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Removal of Lead from Wastewater Contaminated with Chemical Synthetic Dye by *Aspergillus terreus*

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Abstract

Novel isolated microorganisms have been demonstrated to efficiently remove lead from wastewater contaminated with chemical synthetic dye. In this study, the physical and chemical parameters of wastewater samples (including Pb concentrations) were analyzed before and after treatment with microorganisms. The highest Pb concentration detected in wastewater was 0.788 mg/l. Investigations of the Pb tolerance and removal capacities of microorganism strains isolated from the wastewater sediment resulted in the selection of three fungal isolates (F102, F203 and F302). Interestingly, isolate F203 had a Pb tolerance of up to 100 mg/l. Using DNA barcoding and morphological characteristics, fungal isolate F203 was identified as *Aspergillus terreus*. Wastewater characteristics before treatment included a grayish black color with pH, TDS, BOD, COD and Pb concentrations higher than the Thailand standard values. Wastewater qualities after treatment with *A. terreus* showed definite improvement; however, the values of certain parameters were still higher than the allowed values based on the Thailand standard. The only improvement that fell within the allowed standard was the Pb concentration. Next, *A. terreus* was used for Pb adsorption in wastewater with an initial Pb concentration of 0.788 mg/l at time points corresponding to 0, 24, 48, 72, 96, 120, 144 and 168 h of incubation. The results showed that *A. terreus* could adsorb and remove higher amounts of Pb from wastewater than the other fungal isolates. Time course adsorption analysis showed the remaining Pb concentrations as 0.788, 0.213, 0.162, 0.117, 0.100, 0.066, 0.042 and 0.032 mg/l, respectively; the percentage of Pb removal could be estimated as 0, 72.97, 79.44, 85.15, 87.31, 91.62, 94.67 and 95.94%, respectively. In conclusion, *A. terreus* possessed the ability to adsorb up to 96% of Pb from chemical synthetic dye within 168 h. Thus, *A. terreus* might be suitable for adaptation and use in Pb treatment.

Keywords: lead removal; fungi; wastewater; chemical synthetic dye; *Aspergillus terreus*

1. Introduction

Heavy metals released into the environment by technological activities pose significant threats to natural ecosystems and public health due to their toxicity, bioaccumulation, non-biodegradation and persistence in the food chain (Wang *et al*., 2006; Chojnacka, 2010; Sousa *et al*., 2010; Mudhoo *et al*., 2012). Thus, excess heavy metals must be removed from wastewater before being discharged into the aquatic environment. As a specific wastewater contaminant, chemical synthetic dye are an important problem in the northeast of Thailand, especially in Chonabot district, Khon Kaen province (Mahachai *et al*., 2009), where wastewater from dyed silk production with high lead (Pb) concentrations has been discharged into the aquatic environment (Volesky and Holan, 1995). Wastewaters from dyeing operations are characterized by color caused by both organic and inorganic compounds containing Pb. Pb is highly toxic to humans, causing damage to the nervous system, reproductive system, and blood circulation system; additionally, Pb is also poisonous to other organisms (Cruz-Olivares *et al*., 2013). Therefore, it is necessary to eradicate the problem (Ahalya *et al*., 2003). There are several methods for wastewater treatment that rely on physical, chemical or biological processes. Each treatment regimen has certain advantages and limitations. Physical and chemical processes have been shown to be the most effective processes. Nevertheless, in practice, the lack of proper procedures renders the process difficult, with a high cost of treatment. Hence, biological processes are a better alternative choice. These techniques are feasible for Pb removal due to their good performance, convenient methods and low cost compared to the physical and chemical methods, although the ability of organisms to remove Pb has not been optimized (Montazer-Rahmati *et al*., 2011). Microorganisms are an accepted alternative, and there has been a great deal of interest in their adoption for wastewater treatment. Many microorganisms have been examined for their biosorption properties, and different types of biomasses have shown high levels of metal uptake (Merroun *et al*., 2001; Bermudez *et al*., 2012; Colin *et al*., 2012). Microorganisms possess different absorption and degradation capabilities depending on several factors, such as ion exchange, adsorption/adsorption, enzyme systems, and control of genes involved in adsorption (Vijayaraghavan *et al*., 2004). Microbial species that have been applied for wastewater treatment include Bacillus sp. (Nourbakhsh *et al*., 2002), *Streptomyces rimosus* (Selatnia *et al*., 2004), *Pseudomonas aeruginosa* (Gabr *et al*., 2008), *Aspergillus flavus* (Akar and Tunali, 2006), *Botrytis cinerea* (Akar and Tunali, 2005), *Mucor rouxii* (Yan and Viraraghavan, 2003) and *Neurospora crassa* (Kiran *et al*., 2005). Several molecular techniques have been used to identify the species of isolated microorganisms and their genetic relationships. For example, molecular identification of fungi through DNA barcoding has become an integrated and essential part of fungal research and has provided new insights into the diversity of many different groups of fungi (Horton and Bruns, 2001; Anderson and Cairney, 2004; Chase and Fay, 2009; Seifert, 2009).

In this work, we studied wastewater quality, the level of Pb contamination, and the bioremediation of Pb in wastewater using microorganisms isolated from wastewater sediments and identified the species of microorganisms for further use in the treatment of wastewater contaminated with chemical synthetic dye.

2. Materials and Methods

2.1. Sampling sites

Wastewater contaminated with chemical synthetic dye is an important problem in the northeast of Thailand. There are many human communities living within the catchment area where the wastewater is situated, with small waterways running from high elevation at the top of the plateau to lower areas. These waterways combine and eventually join the reservoir. Wastewater and sediment samples were collected in the Chonabot district of the Khon Kaen province of Thailand (Fig. 1).

2.2. Wastewater sampling and analysis

Wastewater samples were collected from the small waterways prior to their joining the reservoir in the Chonnabot district, Khon Kaen province, Thailand (Fig. 1). This is normal waterways that receive both wastewater and rain water. Wastewaters were directly discharge from dyed silk activities without further treatment. The physical and chemical parameters of the wastewater samples (Table 1) were analyzed before and after treatment with the effective microorganisms. Pb concentrations in the wastewater samples were determined using a model Analyst 300 atomic absorption spectrometer (AAS) (Correia *et al*., 2003).

2.3. Microorganism screening and species identification

The microorganism strains were isolated from the sediment of wastewater contaminated with chemical synthetic dye that was observed to have the highest Pb concentration (0.788 mg/l). The medium used for isolation was nutrient agar (NA) (0.5% peptone, 0.5% NaCl, 0.2% yeast extract, 0.2% beef extract and 1.5% agar, pH 7.0, all w/v) containing 1 mg/l of Pb(II) acetate. NA was used for primary isolation only. A total of 8 fungal isolates and 25 bacterial isolates were collected after testing to determine their Pb resistance. The testing was conducted in NA supplemented with Pb concentration levels of 10, 100, 500, 1000, 1200, 1400, 1600 and 1800 mg/l. Three bacterial isolates (B206, B308 and B405) and three fungal isolates (F102, F203

Table 1. Methods used for analysis of physical and chemical parameters of wastewater contaminated with chemical synthetic dye

Physical and chemical parameters	Analysis methods
Color	The color chart HTML-RGB
Turbidity	Turbidity meter, model 966, Mettler Toledo
Total Dissolved Solid; TDS	EC meter, model CH-8603, Mettler Toledo
pH	pH meter, model EcoScan pH 5, Eutech
Electrical Conductivity; EC	EC meter, model CH-8603, Mettler Toledo
Biochemical Oxygen Demand; BOD	Azide Modification method (APHA, 2005)
Chemical Oxygen Demand; COD	Azide Modification method (APHA, 2005)
Pb concentrations	AAS, model Aanalyst 300, Perkin-Elmer (Correia *et al*., 2003).

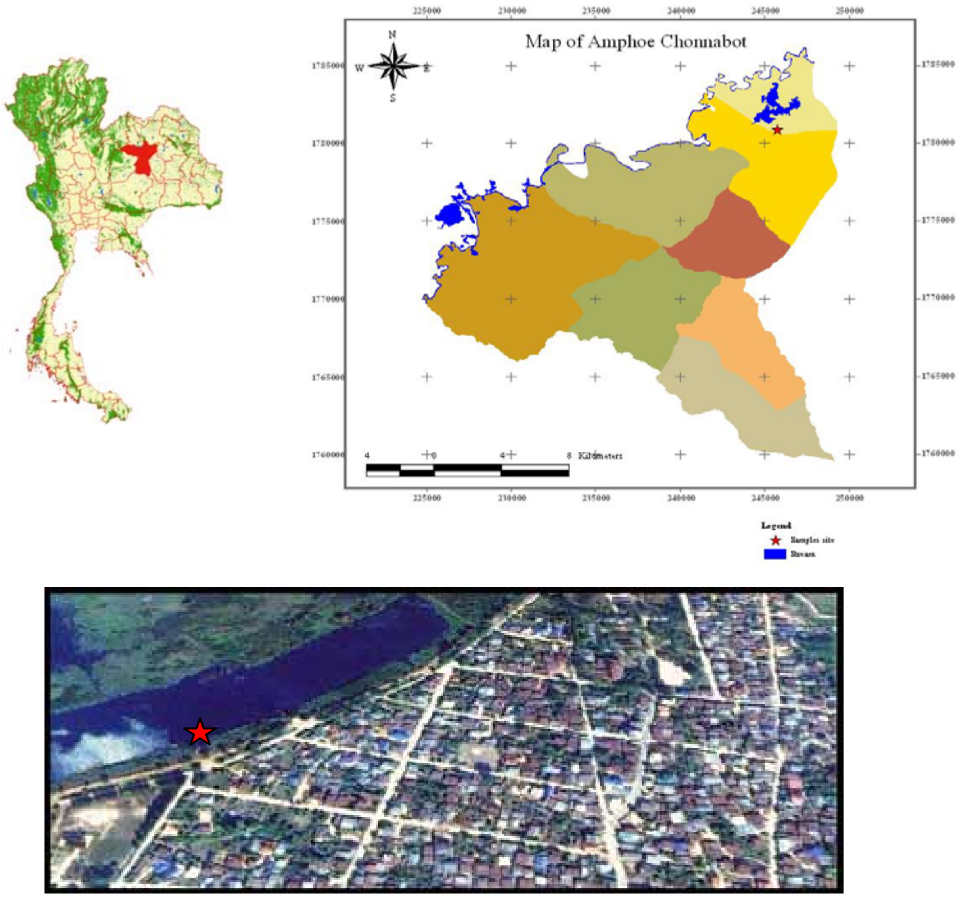

Figure 1. Overview of the Chonabot district area, The location of the sampling collection site is labeled with a star.

and F302) exhibited growth in the presence of high Pb concentrations, although the three fungi were observed to be more tolerant of Pb than the bacteria. The most effective fungi was identified by gene sequencing modified in according to Gostincar *et al*. (2012) and specific primers (50-AATGCAAAAACTGCATA-ACCAC-30 and 50-GGGATAGGCTTACCTTC-GAAG-30). The acquired sequences were compared in dendrogram construction using the MEGA5 program (Tamura *et al*., 2011) and comparisons to genetic similarity values at the Collaborative Unit of Biological Science and Biotechnology of Mahidol University and Osaka University. Additionally, morphological characteristics were examined by scanning electron microscopy (SEM) using a LEO 1450VP apparatus. Specimens were prepared by fixation, dehydration and coating with a very thin layer of carbon (Lyman *et al*., 1990).

2.4. Pb resistance and absorption/adsorption of three fungal isolates in culture media

The highest levels of Pb resistances were found in 3 fungal isolates (F102, F203 and F302). The isolates were cultured in potato dextrose agar (PDA) using a cork borer to remove colonies onto new PDA agar plates, where they were incubated at 30°C for 7 days. Then, a sterile spreader was used to scrape the spores of the fungus with deionized distilled water into a petri dish. The spores were filtered and counted using a hemacytometer. A 0.2 ml spore suspension from the 3 fungal isolates (5×10^6 spores/ml) (Chatterjee *et al*., 2010) was inoculated into a 250 ml Erlenmeyer flask, incubated at 30°C on an orbital shaker (120 rpm) for 72 h and washed with deionized distilled water (Tsekova *et al*., 2010). Three fungal isolates from the fungal preparation process were used as living cells by suspending 0.35 g dry cell weight (the dry weight of cells obtained by baking at 70°C until a constant weight was obtained) into malt yeast extract broth (MYB) mixed with Pb concentrations of 50, 60, 70, 80, 90, 100, 110 and 120 mg/l in a volume of 100 ml. Triplicate experiments were incubated on a shaker at 30°C with agitation (120 rpm) (Majumdar *et al*., 2010). The samples were collected at 24, 48, 72, 96, 120, 144 and 168 h. The samples were centrifuged at 5,000 rpm at 4°C for 10 min, and Pb concentrations in the supernatant were determined by AAS model AAnalyze 300 (Perkin-Elmer). Fungal growth after treatment with Pb in MYB was determined for each

Pb concentration at 168 h.

2.5. Testing of Pb absorption/adsorption in wastewater contaminated with chemical synthetic dye by fungi

A total of 0.35 g of each fungal isolate was mixed with 50 ml of wastewater contaminated with chemical synthetic dye in an Erlenmeyer flask. The Erlenmeyer flasks were incubated in a shaker incubator at 30°C with agitation (120 rpm) (Majumdar *et al*., 2010), and samples were collected at 0, 24, 48, 72, 96, 120, 144 and 168 h. The samples were centrifuged at 5,000 rpm at 4°C for 10 min, and the concentrations of lead in the culture supernatants were determined by AAS. All measurements were performed in triplicate. Additionally, fungal growth after treatment with wastewater contaminated with chemical synthetic dye was determined as described below.

2.6. Testing of Pb remediation and release of Aspergillus terreus (F203)

2.6.1. Pb absorption/adsorption of A. terreus in wastewater contaminated with chemical synthetic dye

A total of 0.35 g of *A. terreus* was mixed with 50 ml of wastewater contaminated with chemical synthetic dye in an Erlenmeyer flask. The Erlenmeyer flasks were incubated at 30°C on an orbital shaker (120 rpm) for 168 h. Then, the samples were centrifuged at 10,000 rpm at 4°C for 15 min, and Pb concentrations in the supernatants were determined by AAS. All measurements were performed in triplicate. The physical and chemical parameters of the wastewater contaminated with chemical synthetic dye after treatment with *A. terreus* were analyzed as described above.

2.6.2. Pb release of A. terreus into HCl

After Pb absorption/adsorption, *A. terreus* were mixed with 50 ml of 0.1 M HCl in an Erlenmeyer flask. The Erlenmeyer flasks were incubated on a shaker at 30°C with agitation (120 rpm) for 3 h. Then, the samples were centrifuged at 10,000 rpm at 4°C for 15 min and Pb concentrations in the supernatants were determined by AAS. All measurements were performed in triplicate (Majumdar *et al*., 2010).

2.7. The growth characteristics of A. terreus

Spore suspensions of *A. terreus* (5×10^6 spores/ml) were inoculated into 100 ml of potato dextrose broth (PDB) in a 250 ml Erlenmeyer flask. The spore cultures were incubated at 30°C in an orbital shaker (120 rpm), and samples were collected at 0, 24, 48, 72, 96, 120, 144 and 168 h. At the end of the incubation period mycelia were harvested by filtration, washed with deionized water, dried by soaking on blotting paper, cut into small pieces, and used as a live biomass for study. A dead biomass was prepared by autoclaving the flasks at 121°C for 15 min before harvesting the mycelia. The moisture content of the biomass was determined by drying at 70°C until a constant weight was obtained (Tsekova *et al*., 2010).

2.8. Statistical analysis

The data were analyzed using descriptive statistic.

3. Results and Discussion

3.1. Wastewater quality and Pb concentrations

The wastewater quality and Pb concentrations are shown in Table 2. The wastewater before treatment with *A. terreus* was grayish black in appearance. Additionally, the turbidity, pH, BOD, COD and Pb

Table 2. Physical and chemical parameters of wastewater contaminated with chemical synthetic dye

Physical and chemical parameters	Wastewater quality	
	Measurement value	Standard value
Color	grayish black	-
Turbidity (Formazin Nephelometric Units; FNU)	183.0	-
TDS (mg/l)	6.88	<3.0
pH	9.40	5.5-9.0
EC (µS/cm)	14.81	-
BOD (mg/l)	4,228	< 60
COD (mg/l)	7,048	< 400
Pb (mg/l)	0.788	< 0.2

Remark: Thailand's wastewater quality standard (Pollution Control Department of Thailand, 2001) concentration were all higher than Thailand's wastewater quality standard level (Pollution Control Department of Thailand, 2001).

3.2. Microorganism screening and species identification

We identified three bacterial isolates (B206, B308 and B405) that could grow in the presence of 10 mg/l of Pb and three fungal isolates (F102, F203 and F302) that could grow in the presence 100 mg/l of Pb. As fungal isolates were previously found to be more durable than the bacterial isolates (data not shown), the fungal isolates were selected for further study of the absorption/adsorption process. Importantly, fungal isolate F203 was the most effective at treating wastewater contaminated with chemical synthetic dye.

Colonies of fungal isolate F203 are shown in Fig. 2. The colonies contained yellowish-brown fibers surrounding cream-colored colonies that had the appearance of cotton on the nutrient agar. The morphology of fungal isolate F203 was further studied using SEM (Fig. 3). Based on a dendrogram of the genetic relationships of fungal isolate F203 compared with other fungal sequences downloaded from the National Center for Biotechnology Information (NCBI) database, fungal isolate F203 was found to possess 98% gene sequence similarity with *A. terreus* (Fig. 4).

3.3. Pb resistance, absorption/adsorption and fungal growth after treatment with Pb in MYB

We observed that the three fungal isolates F102, F203 and F302 could grow in MYB in the presence of Pb concentrations of 50, 60, 70, 80, 90 and 100 mg/l, but not with Pb concentrations of 110 and 120 mg/l. The Pb concentrations in MYB after treatment with F102, F203 and F302 are shown in Fig. 5. The percentages of Pb absorption/adsorption demonstrated that fungal isolate F203 adsorbed/absorbed higher concentrations of Pb than fungal isolates F302 and F102. The percentages of Pb adsorbed/absorbed were 97.47, 95.47, 88.97, 58.95, 18.36 and 14.76% for F203, 96.63, 21.87, 20.51, 16.20, 10.44 and 7.96% for F302, and 83.92, 11.87, 11.89, 9.60, 12.89 and 7.68% for F102, respectively. When fungal growth was compared with dry weight after treatment with Pb in MYB (Fig. 6), fungal isolate F203 exhibited better growth than F302 and F102 .

3.4. Pb adsorption/absorption by fungal isolates in wastewater contaminated with chemical synthetic dye

Three fungal isolates were tested for adsorption/absorption in wastewater contaminated with chemical synthetic dye at multiple time points (0, 24, 48, 72, 96, 120, 144 and 168 h). The results shown that all three fungal isolates decreased the Pb concentrations in the contaminated wastewater. However, less residual Pb was detected in the wastewater incubated with fungal isolate F203 compared with F302 and F102 (Fig. 7).

3.5. Fungal growth after treating Pb in wastewater contaminated with chemical synthetic dye

Fungal growth was compared based on the dry weights of the fungal isolates following treatment of wastewater contaminated with chemical synthetic dye for 0, 24, 48, 72, 96, 120, 144 and 168 h. The results showed that the dry weights of F102 were 0.3516, 0.2739, 0.2900, 0.2910, 0.2987, 0.3070, 0.3130 and 0.3175 g, F203 were 0.3551, 0.3201, 0.3508, 0.3765, 0.3824, 0.3897, 0.3935 and 0.4164 g; F302 were 0.3539, 0.3210, 0.3414, 0.3776, 0.3821, 0.3900, 0.3982 and 0.4131 g, respectively (Fig. 8). Thus, fungal isolates F203 and F302 had better growth than F102.

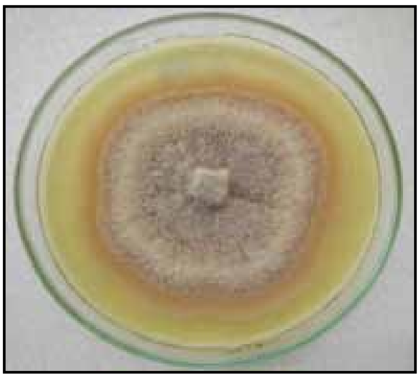

Figure 2. Colonies of fungal isolate F203 on PDA

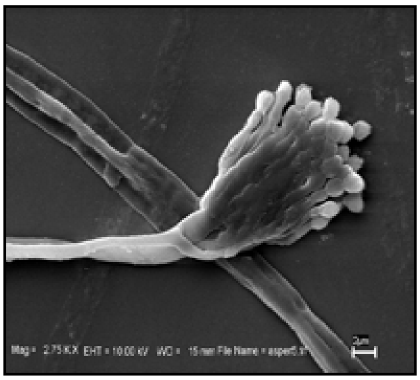

Figure 3. SEM images showing surface morphologies of fungal isolate F203

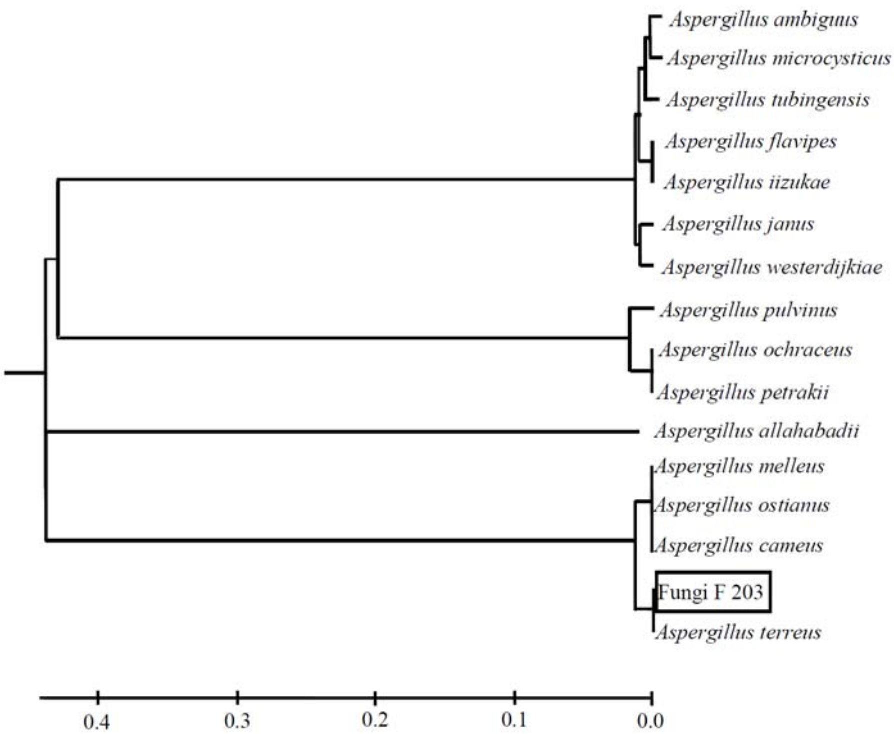

Figure 4. Dendrogram of the genetic relationship of F203 compared with other bacteria from the NCBI database

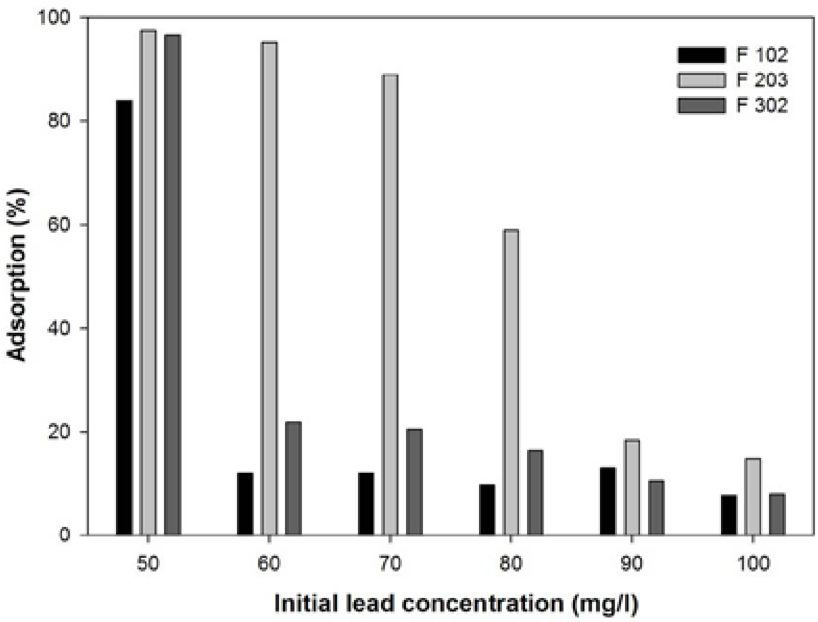

Figure 5. The percentage of Pb adsorbed/absorbed after treatment with F102, F203 and F302

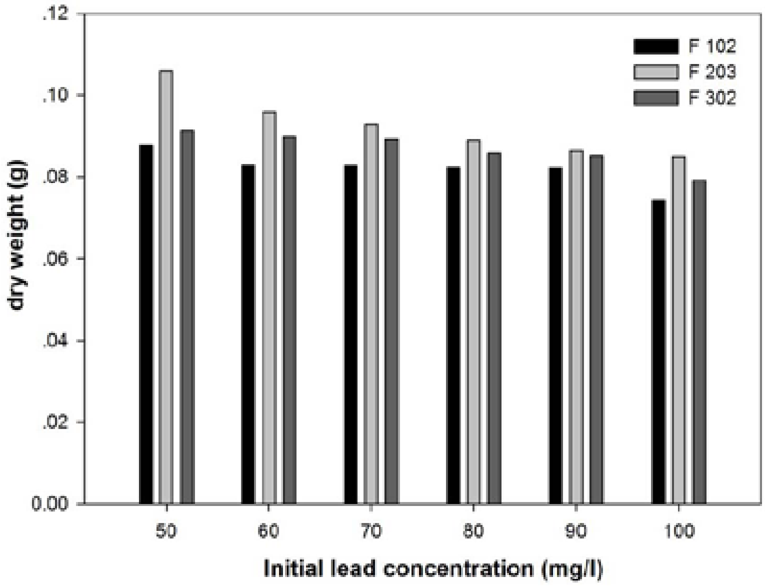

Figure 6. The dry weight of F102, F203 and F302 grown in the presence of Pb concentrations of 50, 60, 70, 80, 90 and 100 mg/l for 168 h in MYB

3.6. The physical and chemical parameters of wastewater contaminated with chemical synthetic dye after treatment with A. terreus (F203)

We demonstrated that *A. terreus* is effective for treating wastewater contaminated with chemical synthetic dye. The physical and chemical parameters of wastewater samples after treatment with *A. terreus* are shown in Table 3. The color of the wastewater after treatment was brown. Importantly, the TDS, pH, BOD and COD were higher than the Thailand standard values, while the Pb concentration was lower than the standard.

3.7. Pb absorption/adsorption and release by A. terreus

The Pb concentration in wastewater contaminated with chemical synthetic dye before treatment with *A. terreus* was 0.788 mg/l. Following incubation of *A. terreus* with wastewater for 168 h, we tested the Pb release in HCl for 3 h (Fig. 9). The results showed

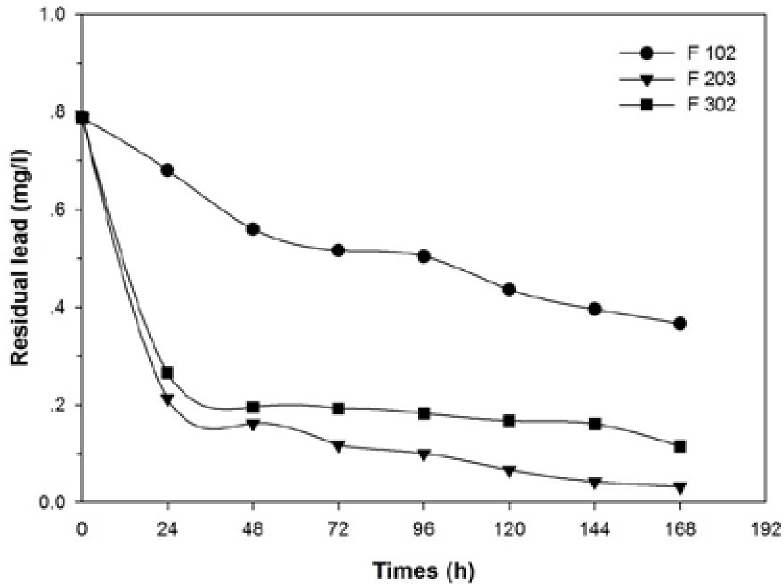

Figure 7. Residual Pb in wastewater contaminated with chemical synthetic dye after treatment with F102, F203 and F302 at 0, 24, 48, 72, 96, 120, 144 and 168 h

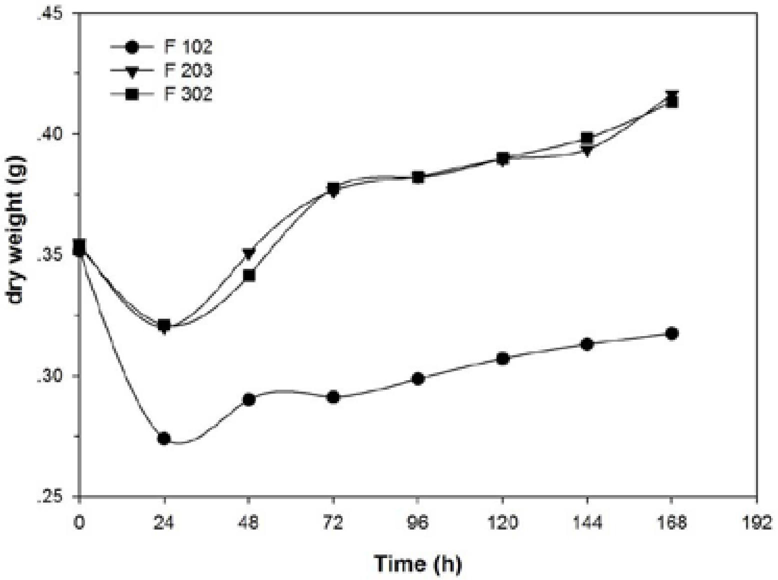

Figure 8. Dry weights of F102, F203 and F302 after treatment with Pb in wastewater contaminated with chemical synthetic dye after 0, 24, 48, 72, 96, 120, 144 and 168 h of incubation

that *A. terreus* absorbed 0.756 mg/l (96%) of Pb, with the total amount of Pb desorbed in HCl equal to 0.702 mg/l (89%).

3.8. Growth characteristics of A. terreus

The growth characteristics of *A. terreus* were compared using the dry weights measured following 0, 24, 48, 72, 96, 120, 144 and 168 h of incubation (Fig. 10). The results showed that *A. terreus* was well adapted to environmental conditions in the time frame of 0-12 h. Then, *A. terreus* exhibited rapid cell division at a constant rate during the 12-72 h period. This phase represented the maximum growth rate, with each cell division cycle taking an equal amount of time. Finally, the dry weight of A. terreus did not increase during the 72-168 h time period.

In this study, lead was removed from wastewater contaminated with chemical synthetic dye using microbial isolation from wastewater sediment with a problematic Pb contamination. Three bacterial isolates (B206, B308 and B405) grew at high Pb concentrations of up to 10 mg/l, and three fungal isolates (F102, F203 and F302) grew at Pb high concentrations of up to 100 mg/l. The results of this study indicated that fungi can tolerate higher Pb concentration than bacteria, and native fungi can absorb Pb in wastewater contaminated with chemical synthetic dye due to the large fibrous structure that bacteria do not possess. The study of fungi used for the absorption/adsorption process by Kapoor and Viraraghavan (1995) reported that common filamentous fungi can absorb heavy metals from aqueous solutions. In this study, the isolation and selection of microorganisms tolerating Pb concentrations revealed

Table 3. Physical and chemical parameters of wastewater before and after treatment with *A. terreus*

Physical and chemical parameters	Wastewater quality		
	before	after	Thailand Standard
Color	grayish black	brown	-
Turbidity (Formazin Nephelometric Units; FNU)	183.0	31.6	-
TDS (mg/l)	6.88	5.96	<3.0
pH	9.40	9.37	5.5-9.0
EC (μS/cm)	14.81	11.86	-
BOD (mg/l)	4,228	3,190	< 60
COD (mg/l)	7,048	5,413	< 400
Pb (mg/l)	0.788	0.032	< 0.2

Remark: Thailand's wastewater quality standard (Pollution Control Department of Thailand, 2001)

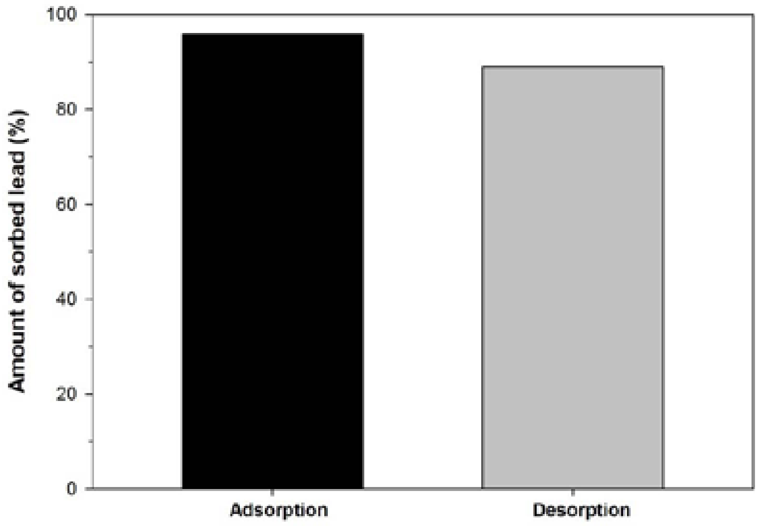

Figure 9. Pb absorption/adsorption and release by *A. terreus*

three fungal isolates (F102, F203 and F302). Fungal isolate F203 had a Pb tolerance of up to 100 mg/l. The DNA barcoding technique is frequently used for fungal identification. Based on the analysis of all of the data, including the morphological characteristics assessed by SEM and DNA sequencing, this fungal isolate was concluded to be *Aspergillus terreus*. The percentages of absorption/adsorption of Pb showed that *A. terreus* could adsorb/absorb Pb with much higher efficiency than the other two fungal isolates. Pb adsorption/absorption by different fungal species depends on cell size and the presence of morphological and functional groups that act to absorb Pb into the fungal cell walls. The fungus absorbs lead from the environment through the wall of the mycelium, which effectively absorbs depending on the composition of its functional groups, including carboxylate, phosphate, and amino groups in the cell wall. Furthermore, the fungal cell wall contains negative ions that are exchanged with the cations of Pb (Iskandar *et al*., 2011). *A. terreus* demonstrated better growth characteristics on MYB containing Pb compared to the other fungal isolates in this study. Fungal biomass also contributes to effective Pb removal capability. The potential of a fungal biomass to function as a sorbent is partly dependent on the cell-wall fraction of the biomass, which plays an important role in the sorption of heavy metals (Kapoor and Viraraghavan, 1995). After employing *A. terreus* in wastewater treatment, the wastewater qualities improved. However, the values of certain parameters were still higher than those allowed

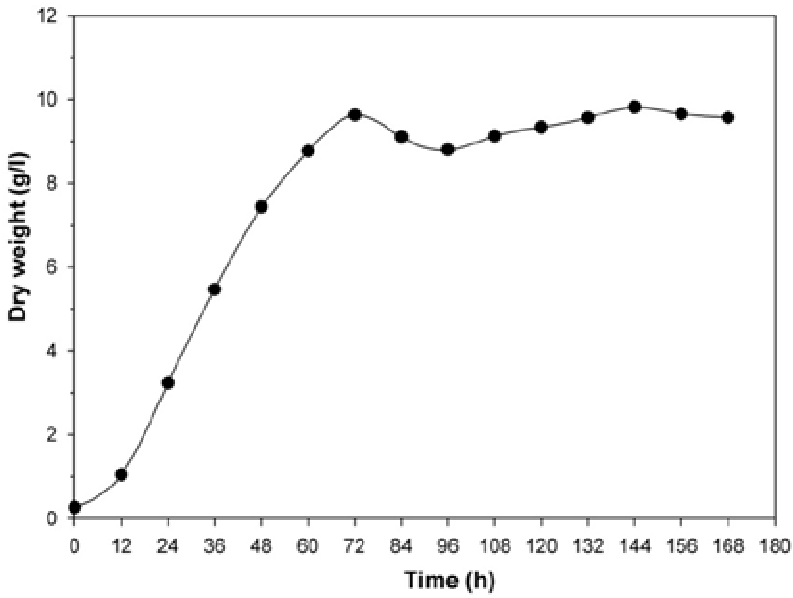

Figure 10. Growth characteristics of *A. terreus*

based on Thailand's wastewater quality standard. The only improvement was the Pb concentration, which was within the allowed standard. *A. terreus* was found to be the most efficient in this respect and removed up to 96% of Pb from wastewater contaminated with chemical synthetic dye. Moreover, Pb desorption in HCl was up to 89%, indicating that the Pb was degraded by *A. terreus*. Because increasing amounts of HCl result in increased release of Pb, pH changes may dictate functional changes in the cell (Gupta *et al*., 2000). Thus, our results showed that *A. terreus* could adsorb and remove Pb from wastewater contaminated with chemical synthetic dye.

4. Conclusions

Microorganisms isolated from the sediment samples in wastewater contaminated with chemical synthetic dye were tested for their Pb removal capacity. Fungi were found to be more tolerant of Pb than bacteria. One of the fungal isolates was identified by gene sequence analysis and morphological characteristics as *Aspergillus terreus*. After employing *A. terreus* in wastewater, the qualities of the wastewater were improved; however, the only improvement that fell within the allowed standard was the Pb concentration. *A. terreus* has the capacity to remove Pb and showed improved growth compared to the two other fungi. *A. terreus* adsorbed up to 96% of Pb from wastewater contaminated with chemical synthetic dye within 168 h, suggesting that *A. terreus* might be suitable for adaptation and use in Pb treatment. *A. terreus* has a high efficiency and can be used for wastewater Pb treatment due to its ability to tolerate a Pb concentration of 0.788 mg/l and as high as 100 mg/l in MYB. Therefore, *A. terreus* is considered to be a good target for the absorption/adsorption of Pb contamination for wastewater treatment. These results may be useful for research into the amelioration of hazardous Pb-contaminated areas worldwide.

Acknowledgments

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