Effective removal of fly ash by *Penicillium chrysogenum* and determination of direct fly ash toxicity with *Daphnia magna*

Burcu Ertit Taştan

**ABSTRACT**

This study demonstrates the removal of fly ash with *Penicillium chrysogenum*, a newly isolated species of fungus, and acute toxicity assessment with *Daphnia magna*. In the study, two different removal mechanisms were compared, both bio-removal and bio-sorption. Six different ash and three different biomass concentrations were used simultaneously. Although other fungal species in the literature failed at such a high concentration of fly ash, *P. chrysogenum* was able to tolerate it even at 10% concentration. The highest bio-removal yield was recorded as 100% at 0.5% fly ash concentration. Maximum bio-sorption yield was 95.27% after 24th hour. The evaluation results of fly ash bio-toxicity by *D. magna* showed that the no observed effect level (NOEL) was 0.2 mg/L and the low observed effect level (LOEL) was 0.5 mg/L. The element analysis, determined by ED-XRF, clarified that Ca, Si, Fe and S were the common elements in this ash. This is the first study in the literature where fly ash removal was carried out using *P. chrysogenum* for both bio-removal and bio-sorption and needs to be developed in the future.

**Key words** | ash bio-removal, bio-sorption, bio-toxicity, *Daphnia magna*, fly ash, *Penicillium chrysogenum*

**HIGHLIGHTS**

- Fly ash removal by *P. chrysogenum* was investigated.
- The fungus achieved the highest removal yields.
- Highest pulp densities of fly ash were tolerated by the fungus in the literature.
- Highest biosorption yield was determined in the literature as 95.27% after 24 hours.
- Evaluation of fly ash bio-toxicity by *D. magna* was revealed for the first time.
GRAPHICAL ABSTRACT

INTRODUCTION

Fly ash is the largest byproduct of coal plants. Approximately 80% of the total coal ash is fly ash and 20% is bottom ash (Querol et al. 1995). Fly ash is produced at 1,200–1,700 °C from a variety of organic and inorganic compounds (Vassilev & Vassileva 2005). The geological factors and operating conditions are important parameters that affect the chemical characterization of fly ash (Siddique 2013). Also, the pH of fly ash varies from 4.5 to 13.25 depending on the sulfur and CaO content of the coal (Riehl et al. 2010). SiO₂, Al₂O₃, Fe₂O₃ and CaO are the main components of the fly ash.

Fly ash is toxic to human health due to its small particles (Borm 1997; Ahmaruzzaman 2010). Money spent on health problems caused by thermal power plants using coal was calculated as 55 trillion Turkish lira (Querol et al. 1995). The fly ash produced worldwide is estimated to be around 500 million tonnes (Ahmaruzzaman 2010). Turkey is one of the countries in the world with the highest coal consumption.

Cost effective biological methods are used to reduce hazardous materials. One of the most effective microorganisms used in biological treatments are fungi, which are very effective microorganisms in heavy metal accumulation (Leung et al. 2010; Taştan et al. 2010). Therefore, they are frequently preferred in biotechnological applications.

The use of fungi in the bio-removal process of heavy metals from fly ash is one of the most popular research areas in recent years (Xu & Ting 2009). Penicillium chrysogenum, a species of fungus mostly found in damp environments, is preferred in treatment studies due to its ease of culture, availability, low cost and adaptation to adverse conditions (Aranciaga et al. 2012; Ferreira-Guedes & Leitao 2018). However, according to our best knowledge there is no study on the bio-removal and bio-sorption of fly ash by Penicillium genus. Previous studies have shown that Aspergillus species fail at high fly ash concentrations. The aim of the present study is to reveal the potential usability of P. chrysogenum at high fly ash concentrations with a low cost culture media, minimum operation time and minimum biomass concentrations.

There are quite a limited number of studies in the literature investigating fly ash toxicity with Daphnia magna (Blinova et al. 2012; Rodrigues et al. 2020), and they the acute toxicity of fly ash for D. magna. Millions of tonnes of fly ash are produced by factories every year, and this is a very serious global environmental problem. This study adds much new information to the literature to provide a solution to this global problem.
MATERIALS AND METHODS

Fly ash

Incineration fly ashes of different sizes (116, 232, 248, 265, 348 and 809 μm) were obtained from power plants in the Central Anatolia Region in Turkey. Fly ashes were quantified by preparing 10,000 ppm stock solutions continuously mixed with a magnetic stirrer, after which they were autoclaved at 121 °C for 15 min before use (Taştan 2017).

Culture conditions

The aim was to use a low cost culture medium in this study. Therefore, fungal biomass was inoculated into 250 mL Erlenmeyer flasks containing sterile fly ash in 100 mL T6 nutrimedia (Maysa, Turkey Rev: NMT6.01.0113) as a low cost industrial medium (Taştan 2017). The pH of the growth medium was adjusted to 6.0 by adding diluted (0.01 M) and concentrated (1 M) sulfuric acid or sodium hydroxide solutions. The flasks were incubated at 25 ± 2 °C on a rotary shaker (VWR 5000 Model Orbital Shaker) at 100 rpm for 7 days. In order to quantify the initial fungal biomass concentration, 0.5 g/L dry weight of fungal biomass was inoculated in 100 mL culture medium.

In some treatment studies, a more effective removal process was tested by using two types of culture stages (Wang et al. 2009). In the first type of culture stage, the fungus was inoculated into culture media at the start time (t = 0 day) and at the same time sterile fly ash was added to the media (t = 0 day), then they were incubated for 7 days. In the second type of culture stage, first the fungus was pre-incubated for 5 days (t = 5 days) without fly ash and then sterile fly ash was added on the fifth day (t = 5 days) and incubated for another 2 days. A schematic of the experimental setup is shown in Figure 1.

Isolation of microorganism

Cheese from Erzurum (Turkey) was selected as the inoculum and it was spread on potato dextrose agar (PDA) in Petri plates and incubated at 30 ± 1 °C. After 7 days of incubation, fungal colonies were isolated and then purified by streaking the cells repeatedly on the PDA. The pure cultures were kept at 4 °C and were transferred to fresh PDA every 3 months (Taştan 2017).

Identification of new isolate with PCR and sequencing

Whole cells from an exponentially growing culture of the isolate were used for 5.8S rRNA gene amplification. The 5.8S rRNA region was amplified with primers as designed by Bokulich & Mills (2013), forward ITS1 5′-TCC GTA GGT GAA CCT GCG G-3′, reverse ITS2 5′- GCT GCG TTC TTC ATC GAT GC-3′. Polymer Polymerase chain reaction (PCR) was carried out in 50 μL reactions and the reaction mix included 0.3 μM of each primer, 0.2 mM of each dNTP, 2.0 mM MgCl₂ and 100 ng of template DNA. Taq DNA polymerase was used in the amplification. Amplification by PCR technique was carried out by an initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds, an extension at 72 °C for 1 min, and a final extension of 72 °C for 5 min. Applied Biosystems Genetic Analyzer 3130 was used in sequencing.

Bio-removal process

Four different culture media compositions were prepared for the fungal ash bio-removal process. (i) T6 nutrimedia (12 g/L),
(ii) T6 nutrimedia (12 g/L) + corn syrup (5 mL/L) (Vanikoy, Turkey), (iii) T6 nutrimedia (12 g/L) + whey powder (2.5 g/L) (Maybi, Turkey) and (iv) T6 nutrimedia (12 g/L) + corn syrup (5 mL/L) + whey powder (2.5 g/L). To assess the effect of incineration fly ashes of different sizes (116, 232, 248, 265, 348 and 809 μm) on fungal ash bio-removal, the fungus was cultivated separately and simultaneously in the first type of culture stage and the second type of culture stage.

The effect of pH on fungal ash bio-removal was investigated at pH 4.0, 5.0, 6.0, 7.0 and 8.0 at 1% fly ash concentration in the second type of culture stage. To detect the effect of fly ash concentration, 0.5, 1, 5, 7.5 and 10 g fly ash was included in 100 mL culture media in the second type of culture stage. The concentration of fly ash was expressed as 0.5, 1, 5, 7.5 and 10%. The effect of increasing fungal biomass concentrations was investigated at 1% concentration of selected optimum fly ash in the second type of culture stage. The initial fungal biomass concentrations corresponded to the dry weights $X = 0.25$ g/L, $X = 0.5$ g/L and $X = 1$ g/L.

**Bio-sorption process**

The fungal biomass was centrifuged at 5,000 rpm for 10 min at the end of the logarithmic growth period. After washing the pellet twice with dH2O it was autoclaved (121 °C, 15 min, ALP CLG-40M, Japan). The wet cells were converted to dry weight using the wet/dry weight standardization method ($R^2 = 98.5\%$). Bio-sorption studies were carried out in 100 mL aqueous solution in 250 mL Erlenmeyer flasks as described by Laçin et al. (2015) at 5, 15 and 30 min, and 1, 2, 4, 6, and 24 hours with 1% fly ash concentration. The fungal ash bio-sorption yield ($Y\%$) was calculated using Equation (1) as described below. An ash-free control group was used as the blank.

$Y\% = \frac{(C_0 - C_i)}{C_0} \times 100$ \hspace{1cm} (1)

$Y\%$ is the maximum amount of fly ash taken up per unit dry weight of fungal cells (mg/g), $X$ is dry biomass (g/L), $C_0$ is the initial fly ash concentration (mg/L) and $C_i$ is the final fly ash concentration (mg/L), respectively (Taşran et al. 2010, 2012).

In these equations, $q_m$ is the maximum amount of fly ash taken up per unit dry weight of fungal cells (mg/g). $X$ is dry biomass (g/L), $C_0$ is the initial fly ash concentration (mg/L) and $C_i$ is the final fly ash concentration (mg/L), respectively (Taşran et al. 2010, 2012).

The biomass was calculated by determining the dried cell bulk. The cell bulk was calculated by measuring the centrifuged pellets, which were dried at 80 °C overnight (Taşran et al. 2016). The dried cell bulk was expressed as the ash-free immobilization of fly ash, daphnids were used as the test species (Balusamy et al. 2013). The daphnids were cultivated at a stable temperature of 20 ± 1 °C in 16:8 hours light:dark periods. Increasing concentrations of fly ash at 0, 0.2, 1, 5 and 10 mg/L were tested in the test medium. Bio-toxicity studies were conducted as three replicates and five daphnids were used for each concentration and for the control group. After the 24, 48 and 72 hours, daphnids in each group were tested for immobilization.

**Analytical methods**

In the experiments 3 mL samples were taken daily for bio-removal experiments and taken hourly for bio-sorption experiments during the incubation period. Erlenmeyer flasks not containing fly ash were used as the control media.

The fungi to be used during calculations was passed through a series of steps as follows:

i. After the incubation period was completed, the fungi were filtered through coarse filter paper.

ii. The fungi were washed to allow cells to separate from medium residues.

iii. The fungi containing ash and the fungi without ash (control media) were weighed separately according to their purpose as wet or dry weight and tared.

Fungal ash bio-removal and bio-sorption yields ($Y\%$) and maximum specific fly ash uptake ($q_m$) was calculated using Equations (1) and (2);

$Y\% = \frac{(C_0 - C_i)}{C_0} \times 100$ \hspace{1cm} (1)

$q_m = \frac{(C_0 - C_i)}{X}$ \hspace{1cm} (2)

EC50 is the concentration estimated to immobilize 50% of D. magna (daphnids) within a stated exposure period. Immobilization is taken to mean those animals that are not able to swim within 15 seconds, after gentle agitation of the test vessel (OECD 2004). To detect the acute immobilization of fly ash, daphnids were used as the test species (Balusamy et al. 2013). The daphnids were cultivated at a stable temperature of 20 ± 1 °C in 16:8 hours light:dark periods. Increasing concentrations of fly ash at 0, 0.2, 1, 5 and 10 mg/L were tested in the test medium. Bio-toxicity studies were conducted as three replicates and five daphnids were used for each concentration and for the control group. After the 24, 48 and 72 hours, daphnids in each group were tested for immobilization.
control group ($X_c$) and the ash-containing group ($X_s$). The fly ash concentration was measured as 0.5, 1, 5, 7.5 and 10 g fly ash in 100 mL culture media and expressed as 0.5, 1, 5, 7.5 and 10%.

An energy dispersive X-ray fluorescence spectrometer (ED-XRF, Shimadzu) was used for element analyses. Shimadzu reference materials (standards) were used for calibrating the ED-XRF instrument. The samples were dried at 110 °C for 12 hours before the analyses and detected with a silicon drift detector (Taştan 2017).

All of the tests were carried out three times. All data were calculated with corresponding standard error as formulated and described by Kenney & Keeping (1962), Equation (3); $\sigma$ is the square root of the estimated error variance of the quantity.

$$SE = \sqrt{\sigma^2}$$

(3)

**RESULTS AND DISCUSSION**

**Effect of growth medium on fungal ash bio-removal**

As a result of PCR and sequencing, the isolated new strain was identified as *P. chrysogenum*. The effect of different media compositions were tested for increasing the performance of fungal fly ash uptake and summarized in Table 1. The bio-removal yield obtained in T6 nutrimedia was higher than the yield of T6 nutrimedia + corn syrup media, but it was lower than the yields that were obtained in T6 nutrimedia + whey powder. In this step, dry biomass amounts of the ash-free control group ($X_c$) and ash-containing sample groups ($X_s$) were compared. The lowest yield was obtained in T6 nutrimedia + corn syrup + whey powder media, while the lowest $X_s$ was obtained in T6 nutrimedia. Therefore T6 nutrimedia + whey powder media was more efficient in fly ash removal process and also the $X_c$ value (2.65 g/L) recorded in this medium was only 21.6% lower than of $X_s$ (5.38 g/L). By contrast, the maximum $X_c$ was obtained in T6 nutrimedia + corn syrup media, while 30.8% decrease was observed in $X_s$ amount of fungus. The highest difference between $X_s$ and $X_c$ amounts was observed in T6 nutrimedia as 34.6%. In a summary, the highest bio-removal yield, highest $X_s$ amount and the minimum difference between $X_s$ and $X_c$ amounts were obtained in T6 nutrimedia + whey powder media.

In the removal of various contaminants, it was found that different culture media had a direct effect on fungal removal efficiency (Marcial et al. 2006; Taştan et al. 2016). The use of this inexpensive and commercial T6 nutrimedia culture medium in the literature is very recent. Studies have shown that the medium is effective in ash removal by different types of fungi (Taştan 2017). In this study, *P. chrysogenum* was able to tolerate ash up to 10% concentration using T6 nutrimedia culture medium as can be seen in Table 1. In a study using the same medium, Fusarium oxysporum and *P. glabrum* removed 6.36 and 18.65% fly ash at 7.5% pulp concentration, respectively (Taştan 2017). In another study, standard sucrose medium was used as the culture medium and *A. niger* did not show any growth at 6% ash concentration (Xu & Ting 2009). The results show that this commercial medium is a highly productive medium that will contribute to the literature.

**Effect of different fly ashes on fungal ash bio-removal**

The fungus was cultivated in the two independent types of bio-removal process with six different fly ashes (Table 2). In the first type of culture stage, fungus was first cultivated

| Culture media | $Y$ (%) | $X_c$ (g/L) | $X_s$ (g/L) |
|---------------|---------|------------|-------------|
| T6 nutrimedia | 76.79 ± 1.11 | 2.06 ± 0.28 | 3.15 ± 0.71 |
| T6 nutrimedia + corn syrup | 71.60 ± 0.41 | 2.47 ± 0.37 | 3.57 ± 0.40 |
| T6 nutrimedia + whey powder | 83.64 ± 0.55 | 2.65 ± 0.32 | 3.38 ± 0.22 |
| T6 nutrimedia + corn syrup + whey powder | 42.25 ± 0.28 | 2.57 ± 0.11 | 3.33 ± 0.15 |

### Table 1

Effects of different media on bio-removal yield ($Y$ %) of fly ash by *P. chrysogenum* ($X_c$; dry biomass amounts of ash-free control group, $X_s$; and ash-containing sample groups)
with incineration fly ashes of different sizes (116, 232, 248, 265, 348 and 809 μm) at 1% fly ash concentration. According to results, the maximum bio-removal yield, 82.32%, was obtained with fly ash of 248 μm size and the minimum bio-removal yield, 45.25%, was obtained with fly ash of 809 μm size, due to the larger size of this fly ash. In the second type of culture stage, fungus was introduced with fly ash after 5 days of pre-incubation. According to these results, the maximum bio-removal yield was obtained with fly ash of 116 μm size. The minimum bio-removal yield, 100%, was obtained again in fly ash of 809 μm size. The second type of culture stage allowed the rapid transformation of fungal spores into micelles and removed the fly ash with higher yields in a shorter time (2 days). By contrast, while the ash size was 26.3 μm, A. niger showed an improvement at 0–5% fly ash pulp density, but did not show an improvement at 6% (Xu & Ting 2009). In another study, more effective treatment was obtained by using two types of culture stages (Wang et al. 2009), and 45% decrease in lag phase was realized by pre-washing before the fungal bioleaching process and thus the bioleaching process was accelerated by 30%. When all results are taken into consideration, 116 μm size of fly ash was selected for further testing in the second type of culture stage (t = 5 days).

### Effect of pH on fungal ash bio-removal

The effect of pH on fungal ash removal yield by *P. chrysogenum* is presented in Table 3. Although the same removal yields were observed at pH 4.0, 5.0 and 6.0, yields decreased up to 55.61% at pH 8.0. The maximum X (dry weight g/L) values were obtained as 3.08 g/L at pH 6.0 despite these equal removal yields. Therefore, the optimum pH was selected as 6.0 (Table 3).

In another study, two types of culture stages were used. In the first one, fungus and ash were incubated at the same time, in the second, fly ash was added after the fungus developed for 2 days. At the end of day 15 in the first type of culture stage, the pH decreased to about 6.0 and in the second type of culture stage pH decreased to about 4.0 (Xu et al. 2014), and these results support this study.

### Effect of increasing fly ash concentrations on fungal ash bio-removal

The results of increasing fly ash concentrations from 0.5 to 10% are summarized in Figure 2. As seen in the Figure 2, *P. chrysogenum* tolerated the fly ash well up to high concentrations. The maximum fungal ash removal yields were obtained at the lowest two concentrations of fly ash (0.5 and 1%). The fungus was able to completely remove low concentrations, and it was also able to tolerate high concentrations up to 10%. In another study the bioleaching kinetics of A. niger in the presence of fly ash at 1–6% concentrations in a bioleaching process was investigated. According to the results, fungal growth was not observed at higher than 6% of fly ash concentrations (Xu & Ting 2009).

### Table 2 | Fungal ash removal yields (Y %) with different types of culture stages

| Sizes of fly ash (μm) | 116 | 232 | 248 | 265 | 348 | 809 |
|-----------------------|-----|-----|-----|-----|-----|-----|
| Type of culture stage |     |     |     |     |     |     |
| First                 |     |     |     |     |     |     |
| First                 | 78.06 ± 1.74 | 46.49 ± 6.01 | 82.32 ± 0.67 | 14.75 ± 3.77 | 62.67 ± 1.44 | 45.25 ± 0.52 |
| Second                | 100 ± 0 | 63.52 ± 0.74 | 62.62 ± 4.37 | 49.81 ± 1.56 | 51.40 ± 4.34 | 22.97 ± 3.75 |

### Table 3 | Selection of pH in fungal fly ash bio-removal (Y %)

| pH | 4.0 | 5.0 | 6.0 | 7.0 | 8.0 |
|----|-----|-----|-----|-----|-----|
| Y %   | 100 ± 0 | 100 ± 0 | 100 ± 0 | 94.06 ± 4.10 | 55.61 ± 7.67 |
| Xₚ (g/L) | 2.36 ± 0.06 | 2.88 ± 0.19 | 3.08 ± 0.12 | 2.47 ± 0.56 | 2.00 ± 0.23 |
In a study by Xu & Ting (2004), different parameters were investigated on the bioleaching of fly ash by *A. niger*. Results showed that fly ash concentration was more important than the spore concentration and the time of addition of fly ash. In another study the scanning electron microscope (SEM) results of *A. niger* emphasized that the fungal hyphae were impacted negatively by increasing fly ash concentrations (Xu et al. 2014).

According to Figure 2, the maximum specific fly ash uptake \( q_m \) was 4.08 mg/g at 0.5% fly ash concentration. By contrast, the fungus was able to uptake fly ash at 0.08 mg/g at the highest fly ash concentration (10%). The mechanism of fungal cellular uptake was expressed in this stage of experiments. Therefore bio-sorption experiments were also conducted. Further studies are needed to better understand the mechanism, such as genetic, enzymatic and molecular analyses and advanced microscopic studies using transmission electron microscopy (TEM) and SEM.

Shortening the time factor in bio-removal studies affects profits at industrial scales. In the present study *P. chrysogenum* grew in 0.5–10% concentrations of fly ash with the help of adding fly ash after an incubation period, meaning that fungal growth was not inhibited by increasing concentrations of fly ash. Similar to the results of the present study, Xu & Ting (2004) added fly ash after 2 days of fungal incubation and studied 1–8% concentrations. They noted that the fungus *A. niger* was able to grow in the presence of 4% fly ash.

### Table 4 | Effect of initial biomass concentrations on the fungal fly ash bio-removal yields (Y%)

| Initial fungal biomass concentrations \( X_0 \) (g/L) | 0.25 | 0.5  | 1  |
|-----------------------------------------------|------|------|----|
| 6 hours                                      | 97.42 ± 0.51 | 98.71 ± 0.32 | 100 ± 0 |
| 24 hours                                     | 100 ± 0   | 100 ± 0   | 100 ± 0 |
| 48 hours                                     | 100 ± 0   | 100 ± 0   | 100 ± 0 |

### Table 5 | Bio-sorption yields of fly ash by *P. chrysogenum*

| Time  | 1 min  | 5 min  | 15 min | 30 min | 1 hour  | 2 hours  | 4 hours  | 6 hours  | 24 hours |
|-------|--------|--------|--------|--------|---------|----------|----------|----------|----------|
| Y %   | 50.35 ± 3.16 | 51.48 ± 2.6 | 57.93 ± 0.02 | 64.04 ± 2.07 | 57.88 ± 1.45 | 59.62 ± 4.76 | 64.64 ± 3.58 | 66.78 ± 0.12 | 95.27 ± 3.60 |

Effect of increasing biomass concentrations on fungal ash bio-removal

To find a suitable biomass concentration of *P. chrysogenum*, 0.25, 0.5, 1 g/L initial biomass concentrations were tested.
As seen in Table 4, the minimum fungal ash removal yield, 97.42%, was obtained at 0.5 g/L initial biomass concentration in the sixth hour of incubation. When the initial biomass concentration increased to 1 g/L, the yield increased to 100% in the sixth hour. All of the fungal fly ash removal yields increased to 100% after 24 hours at all biomass concentrations. As can be seen from the results, the fungus can be recorded as a very active species for ash removal. Regardless of the initial fungal biomass concentrations within the studied ranges, ash removal yields reached to 100% after 24–48 hours. This led to the result that *P. chrysogenum* is a highly effective biological material that can be used for ash removal in further studies. To the best of our knowledge, investigating the effect of increasing fungal biomass concentrations on ash removal has not been conducted before in the literature. However, ash removal studies in the current literature showed that increasing biomass is associated with increased removal efficiency (Taştan et al. 2017; Taştan & Dönmez 2015).

**Bio-sorption experiments**

To better understand the mechanism of fly ash removal by fungi, the effect of bio-sorption on fungal ash uptake were tested. The data obtained after 24 hours of incubation with autoclaved (dead) fungus are summarized in Table 5. The maximum bio-sorption yield was 95.27% after 24 hours. Moreover half of fly ash was biosorbed in the first hour of the incubation. Therefore bio-sorption of fly ash by *P. chrysogenum* biomass can be recommended as fast way to remove fly ash, a harmful pollutant, from the environment.

In another study, the bio-sorption of heavy metals was carried out by *A. niger* biomass in bioleaching of fly ash. The fungal biomass was in contact with a heavy metal solution extracted from fly ash. This research showed that the bio-sorption of metal ions decreased clearly in the presence of fly ash compared to that in the absence of fly ash (Yang et al. 2009). It was determined that the hours-long bio-sorption process was more effective in ash removal than the days-long fungal cellular uptake mechanism.

**Element analyses**

The element analysis results conducted by ED-XRF are shown in Table 6. In the samples, Ca, Fe and Si were the

| Elemental analyses of fly ash, growing media and biomass of *P. chrysogenum* with fly ash analyzed by ED-XRF |
|---------------------------------------------------------------|
|                        | Ca% | Fe% | Si% | S% | K% | Ti% | Sr% | Mn% | Ni% | Cr% | Cu% | Zr% | V% | Zn% | Rb% | P% | Mo% |
|------------------------|-----|-----|-----|----|----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|---|-----|
| Fly ash                | 38.49±0.16 | 16.52±0.10 | 35.40±0.28 | 5.85±0.03 | 1.67±0.02 | 1.18±0.01 | 0.31±0.00 | 0.22±0.01 | 0.04±0.00 | 0.05±0.00 | 0.06±0.01 | 0.07±0.00 | 0.08±0.00 | 0.09±0.00 | 0.14±0.00 | 0.17±0.00 | 0.02±0.00 | 0.00±0.00 |
| Growing media          | 77.99±0.09 | 0.16±0.03 | 0.78±0.02 | 0.72±0.02 | 2.74±0.02 | 0.11±0.01 | 0.02±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| Biomass of *P. chrysogenum* with fly ash | 39.12±0.23 | 17.74±0.15 | 30.24±0.23 | 5.19±0.18 | 2.53±0.01 | 1.21±0.01 | 0.32±0.00 | 0.25±0.01 | 0.04±0.00 | 0.05±0.00 | 0.06±0.01 | 0.07±0.00 | 0.08±0.00 | 0.09±0.00 | 0.10±0.00 | 0.14±0.00 | 0.17±0.00 | 0.02±0.00 | 0.00±0.00 | 0.00±0.00 |
common elements in the fly ash used in this study. The Ca concentrations were higher than the other major elements in all samples. The reason why the Ca amounts were higher in the biomass of *P. chrysogenum* was due to the high Ca levels observed in growth medium (77.99%). As stated before, the Ca concentration probably was related to the organic matter in the coal samples (Sutcu & Karayigit 2015). Therefore all the samples had high Ca concentrations. The average yield of Si in fly ash was 35.40%. Fe, S, K and Ti were the other remediated elements in fly ash, with the yields of 16.52, 5.85, 1.67 and 1.18%, respectively (Table 6). Elemental analysis results also show that this application can be useful in bio-removal of fly ash. Therefore the isolated fungi, *P. chrysogenum*, could be suggested as efficient bio-material in the process of remediation of metals from fly ash when it is scaled up.

Bayat (2002) examined the removal of Cr and Cd from two fly ashes. The adsorption of Cd was found to be higher than Cr. In another study, some properties of various fly ashes in Turkey were detected and high Ca levels were found (Bayat 1998). In the present study, the reason for high fungal tolerance to increasing fly ash concentrations may be to the high Ca levels in fly ash and in the growth medium.

**Acute immobilization test**

The EC50 concentration was found to be 10 mg/L. However, no LD50 value was determined by *D. magna* after 72 hours of fly ash exposure time. The no observed effect level (NOEL) was found at 0.2 mg/L and low observed effect level (LOEL) was found at 0.5 mg/L fly ash (Figure 3).

There are only a few studies in the literature on the toxicity tests of fly ash using crustaceans. In a study, the toxicity evaluation of municipal solid waste incineration fly ash was evaluated with the role of cadmium by a clone of *D. pulex*. They explained that the leachate was more than 20 times more toxic than expected from its cadmium concentration for survival of *D. pulex* (Kaneko 1996). There are some studies about evaluation of the toxicity of lanthanum oxide (Balusamy et al. 2018), acid dyes (Dehghani et al. 2019), graphene oxide (Zhang et al. 2019), and pesticides (Taştan et al. 2017). But according to our best knowledge there is a limited number of studies in the literature investigating the direct effects of fly ash toxicity. In one of the limited number of studies conducted, it was found that the EC50 value of *D. magna* in fly ash toxicity was 30.80% (Rodrigues et al. 2020), and in another, the inhibition was between 10 and 65% in different fly ash water eluates (Blinova et al. 2012).

**Figure 3** Effect of increasing fly ash concentrations on the mobility of *D. magna* after 72 hours of exposure.
Therefore the present study is the first to reveal the bio-toxicity evaluation of fly ash with above mentioned requirements.

**CONCLUSIONS**

This study highlights the potential usage of *P. chrysogenum* in the bio-removal of fly ash. Six different sizes of fly ash, two different culture stage techniques and three different fungal biomasses were used. In the first type of culture stage, the maximum removal rate was 82.32%, and in the second type of culture stage the maximum removal rate was 100%. The second type of culture stage allowed the rapid transformation of fungal spores into micelles. Thus, the fungal biomass developed rapidly. Fungal biomass could tolerate 10% fly ash concentration, which to the best of our knowledge, is the highest ash concentration in the literature. ED-XRF results helped to show the fate of metals in fly ash. In this study, in which both bio-sorption and bio-removal mechanisms were investigated, bio-sorption was found to be a highly effective mechanism, as well as bio-removal. *P. chrysogenum* could serve as an effective bio-agent for bio-removal and bio-sorption of fly ash from aqueous media. The present method was found to be advantageous as compared to other reported bio-removal studies, as it allowed the fungus to tolerate high concentrations of fly ash.

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**DATA AVAILABILITY STATEMENT**

All relevant data are included in the paper or its Supplementary Information.

**REFERENCES**

Ahmaruzzaman, M. 2010 A review on the utilization of fly ash. *Progress in Energy and Combustion Science*. https://doi.org/10.1016/j.pecs.2009.11.003.

Aranciaga, N., Durruty, I., Gonzalez, J. F. & Wolski, E. A. 2012 Aerobic biotransformation of 2, 4, 6-trichlorophenol by *Penicillium chrysogenum* in aqueous batch culture: degradation and residual phytotoxicity. *Water SA*. https://doi.org/10.4314/wsa.v38i5.5.

Bayat, O. 1998 Characterisation of Turkish fly ashes. *Fuel*. https://doi.org/10.1016/S0016-2361(97)00274-3.

Balusamy, B., Taştant, B. E., Ergen, S. F., Uyar, T. & Tekinay, T. 2015 Toxicity of lanthanum oxide (*La_2O_3*) nanoparticles in aquatic environments. *Environmental Sciences: Processes and Impacts*. https://doi.org/10.1016/j.ejps.2015.09.059.

Bayat, B. 2002 Comparative study of adsorption properties of Turkish fly ashes: II. The case of chromium (VI) and cadmium (II). *Journal of Hazardous Materials*. https://doi.org/10.1016/S0304-3894(02)00141-3.

Blinova, I., Bityukova, L., Kasemets, K., Ivask, A., Käkinen, A., Kurvet, I., Bondarenko, O., Kanarbik, L., Sihtmae, M., Arujo, V., Schvede, H. & Kahru, A. 2012 Environmental hazard of oil shale combustion fly ash. *Journal of Hazardous Materials*. http://dx.doi.org/10.1016/j.jhazmat.2012.05.095.

Bokulich, N. A. & Mills, D. A. 2013 Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.03870-12.

Borm, P. J. A. 1997 Toxicity and occupational health hazards of coal fly ash (CFA). A review of data and comparison to coal mine dust. *Annals of Occupational Hygiene*. https://doi.org/10.1016/S0003-4878(97)00026-4.

Dehghani, M. H., Mahdavi, P., Tyagi, I., Agarwal, S. & Gupta, V. K. 2016 Investigating the toxicity of acid dyes from textile effluent under UV/ZNO process using *Daphnia magna*. *Desalination and Water Treatment*. https://doi.org/10.1007/s11281-016-11432-7.

Ferreira-Guedes, S. & Leitao, A. L. 2018 Simultaneous removal of dihydroxybenzenes and toxicity reduction by *Penicillium chrysogenum* var. *halophenolicum* under saline conditions. *Ecotoxicology and Environmental Safety*. https://doi.org/10.1016/j.ecoenv.2017.12.046.

Kaneko, H. 1996 Evaluation of municipal waste incinerator fly ash toxicity and the role of cadmium by two aquatic toxicity tests. *Waste Management*. https://doi.org/10.1016/S0956-053X(96)00097-9.

Kenney, J. F. & Keeping, E. S. 1962 Linear Regression and Correlation. In *Mathematics of Statistics*. Part 1, 3rd edition. Van Nostrand, Princeton, NJ, pp. 252–285.

Laçin, B., Taştant, B. E. & Dönmez, G. 2015 Detection of boron removal capacities of different microorganisms in wastewater and effective removal process. *Water Science and...
Technology 72 (10), 1832–1839. https://doi.org/10.2166/wst.2015.409.

Leung, H. M., Wu, F. Y., Cheung, K. C., Ye, Z. H. & Wong, M. H. 2010 Synergistic effects of arbuscular mycorrhizal fungi and phosphate rock on heavy metal uptake and accumulation by an arsenic hyperaccumulator. Journal of Hazardous Materials. https://doi.org/10.1016/j.jhazmat.2010.05.042.

Marcial, J., Barrios-Gonzalez, J. & Tomasini, A. 2006 Effect of medium composition on pentachlorophenol removal by Amylomyces rouxii in solid-state culture. Process Biochemistry. https://doi.org/10.1016/j.procbio.2005.07.010.

OECD 2004 Guideline 202: Daphnia sp., Acute Immobilisation Test. OECD guidelines for testing of chemicals.

Querol, X., Fernández-Turiel, J. & López-Soler, A. 1995 Trace elements in coal and their behaviour during combustion in a large power station. Fuel. https://doi.org/10.1016/0016-2361(95)03464-0.

Riehl, A., Elsass, F., Duplay, J., Huber, F. & Trautmann, M. 2010 Changes in soil properties in a fluvisol (calcaric) amended with coal fly ash. Geoderma. https://doi.org/10.1016/j.geoderma.2009.11.025.

Rodrigues, P., Silvestre, J. D., Flores-Colen, I., Viegas, C. A., Ahmed, H. H., Kurda, R. & de Brito, J. 2020 Evaluation of the ecotoxicological potential of fly ash and recycled concrete aggregates use in concrete. Applied Sciences. http://dx.doi.org/10.3390/app10010351.

Siddique, R. 2010 Utilization of coal combustion by-products in sustainable construction materials. Resources, Conservation and Recycling. https://doi.org/10.1016/j.yreccon.2010.06.011.

Sutcu, E. C. & Karayigit, A. I. 2015 Mineral matter, major and trace element content of the Afsin-Elbistan coals, Kahramanmaras, Turkey. International Journal of Coal Geology. https://doi.org/10.1016/j.coal.2015.04.007.

Taştan, B. E. 2017 Clean up fly ash from coal burning plants by new isolated fungi Fusarium oxysporum and Penicillium glabrum. Journal of Environmental Management. https://doi.org/10.1016/j.jenvman.2017.05.062.

Taştan, B. E. & Dönmee, G. 2015 Biodegradation of pesticide triclosan by A. versicolor in simulated wastewater and semi-synthetic media. Pesticide Biochemistry and Physiology. http://dx.doi.org/10.1016/j.pestbp.2014.11.002.

Taştan, B. E., Erteğrul, S. & Dönmee, G. 2010 Effective bioremoval of reactive dye and heavy metals by Aspergillus versicolor. Bioresource Technology. https://doi.org/10.1016/j.biortech.2009.08.099.

Taştan, B. E., Duygu, E. & Dönmee, G. 2012 Boron bioremoval by a newly isolated Chlorella sp and its stimulation by growth stimulators. Water Research. https://doi.org/10.1016/j.watres.2011.05.045.

Taştan, B. E., Özdemir, C. & Tekinay, T. 2016 Effects of different culture media on biodegradation of triclosan by Rhodotorula mucilaginosa and Penicillium sp. Water Science and Technology. https://doi.org/10.1016/wst.2016.221.

Taştan, B. E., Tekinay, T., Çelik, H. S., Özdemir, C. & Cakir, D. N. 2017 Toxicity assessment of pesticide triclosan by aquatic organisms and degradation studies. Regulatory Toxicology and Pharmacology. https://doi.org/10.1016/j.jytph.2017.10.030.

Vassilev, S. V. & Vassileva, C. G. 2005 Methods for characterization of composition of fly ashes from coal-fired power stations: a critical overview. Energy and Fuels. https://doi.org/10.1021/ef049694d.

Wang, Q., Yang, J., Wang, Q. & Wu, T. 2009 Effects of water-washing pretreatment on bioleaching of heavy metals from municipal solid waste incinerator fly ash. Journal of Hazardous Materials. https://doi.org/10.1016/j.jhazmat.2008.05.125.

Xu, T. J. & Ting, Y. P. 2004 Optimisation on bioleaching of incinerator fly ash by Aspergillus niger – Use of central composite design. Enzyme and Microbial Technology. https://doi.org/10.1016/j.enzmictec.2004.07.003.

Xu, T. J. & Ting, Y. P. 2009 Fungal bioleaching of incineration fly ash: metal extraction and modeling growth kinetics. Enzyme and Microbial Technology. https://doi.org/10.1016/j.enzmictec.2009.01.006.

Xu, T. J., Ramanathan, T. & Ting, Y. P. 2014 Bioleaching of incineration fly ash by Aspergillus niger – precipitation of metallic salt crystals and morphological alteration of the fungus. Biotechnology Reports. https://doi.org/10.1016/j.btre.2014.05.009.

Yang, J., Wang, Q., Luo, Q., Wang, Q. & Wu, T. 2009 Bio-sorption behavior of heavy metals in bioleaching process of MSWI fly ash by Aspergillus niger. Biochemical Engineering Journal. https://doi.org/10.1016/j.bej.2009.05.022.

Zhang, Y., Meng, T., Shi, L., Guo, X., Si, X., Yang, R. & Quan, X. 2019 The effects of humic acid on the toxicity of graphene oxide to Scenedesmus obliquus and Daphnia magna. Science of the Total Environment. https://doi.org/10.1016/j.scitotenv.2018.08.280.

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