 15s per rep), with a ratio of 50 mg mycelia/280 mL of the extraction buffer provided in the kit. DNA was quantified at 260/280 (IMPLEN NP80) and used as a template for Polymerase Chain Reaction (PCR). The PCR was carried out using the forward primer ITS1F (5'-TCCGTAGGTGAACCTGCGGG-3') and the reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') targeting the internal transcribed spacer (ITS) regions 1 and 4 of fungal DNA. The thermal cycling program was optimized to: initial denaturation 94°C, 2 min followed by 31 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, with a final extension of 72°C for 7 min. Each of the amplicons was run on a 2% w/v agarose gel stained with 0.5 μg/mL EtBr alongside 1 Kb and 100 bp molecular weight markers at 45 min, 100V (Vivantis) and were visualized under a UV transilluminator (SynGene).

All PCR products were purified using the Wizard SV Gel and PCR Cleanup System (Promega) following the manufacturer's instructions and were sent to Eurogenetec Asia (Singapore) for standard sequencing. Electropherograms were reviewed manually using Serial Cloner 2.6 (http://serialbasics.free.fr/Serial_Cloner.html), with extraneous sequences of low signature being removed manually. BLAST analysis was performed to compare resulting sequences with known fungal ITS sequences against the NCBI database.

2.4 Growth Rates and Tolerance Indexes

Growth rates (GR) and TI measurements were generated via a plate assay. PDA/tet plates were supplemented with Pb(II) to final plate concentrations of 500, 1000, 2000 and 3000 μg/mL. These plates were inoculated with $\sim$1.0 x 10^5 spores in a 1% Tween20/Phosphate-buffered saline solution

(Tween-PBS) from each of the fungal isolates coming from the stock cultures. These were then incubated at 30°C for a maximum of 15 days. The diameter (mm) of the mycelial mass was measured daily for the 15-day duration using a digital caliper to generate GRs. Mycelial morphology was photo documented every 24 h. TIs were generated using the GR measurements of all species tested following Dey et al., (2016). The TI shows a ratio of relative growth of each species grown on the Pb(II) supplemented medium (MSM) against growth of the same species on a non-Pb(II) supplemented plate. The TI was measured as:

$$TI = \frac{Fungal\ growth\ on\ MSM/tet}{Fungal\ growth\ on\ PDA/tet} \quad (1)$$

Tolerance index values <1 indicate growth was suppressed by Pb(II) supplementation; a value of 1 indicates non-influence of growth by Pb(II) and a value >1 one indicates that growth is enhanced by Pb(II) supplementation.

2.5 Pb(II) Removal Efficiency and Uptake in Liquid Media

The impact of initial Pb(II) concentration on the uptake potential of the fungal species was tested using a modified liquid culture set-up based on Mohsenzadeh and Shahrokhi, (2014). Flasks containing 50 mL of the Potato Dextrose Broth (PDB) were supplemented with Pb(II) with final concentrations of 500, 1000, 2000, and 3000 μg/mL and 100 μg/mL of tetracycline adjusted with Na-P buffer at 6 pH. These were inoculated with 100 μL of a $\sim$1.0×10^5 spores in Tween-PBS as previously described and then incubated at 30°C, 180 rpm for 15 days. After the incubation duration, the fungal biomass was separated from the liquid medium via gravity filtration using a Whatman 2 (47 mm) filter as described by Kiene and Slezak, (2006), letting the media drip into a sterile 50 mL conical tube. The filtrate, ranging from 45-50 mL, was analyzed using inductively coupled plasma atomic emission spectroscopy (ICP-AES) and the fungal biomass was kept at -20°C. Three conical tubes with 50 mL of sterile PDB/tet supplemented with Pb(II) concentrations of 500, 1000, 2000, and 3000 μg/mL were also sent as standards for measurement of removal efficiency. The remaining fungal biomass after the filtration was placed in similar containers and was oven dried at 60°C prior to measurement of dry weight. All trials were done in triplicate.

Removal efficiency, the percentage of metal removed from the solution (R,%) was computed using the values obtained from the samples processed through ICP/AES using the following formula (Iskandar et al., 2011):

$$R = \frac{(C_o - C_f)}{C_o} \times 100 \quad (2)$$

where R is the Removal Rate, C_o is the predetermined initial Pb(II) concentration (μg/mL) and C_f is the final Pb(II) concentration (μg/mL) determined from the ICP/AES measurements.

The amount of metal uptake by the fungi (q, mg/g) was quantified as follows (Chen et al., 2017):

$$q = \frac{(C_o - C_f)v}{M} \quad (3)$$

where q is the uptake capacity, C_o is the initial Pb(II) concentration (mg/L); C_f the final Pb(II) concentration (mg/L); V the volume of solution (L); M the dry weight of biomass (g).

2.6 Statistical Analysis

Triplicates were prepared for all experiments. The data obtained for GR, TI, R and q was analyzed with Two-Way Analysis of Variance (ANOVA). Means were compared with Tukey's Honestly Significant Difference test (P<0.05) using a repeated-measures multiple-comparisons test (MCT). All statistical analyses were performed using GraphPad (v6.01).

3 Results

3.1 Fungal Identification

Four fungal species from the mixed soil and water samples survived on 500 μg/mL of Pb(II) were identified and screened. The ITS-based PCR identified the fungi belonging to the *Penicillium* and *Talaromyces* genera. The isolates had closest matches to *P. canescens* (KX258810.1), *P. simplicissimum* (KM613146.1), *T. macrosporus* (KU204425.1) and *Talaromyces sp.* strain Fi5 (MG098734.1) (Table 1).

Table 1. Taxonomic identification of fungal species with similarities on the NCBI database.

Lab ID	Closest Species Match	Accession number	Sequence Identity (%)	Query Coverage (%)
Sp AF-01	*Penicillium canescens*	KX258810.1	99	97
Sp F-01	*Penicillium*	KM613146.1	98	75
Sp A-01	*Talaromyces macrosporus*	KU204425.1	98	98
Sp C-01	*Talaromyces sp.* strain Fi5	MG098734.1	92	99

3.2 Tolerance Responses to Pb(II)

The mycelial growth responses and tolerance indices of *P. canescens*, *P. simplicissimum*, *T. macrosporus*, and *Talaromyces sp.* strain Fi5 varied across different concentrations of Pb(II), with all species capable of growth at 500, 1000, and 2000 μg/mL of Pb(II). Only *P. simplicissimum* and *Talaromyces sp.* strain Fi5 grew at 3000 μg/mL of Pb(II). The tolerance indices derived from the GRs measured over a 15-day interval compared with that of control plates without Pb(II) supplementation are seen in Figure 1.

The highest TIs for all isolates are generally exhibited at 500 μg/mL. The tolerance graphs for both *Penicillium* species (Fig 1A-B) show that their highest TI is achieved at the early onset of exposure to Pb(II) at 1-2 days, showing enhanced growth compared to the control, with subsequent decline. *P canescens* achieves its highest TI (1.59 ± 0.17) at

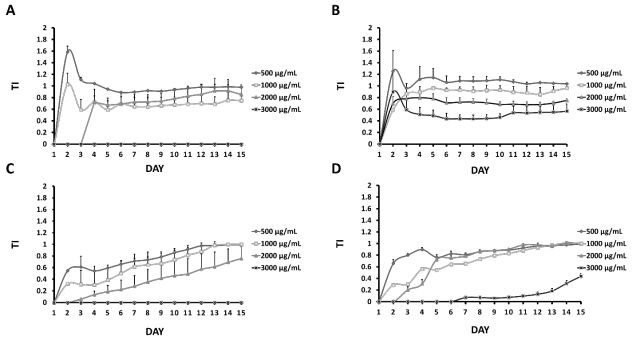

Figure 1. Tolerance index curves of all species at 500 μg/mL, 1000 μg/mL, 2000 μg/mL and 3000 μg/mL of Pb(II) supplementation for *P. canescens*, (A) *P. simplicissimum*, (B) *T. macrosporus*, (C) and *Talaromyces sp.* strain Fi5 (D). Bars indicate standard deviation of at least three replicates.

day 2 at 500 μg/mL exposure, while *P. simplicissum* obtains its peak TI (1.25 ± 0.62) at day 2 at 500 μg/mL exposure. *P. canesens* does not reach TI = 1 after day three, while *P. simplicissimum* has TI>1 only for 500 μg/mL from day 1-15. A decreasing trend in TI is seen with increasing Pb(II) concentration for both species, with *P. canescens* being completely inhibited at 3000 μg/mL Pb(II).

Both *Talaromyces* isolates exhibit slow relative growth on Pb(II) supplemented media reaching their peak growth (TI = 1) only at day 15 (Fig 1C-D). *T. macrosporus* reaches TI = 1 for 500 μg/mL and 1000 μg/mL, while *Talaromyces sp.* reaches TI = 1 for all concentrations except 3000 μg/mL. *T. macrosporus* shows decreasing TI as Pb(II) concentrations increase, being completely inhibited at 3000 μg/mL Pb(II). *Talaromyces sp.* shows irregular TI fluctuation from days 1-4, with TI at 2000 μg/mL matching and exceeding TIs of the 500 μg/mL and 1000 μg/mL treatment groups starting day 6. Additionally, the growth of *Talaromyces sp.* is inhibited from days 1-6 but reaches a maximum TI of 0.57 ± 0.04 at day 15.

Varying relative growth responses were shown by each isolate in response to varying concentrations of Pb(II) at day 5, 10 and 15. Differences in TI for each isolate exposed to similar Pb(II) concentrations are seen in Figure 2, while differences in TI per isolate exposed to increasing Pb(II) concentrations are seen in Figure 3.

Assessing day five (Figure 2A), there are no significant differences (P>0.05) in TI between isolates exposed to 500 μg/mL. *T. macrosporus* shows a significantly (P>0.05) lower TI compared to all the other isolates at 500 to 2000 μg/mL Pb(II). The growth of all isolates except *P. simplicissimum* are completely inhibited at 3000 μg/mL. Progressing to day 10 (Figure 2B), significant (P>0.05) differences in TI are seen in 2000 μg/mL where *Talaromyces sp.* has significantly (P>0.05) higher TI than all other isolates. *Talaromyces sp.* also begins to show growth at 3000 μg/mL, albeit still significantly (P>0.05) lower than *P. simplicissimum*. Finally, at day 15 (Figure 2C), both *P. simplicissimum*

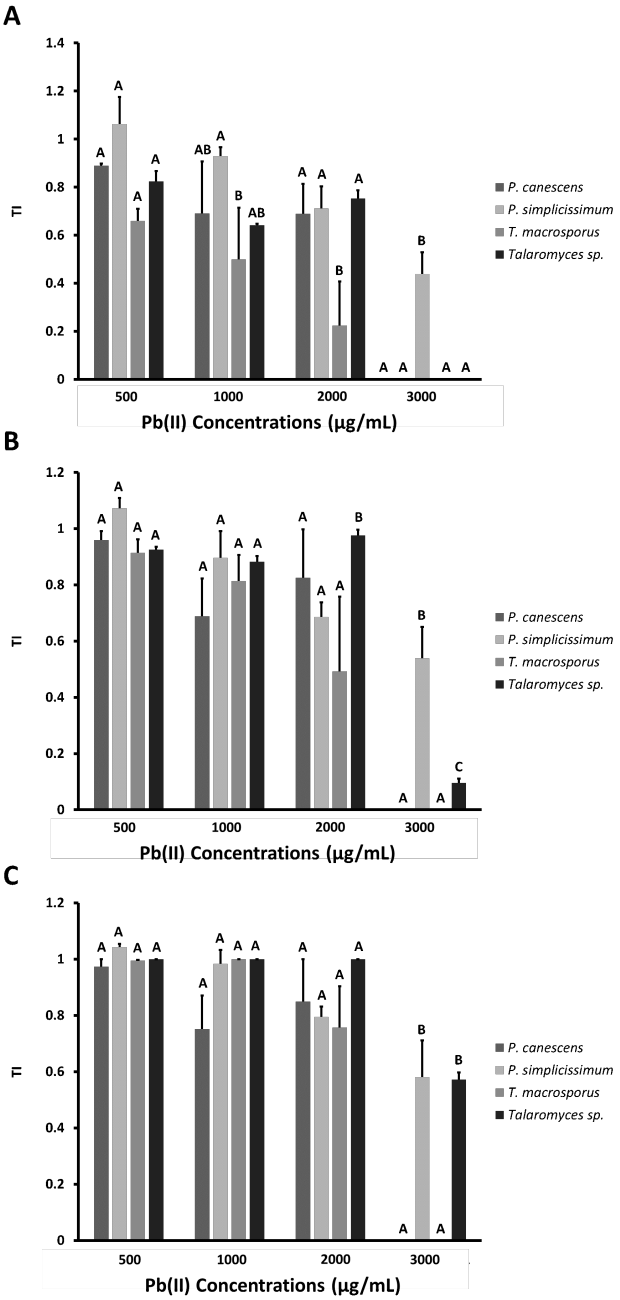

Figure 2. Tolerance Index comparison of all isolates at 500, 1000, 2000, and 3000 μg/mL of Pb(II) exposure at 30°C on day 5 (A), 10 (B) and 15(C). Means that share at least one similar letter are not significantly different at Honestly Significant Difference ($HSD_{(0.05)}$), P<0.05. Bars indicate standard deviation of at least three replicates.

and *Talaromyces sp.* have significantly (P>0.05) higher TIs compared to *P. canescens* and *T. macrosporus* that are inhibited at 3000 μg/mL Pb(II).

Comparing TI performance for each species as a response to different Pb(II) concentrations, it can be seen that on day 5 (Figure 3A), TI at 500-2000 μg/mL are not significantly (P>0.05) different from one another, with the 3000 μg/mL test concentration completely inhibiting all isolates other than *P. simplicissimum*. Continuing to day 10 (Figure 3B), no significant (P>0.05) differences in TI are seen for all isolates at 500 μg/mL and 1000 μg/mL. *P. simplicissimum* and *T. macrosporus* show significantly (P>0.05) lower TI at 2000 μg/mL and 3000 μg/mL compared to other test concentrations, while *P. canescens* and *T. macrosporus* still completely inhibited at 3000 μg/mL. Reaching day 15 (Figure 3C), *P. canescens* and *Talaromyces sp.* have similar TI trends for 500 to 2000 μg/mL Pb(II), while *P. simplicissimum* and *T. macrosporus* have significantly (P>0.05) higher TI at 500-1000 μg/mL versus 2000-3000 μg/mL, with *P. canescens* and *T. macrosporus* still completely inhibited at 3000 μg/mL.

3.3 Removal Efficiency

The removal efficiency for Pb(II) for each isolate exposed to the test Pb(II) concentrations grown on liquid PDB (Table 2) reveals increasing R as Pb(II) increases from 500-2000 μg/mL for all species except *P. simplicissimum* which has a higher TI at 2000 μg/mL (98.25 ± 0.1) than at 1000 μg/mL (97.37 ± 0.3). All isolates except *T. macrosporus* have their lowest TI at 3000 μg/mL. Removal rates >95% are observed for both *Talaromyces* isolates for Pb(II) concentrations <2000 μg/mL. *T. macrosporus* shows <2% variation in R regardless of Pb(II) concentration, while *Talaromyces sp.* removal is maintained at >98% from 500-2000 μg/mL exposure only lowering to 51.7% at 3000 μg/mL of Pb(II). All isolates except *T. macrosporus* have peak removal efficiencies at 2000 μg/mL. *P. canescens* has the poorest removal efficiency at 21-77%, while *T. macropsorus* consistently tops all other isolates at 97-99.5%.

Table 2. Mean removal efficiency (*R*) rates of *P. canescens*, *P. simplicissimum*, *T. macrosporus* and *T. macrosporus sp.* exposed to 500, 1000, 2000, and 3000 μg/mL Pb(II).

Pb(II) concentration	*R* (%)			
(µg/mL)	500	1000	2000	3000
P. canescens	12.28 ±4.0	49.83 ±8.3	77.78 ±3.7	58.94 ±2.0
P. simplicissimum	87.54 ±6.8	98.25 ±0.1	97.37 ±0.3	35.75 ±5.6
T. macrosporus	97.92 ±0.2	98.56 ±0.3	99.30 ±0.1	99.50 ±0.1
Talaromyces sp	98.34 ±0.6	99.58 ±0.1	99.69 ±0.1	51.69 ±4.2

Comparing isolate performance at each test concentration, *T. macropsorus* has the most consistent removal ability, having no significant (P<0.05) differences in removal percentage from 500 μg/mL to 3000 μg/mL Pb(II) (Figure 4C). *Talaromyces sp.* maintains >90% removal from 500-2000 μg/mL Pb(II) (Figure 4C) but significantly (P<0.05) drops in removal at 3000 μg/mL. *P. canescens*

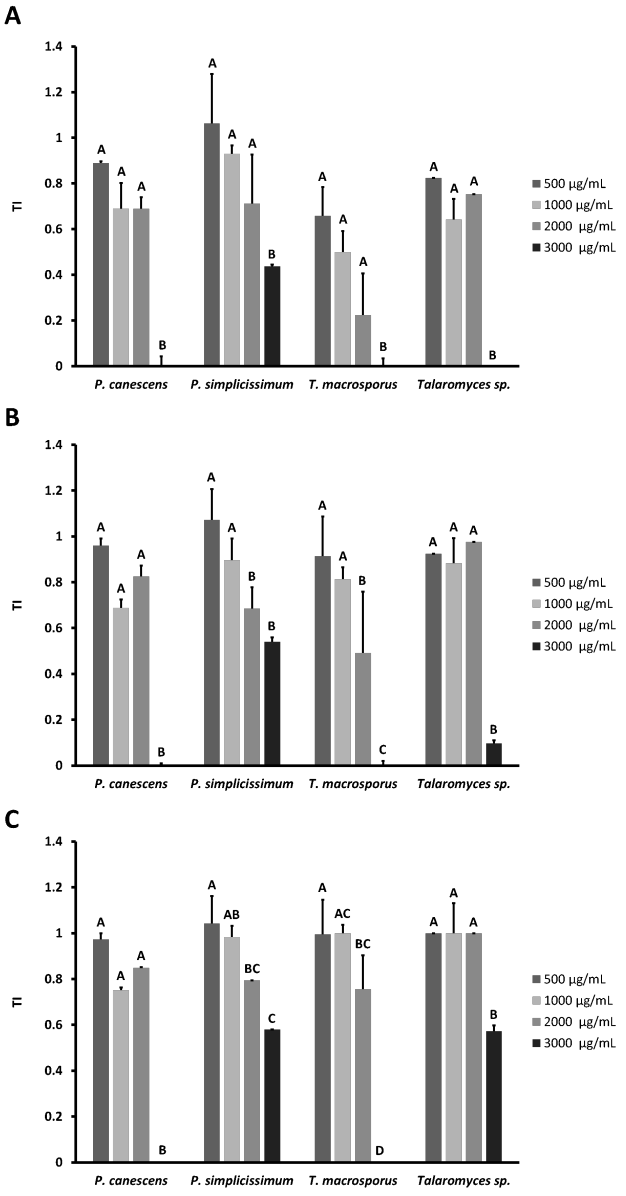

Figure 3. Tolerance Index comparison per isolate exposed to 500, 1000, 2000, and 3000 μg/mL of Pb(II) at 30°C on day 5 (A), 10 (B) and 15(C). Means that share at least one similar letter are not significantly different at Honestly Significant Difference ($HSD_{(0.05)}$), P<0.05. Bars indicate standard deviation of at least three replicates.

has the most variable response to the different in Pb(II) concentrations of all the species (Figure 4A), with different removal rates at all test concentrations. *P. simplicissimum* has a higher R at 1000 μg/mL and 2000 μg/mL, both having significantly (P<0.05) higher compared to 500 μg/mL and 3000 μg/mL of Pb(II) (Figure 4B).

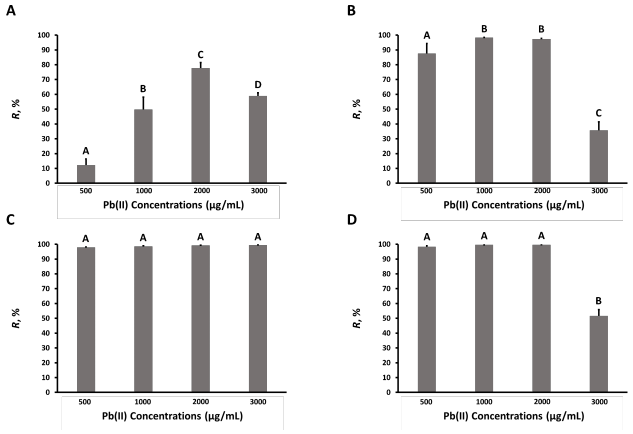

Figure 4. Removal efficiency, *R*, of *P. canescens*(A), *P. simplicissimum* (B), *T. macrosporus* (C) and *Talaromyces sp.* (D) exposed to 500 μg/mL, 1000 μg/mL, 2000 μg/mL, and 3000 μg/mL Pb(II). Means that share at least one similar letter are not significantly different at Honestly Significant Difference ($HSD_{(0.05)}$), P<0.05. Bars indicate standard deviation of at least three replicates.

Assessment of the removal of each species per test concentration (Figure 5) shows that at 500 μg/mL and 1000 μg/mL, both *Penicillium* isolates have significantly lower removal than the *Talaromyces* isolates. At 1000 μg/mL and 2000 μg/mL, only *P. canescens* has significantly (P>0.05) lower removal compared to all isolates. At 3000 μg/mL, each isolate has significantly (P>0.05) different *R* with *T. macrosporus* having the highest computed removal efficiency.

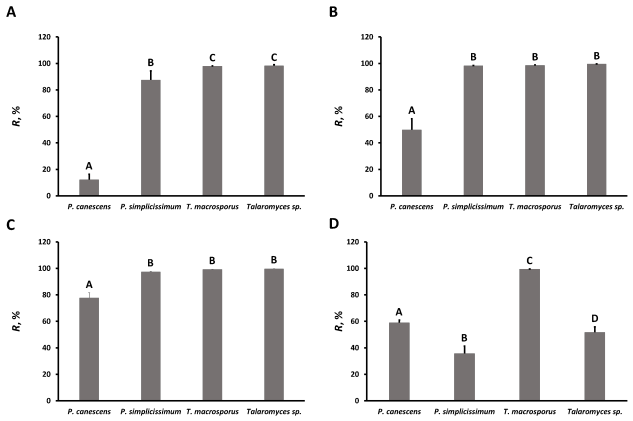

Figure 5. Removal efficiency, *R*, of *P. canescens*, *P. simplicissimum*, *T. macrosporus* and *Talaromyces sp.* exposed to 500 μg/mL (A), 1000 μg/mL (B), 2000 μg/mL (C), and 3000 μg/mL (D) Pb(II). Means that share at least one similar letter are not significantly different at Honestly Significant Difference ($HSD_{(0.05)}$), P<0.05. Bars indicate standard deviation of at least three replicates.

3.4 Uptake of Pb(II) in Liquid Medium

Differences in uptake capacity (q) are seen among the different isolates (Table 3). *P. canescens* had the lowest q among all isolates from 500 μg/mL to 2000 μg/mL Pb(II), while *P. simplicissimum* had the lowest q at 3000 μg/mL. *Talaromyces sp.* had the highest q from 500-2000 μg/mL, while *T. macrosporus* had the highest q (601.0 ± 3.6 mg/g) at 3000 μg/mL.

Table 3. Mean uptake capabilities (q) of *P. canescens*, *P. simplicissimum*, *T. macrosporus* and *T. macrosporus sp.* strain Fi5 exposed to 500, 1000, 2000, and 3000 μg/mL Pb(II).

Pb(II) concentration	q (mg/g)			
(µg/mL)	500	1000	2000	3000
P. canescens	7.03 ±2.6	92.3 ±3.0	407.4 ±5.7	370.6 ±2.9
P. simplicissimum	50.79 ±3.2	223.6 ±3.7	412.6 ±4.9	213.4 ±9.0
T. macrosporus	58.85 ±0.8	226.2 ±1.3	516.1 ±1.2	601.0 ±3.6
Talaromyces sp	60.88 ±2.4	233.5 ±3.9	519.1 ±2.6	402.3 ±7.8

Comparing uptake values (q) between species (Figure 6), all isolates have their lowest and highest q at 500 μg/mL and their highest q at 2000 μg/mL except *T. macrosporus* which has a higher q (601.0 ± 3.6 mg/g) at 3000 μg/mL. There are no significant (P>0.05) differences in q at 500 and 1000 μg/mL Pb(II) aside from *P. canescens* that has significantly (P>0.05) lower uptake compared to all the other isolates. At 2000 μg/mL, both *Penicillium* isolates have significantly lower q compared to both *Talaromyces* isolates. At 3000 μg/mL, all isolates have significantly (P>0.05) different q against each other, with *T. macrosporus* having the highest uptake, followed by *Talaromyces sp.*, *P. canescens*, then *P. simplicissimum*. Comparing individual isolate performance against Pb(II) concentrations (Figure 7), there is a significant (P>0.05) increase in q for all of the isolates from 500 to 2000 μg/mL of Pb(II). All isolates except *T. macrosporus* have a significant decline in q at 3000 μg/mL.

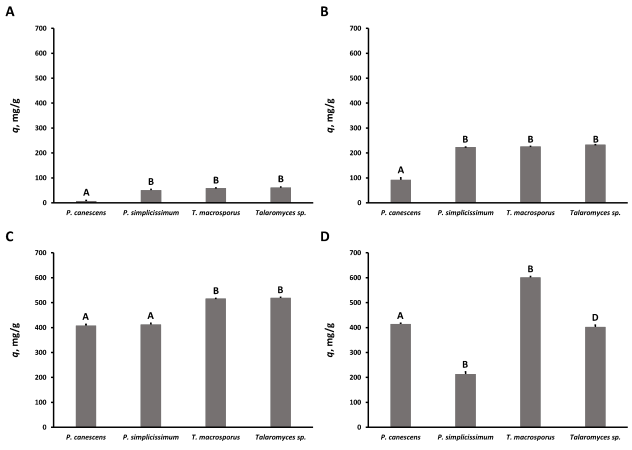

Figure 6. Uptake capacity (q, mg/g) of *P. canescens*, *P. simplicissimum*, *T. macrosporus* and *Talaromyces sp.* exposed to 500 μg/mL (A), 1000 μg/mL (B), 2000 μg/mL (C), and 3000 μg/mL (D) Pb(II). Means that share at least one similar letter are not significantly different at Honestly Significant Difference ($HSD_{(0.05)}$), P<0.05. Bars indicate standard deviation of at least three replicates.

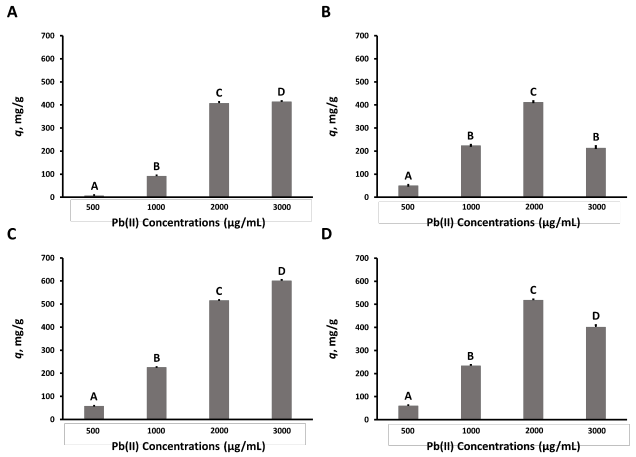

Figure 7. Uptake capacity (q, mg/g) of *P. canescens* (A), *P. simplicissimum* (B), *T. macrosporus* (C)and *Talaromyces sp.* (D) exposed to 500 μg/mL, 1000 μg/mL, 2000 μg/mL, and 3000 μg/mL Pb(II). Means that share at least one similar letter are not significantly different at Honestly Significant Difference ($HSD_{(0.05)}$), $P<0.05$. Bars indicate standard deviation of at least three replicates.

4 Discussion

4.1 Fungal Tolerance on Solid Media

This study reports the variable relative growth of all isolates at up to 2000 μg/mL Pb(II) with *P. simplicissimum* and *Talaromyces sp.* exhibiting growth up to 3000 μg/mL Pb(II), all on solid media. Both *Penicillium* isolates show elevated tolerance indices (TI>1) 1-2 days post inoculation, while *Talaromyces* isolates are relatively slow growing, reaching TI = 1 at day 15 for almost all concentrations. These are very high survival rates, comparable to some of the highest recorded Pb(II) growth tolerance, similar to *P. lilacinus* which can tolerate 1437 μg/mL (Zucconi et al., 2003) and Simplicissimum chinense, capable of growth at 5000 μg/mL (Chen et al., 2017) The ability of survival of fungal species in 2000 μg/mL has also been reported in *A. fumigatus*, *P. janthanellum*, *P. simplicissimum*, and *T. asperellum* (Chen et al., 2017; Iskandar et al., 2011).

Variations in survival rates and TI patterns among isolates of this study reinforce studies that report fungal tolerance as dependent on innate characteristics inherent to the species being studied (Say et al., 2003a; Iskandar et al., 2011), as well as adaptive strategies performed in response to exposure of increased metal concentrations (Valix and Loon, 2003; Zafar et al., 2007). These high tolerances to increased levels of Pb(II) as opposed to most records in literature may be due to constant exposure to Pb(II) in their local environment, which can trigger adaptive changes in as fast as 8 days (Valix et al., 2001). Constant exposure of fungi to HMs has been implicated in their ability to develop various tolerance mechanisms (Gorbushina and Krumbein, 2000) and the training of new biotypes to respond to various HMs has been recorded (Chen et al., 2017; Valix et al., 2001).

The lowering of TI for isolates show increased susceptibility to increasing Pb(II) concentrations on solid media, especially at 3000 μg/mL where *P. canescens* and *T. macrosporus* are completely inhibited. This is to be expected since Pb(II) is a non-essential element and is not required for any fungal physiological activity. The same result has been shown for fungi from various genera (Iram et al., 2009; Iskandar et al., 2011). While there are fluctuations in TI trends, especially evident in *Talaromyces sp.*, there are no significant ($P>0.05$) differences in TI at days 5, 10, and 15 for each species exposed to 500-1000 μg/mL Pb(II) concentrations. This suggests that the Pb(II) tolerance of the isolates may be independent of the HM concentration differences at this range. This is comparable to the static TI change of 13-day old *R. microsporus* grown on 100-400 μg/mL Pb(II) (Oladipo et al., 2018).

The ability of the *Penicillium* genus to survive Pb(II) has been widely established with abilities of various *Penicillium* species showing much variation in terms of Pb(II) tolerance range (Leitão, 2009). Reports for *Penicillium* species range from survival and growth at 40 μg/mL (Iram et al., 2009) to 2000 μg/mL Pb(II) (Chen et al., 2017) The TI values for both *Pencillium* isolates (>0.9) in this study correspond to tolerance values reported by various members of the *Pencillium* genus that have been subjected to Pb(II) tolerance tests ranging from 0.79-1.2 TI (Ezzouhri et al., 2009). *P. canescens* and *P. simplicissimum* follow a characteristic growth pattern similar to other *Penicillium* isolates having 1-2 days of growth lag and a succeeding rapid growth phase when tested against Ni, Co, Fe, Mg and Mn (Valix and Loon, 2003). Increased TI after 7 days of Pb(II) exposure has also been noted in *Penicillium sp.* isolated from marine environments (Jacob et al., 2013).

Elevated TIs (TI>1) within the immediate days of isolation for both *Penicillium* isolates may suggest a predominance of innate biosorptive mechanisms for handling HM influx, affording an abrupt response to HM stress at the early onset of growth. Biosorption rates are faster than bioaccumulation, since these are passive and metabolically independent reactions between the HM and fungal cell walls (Chojnacka, 2010). These cell walls act as the first barrier to the incoming HM, serving to reduce its concentration (Hafez et al., 1997). Support of these results can be found in several studies where Pb(II) biosorption has been described as a major component of increased fungal tolerance mechanisms in *P. chrysogenum*, *P. citrinum* (Oso et al., 2015), *P. janthinellum* (Aytar, 2014; Iskandar et al., 2011), *P. oxalicum* (Svecova et al., 2006), *P. purpurogenum* (Say et al., 2003a) and *P. smplicissimum* (Iskandar et al., 2011). Specifically, dried *P. simplcissimum* has been tested as a biosorbent and has shown to adsorb 76.9 mg/g of Pb(II), with higher sorption rates at higher initial metal inoculation concentrations (Fan et al., 2008). *P canescens* has similarly been studied to have 40.4 mg/g of Pb(II) showing higher affinity for Pb compared with Cd, Hg, and As (Say et al.,

2003b).

Both *Talaromyces* isolates show a similar Pb(II) reaction trend with the *Penicillium* isolates having a slightly elevated TI at the first few days of growth most evident at days 1-3 at 500-1000 μg/mL for *T. macrosporus* and days 1-4 at 1000-2000 μg/mL. The TI of the *Talaromyces* isolates, however, does not reach values >1 during the duration of the study. The slow relative growth of both *Talaromyces* isolates may suggest bioaccumulation as an early dominant mechanism in their tolerance strategy. Bioaccumulation is a slower, metabolism dependent process (Chojnacka, 2010), which may explain the lack of a rapid growth phase and why several days are required to reach TI = 1 for both isolates. Despite this, comparison of TI response with other members of the *Talaromyces* genus is difficult since very few members of the *Talaromyces* genera have been studied for HM tolerance, and data for comparing TI is sparse. However, in terms of tolerance to other HMs, *T. wortmanii* and *T. flavus* were found to survive As concentrations at 65-100 μg/mL (Šimonovičová, 2008), while *T. helicus* resistance to Cu at a maximum of 600 μg/mL (Romero et al., 2006), and to Cd at 10 μg/mL (Massaccesi et al., 2002). A mixture of xanthate-modified thiourea chitosan sponge with *Pseudomonas* putida and *T. amestolkiae* biomass has been shown to significantly reduce Pb(II) in solutions up to 500 μg/mL (Wang et al., 2017), while one member, *T. emersonii*, was found to have survival and biosorption potential for uranium (Bengtsson et al., 1995).

Care must be taken in attributing the speed of TI increase at early stages of growth to a predominance of either biosorption or bioaccumulation alone since the total tolerance ability of a fungal strain to withstand a particular HM is a combination of both processes (Joshi et al., 2011). While biosorption is fast, there are a finite number of binding sites that can accommodate a limited number of HM ions (Ayangbenro and Babalola, 2017; Chojnacka, 2010), and sorption rates are dependent on the state of hyphal development (Yetis et al., 2000). Thus, if TI does not significantly increase as time elapses as seen in all isolates of this study, biosorption alone may not be enough to maintain a stable growth rate under Pb(II) stress. In these cases where sorption sites are filled, it has been suggested that bioaccumulation may take over as the dominant mechanism for tolerance (Jentschke and Godbold, 2000).

4.2 Removal Efficiency in Liquid Media

Lead removal efficiencies (R,%) of each of the isolates show consistent >90% Pb(II) removal for *P. simplicissimum*, *T. macrosporus*, and *Talaromyces sp.* after 15 days of inoculation on liquid media at 500-2000 μg/mL. *T. macrosporus* continues maintaining >90% R until 3000 μg/mL, with Pb(II) concentrations having no significant influence on R. *Similar R* values in response to Pb(II) have been seen in various fungi such as *A. macropsorus* with R = 88.69% in 100 μg/mL alkaline solution after 500 h (Melgar et al., 2007), *S. chinenense* having R = 80.6% at 100 ppm (Chen et al., 2017), and *T. longibrachiatum* having R = 78.84% at 100 ppm after 15 days (Adeogun et al., 2012). The removal values of *P. cansescens*, ranging from 12%-77% are closer to reports in *A. flavus* (Akar and Tunali, 2006) and *P. lilacinus* (Zucconi et al., 2003).

Consistent removal rates independent of HM concentration are shown at 500-2000 μg/mL Pb(II), most especially in *T. macrosporus* and *P. canescens*. *P. simplicissimum* and *Talaromyces sp.* show a drop in R at 3000 μg/mL, most severe in *Talaromyces sp.* that drops by 48% from 2000 μg/mL to 3000 μg/mL. These suggest exemplary performance for Pb removal in liquid media at 500 to 2000 μg/mL for all isolates other than *P. canescens*, and greater susceptibility to Pb(II) at 3000 μg/mL for all isolates other than *T. macrosporus*. This is not surprising since most recorded ranges of removal are done with solutions less than 3000 μg/mL (Ahmad et al., 2011; Leitão, 2009).

4.3 Uptake of Pb(II) in Liquid Medium

Both *P. canescens* and *T. macropsorus* show growth and uptake in liquid media at 3000 μg/mL Pb(II) despite not being able to grow on solid PDA of the same concentration for TI evaluation. This may be explained by the shaking incubation, which allows better air perfusion and agitation of cells, allowing greater access to nutrients in liquid medium (Bulut and Baysal, 2006). Metal precipitation is also common at high concentrations of Pb(II), thus modifying the actual Pb(II) ions available for interaction with the growing fungi (Sun and Shao, 2007). The ability of the fungi to grow on both forms of media at high Pb(II) concentrations compared to known literature heavily suggests not just tolerance but physiological processes that help manage their interaction with lead in liquid media (Acharya et al., 1999).

Increasing uptake is observed in all isolates from 500-3000 μg/mL Pb(II) except for both *P. canescens* and *Talaromyces sp.* that have lower q at 3000 μg/mL than at 2000 μg/mL Pb(II). This agrees with results for *A. niger*, *P. simplicissimum* and *T. asperellum* where a depression in uptake capacity for Pb(II) in liquid medium was reduced from 250 μg/mL to 300 μg/mL Pb(II) (Iskandar et al., 2011). It has been shown that increasing initial HM concentrations result to higher Pb(II) removal in various fungal species grown on liquid medium (Akar and Tunali, 2006; Iram et al., 2015; Melgar et al., 2007). This has been explained heavily by mass transfer biosorption kinetics (Yalçin et al., 2010) where increasing concentrations provide a driving force to overcome transfer resistances (Fan et al., 2008) necessary for metals to bind to various groups in the cell wall (Say et al., 2003b). This is facilitated by increased metal collisions with the fungal wall being more pronounced in higher HM concentrations (Aksu and Tezer, 2005). However, in *Penicillium sp* Psf-2, it was shown that the ability to

respond to increasing Pb(II) concentrations was influenced heavily by bioaccumulation in living tissue (Sun and Shao, 2007). This has also been shown in several genera of fungi such as *A. macrosporus* (Melgar et al., 2007) *C. lacera*, *M. pelagica* (Taboski et al., 2005), *A. fumigatus*, *P. janthanellum* and *P. simplicissimum* (Iskandar et al., 2011) and *T. viride*, (Šimonovičová, 2008) at varying concentrations of Pb(II).

Recorded uptake values for Pb(II) differ from one fungal species to another (Ahmad et al., 2011; Leitão, 2009). While all isolates have relatively comparable q at each test concentration, *P. canescens* has the lowest q for each concentration except at 3000 μg/mL where the Pb(II) uptake of *P. simplicissimum* drops 97.37% to 35.75%. These changes in uptake capacity between species reacting to changes in HM concentration are expected, since the bisorption and bioaccumulation of deleterious HMs is both inter- and intra- species specific, varying even among closely grouped fungal species (Gadd, 2007; Vodnik et al., 1998) being heavily dependent on species potential and the ecology to which the fungus is exposed to (Udochukwu et al., 2014).

The recorded Pb(II) uptake in this study are higher than most recorded values in literature for the *Penicillim* isolates. For *P. canescens*, reports are at 213.2 mg/g (Say et al., 2003b), for *P. simplicissimum* published values range from 17.61 (Iskandar et al., 2011) to 76.90 at 250 μg/mL (Fan et al., 2008), both lower than the maximum reported values for the *P. canescens* (407.4 ± 5.7 at 2000 μg/mL) and *P. simplcissimum* (412.6 ± 4.9 at 2000 μg/mL) in this work. There are no reports of *Talaromyces* species with Pb(II) uptake capacities specifically performed either through biosorption or bioaccumulation. The remarkable uptake of the *T. macrosporus* (601.0 ± 3.6 at 3000 μg/mL) and *Talaromyces sp.* (519.1 ± 2.6 at 2000 μg/mL) show that all four species have potential for use as material for the remediation of Pb(II) from the environment.

5 Conclusions

The remarkable tolerance and uptake of each of *P. canescens*, *P. simplcissimum T. macrosporus* and *Talaromyces sp.* are demonstrated in this study. All isolates can tolerate increased Pb(II) concentrations on both solid and liquid medium, comparable to results for other fungal species in literature. The initial concentration of Pb(II) does not appear to have significant influence on the removal rates of the *Talaromyces* isolates but increases removal in *Penicillium* isolates when grown in liquid medium. Uptake of Pb(II) increases with increasing Pb(II) concentration up to 2000 μg/mL for all isolates except *T. macorsporus*, where uptake is increased until 3000 μg/mL. The uptake rates presented in this study are higher than those recorded in literature. The high removal rates and uptake abilities of all isolates on liquid media compared to current literature make them ideal candidates for Pb(II) biosorption and bioaccumulation studies.

Author Contributions

ZAN Maini is the project leader who was responsible for overseeing the entire work, drafting the manuscript and assisting in the PCR-based identification of the fungal strains as well as performing statistical analyses. KMJ Aribal was responsible for preparation of stock solutions reagents and mixtures, maintaining the fungal cultures, performing additional TI replications and experiments, and assisted in the performance of the statistical analysis. JAD Frejas & LAM were responsible for performing environmental sampling, initial screening, purification of the cultures, as well as performing the PCR-based identification of the fungal strains. Narag RMA & Melad JKUT performed the removal and uptake tests, as well as initial TI measurements. PCG Gulpeo IA Navarrete and Lopez CM assisted with the over-all design of the experiment, screening of Pb(II) tolerant species, morphological identification, and determination of culture conditions on solid and liquid media and provided supervisory capacity on all levels of the work including assistance in reviewing and drafting the manuscript.

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Conflict of Interest

Authors have no conflict of interest in the research reported here.

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