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Isolation and Characterization of Heavy Metal Resistant Fungal Isolates from Industrial Soil in China

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ABSTRACT

The soil contamination with the heavy metals is the growing concern throughout the world as the result of industrial, mining, agricultural and domestic activities. The fungi are the most common and efficient group of the heavy metal resistant microbial family which have potential for metal bioleaching. The present study was aimed on the isolation and phenotypic characterization of the heavy metal resistant fungal strains. All the strains were grown on SDA, CYA, MEA media and genotypic characterization of all the strains were performed by using specific Internal Transcribed Spacers (ITS) sequencing examination. Different strains were identified as *Rhizomucor pusillus* F1 (WF), Rhizomucor *pusillus* F2 (YBF), *Aspergillus flavus* F3 (LG), *Aspergillus terreus* F4 (YF), *Aspergillus tubingensis* F5 (BF) and *Neosartorya hiratsukae* F6 (DG). The present study reveals that the heavy metals contaminated industrial soil might be considered as a precious natural source of resistant fungal strains, which can be used significantly as a bioleaching or bioremediation tool.

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Authors' Contribution
SQ and PC conceived and designed the study. SQ executed the experimental work, analyzed the data and wrote the article. QG helped in sample collection. IK performed PCR analysis. FM and YZ helped in preparing manuscript.

Key words
Heavy metals, bioremediation, metal-resistant fungi, ITS, bioleaching.

INTRODUTION

Soil contamination has become a serious problem with the economy and industry development. It is major sink for heavy metals released into the environment by aforementioned anthropogenic activities and unlike organic contaminants which are oxidized to carbon (IV) oxide by microbial action, most metals do not undergo microbial or chemical degradation (Kirpichtchikova *et al.*, 2006), and their total concentration in soils persists for a long time after their introduction (Adriano, 2003). Heavy metals constitute an ill-defined group of inorganic chemical hazards, and those most commonly found at contaminated sites are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni) (Gwrtac, 1997). In naturally polluted environments, the concentrations and availability of metals and the actions of different factors such as type of metals, nature of medium and microbial species govern the response of microbes to heavy metals toxicity (Herrera-estrella *et al.*, 2001). Metal resistance is defined as the ability of an organism to survive metal toxicity by means of mechanism produced in direct response to metal species concerned. Without any proper treatment process heavy metals release into the environment poses a serious threat to public health. Microbial population in metal polluted environments possesses toxic concentrations of heavy metals and develops resistance Prasenjit *et al.*, 2005). Fungal and yeast biomasses are known to be resistant to heavy metals contamination in soil (Gavrelesca, 2004; Baldrian, 2003). They can adapt and grow under various culture conditions of pH, temperature, nutrient availability and high metal concentrations, therefore these microorganisms are considered as a versatile group. Recently microbial systems like fungi, bacteria and algae have been explored for their role in the removal of heavy metals from polluted environments (Natarajan *et al.*, 2011).

Fungal leaching of heavy metals is an interesting biological treatment method (Yu *et al.*, 2001). It is based on the fungal production of weak organic acids that solubilize metals by forming water soluble complexes with them (Bosecker, 1997; Burgstaller and Schinner, 1993; Burgstaller *et al.*, 1994). *Aspergillus niger* and *Penicillium simplicissimum* have been observed to produce abundant amount of organic acids (Ren *et al.*, 2009). Fungi are known to be resistant and having ability to detoxify the metals by several mechanisms including valence transformation, active uptake, impermeability

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and sequestration, extra and intracellular precipitation, biosorption to cell wall, crystallization and transformation of metals (Mala *et al.*, 2006; Turnau *et al.*, 2006; Islam *et al.*, 2008). As Compared to lithotrophic bacterial leaching, this technique has advantage of being operated at mildly acidic conditions that minimize the loss of sediments properties, eventual foaming and H_2S evolution phenomena that result from a sudden addition of strong mineral acids.

El-Morsy (2004) also studied 32 fungal species which were isolated from polluted water in Egypt for their resistance to metals and found that *Cunninghamela echinulate* biomass could be applied as a biosorbent of metal ions in waste water. Zafar *et al.* (2007) in the same way reported promising biosorption of Cd and Cr by two filamentous fungi, *Aspergillus* sp. and *Rhizopus* sp. isolated from metal contaminated soil. Until now, filamentous fungi have been mostly been used for the leaching of heavy metals from low grade ores, mine tailings and industrial wastes (Ezzouhri *et al.*, 2009; Tzeferis, 1994; Müller *et al.*, 1995).

Thus, this study highlights the isolation, characterization and selection of more resistant fungal strains from polluted terrestrial environment of Weifang Industry which is located in east part of China in weifang city of Shangdong province. These fungal isolates may possess certain survival mechanisms due to their highly contaminated habitat, which may make them much more significant to be used as bioremediation tool.

MATERIALS AND METHODS

Growth media

Fungal isolation, colony and cell morphology for fungal identification were done by using Malt Extract Agar (MEA), Potato dextrose agar (PDA), Czapek yeast extract agar (CYA), and Sabouraud dextrose agar (SDA) media, supplemented with 0.05% chloramphenicol (Khan *et al.*, 2014; Nonaka *et al.*, 2011).

Sampling

The contaminated soil used in this work was collected from the sub soil (0−20 cm) of the sites under a slag heap at the Smelting Industry, which was built in 1954 and located in Weifang city, Shandong Province, China. These samples were brought to laboratory and kept in refrigerator at 4°C for further processing.

The contaminated soil samples were air dried under laboratory conditions for two weeks, ground, sieved through a 2 mm polyethylene sieve and dried to constant mass in an oven at 75°C and kept in a desiccators for further analysis. Some properties like pH, EC and total metal concentration were done initially. Soil pH was measured potentiometrically in 1M KCl with a soil / extractant ratio of 1:5 in three replicates per sample. The digestions were conducted with a mixture of $7molL^{-1}$ of conc. HNO_3, 2 cm^3 of conc. HF and $1cm^3$ of 40% H_2O_2 solution on a sand bath at a temperature of 200 - 2300°C, for the determination of the total metal contents of the soil samples. The AAS analyses for the determination of the total metal contents of the soil samples were done.

Isolation of resistant fungi

Fungal strains were isolated from soil samples by serial dilution method using Sabouraud Dextrose Agar (SDA) containing Pb, and Cr 100 mgL^{-1} individually. A serial dilution of each sample was made up to 10^6 and 1ml of each 10^4 and 10^6dilution were added in sterilized petri plates in duplicate. 20 mL SDA medium containing (100 mgL^{-1}) Pb, and Cr of one of these heavy metals was poured in the petri plates and incubated at 28°C for 96 h. The colonies of predominant genera of fungi were picked up and purified by streak plate method.

All the fungal isolates were further screened by streaking on SDA medium containing 50 and 100 mg of each of the three heavy metals separately. Streaking of fungal isolates on normal SDA medium reserved as control (normal growth) for comparison of growth of fungal isolates on SDA medium containing different concentration of heavy metals. Observations on growth of fungal isolates were made after 96 h of incubation.

Microscopic iidentification/morphology

Colony and cell morphology observation of the fungal strains were performed using the methods described by Khan *et al.* (2014). Briefly young growing fungal hyphae (2 day old) of each strain were inoculated into sterile CYA, SDA and PDA plates supplemented with 0.5 gL^{-1} chloramphenicol and incubated at 30°C for 7 days and observed under fluorescence microscope (Olympus CKX41-A32PH. Furthermore, the different mold colonies were isolated, purified, and then the pure strain preserved on the PDA slants and stored at -80°C.

Molecular identification

The total genomic DNA of the fungal isolates were isolated and purified by using the methods as described by (Rolhf, 1990). The common primers for amplification and sequencing of internal transcribed spacer (of (ITS) from the fungal strains were performed using the primers IT5: 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′ (Pederson *et al.*, 1997). The reaction system (25 µl) was composed of 10 × buffer 2.5 µl, dNTP 0.8 mM, MgCl2 1.5 mM, ITS-F or ITS-R 0.5 mM, Taq DNA polymerase 1.25 U, template DNA 1.0 µl and H_2O 16.6 µl (Khan *et*

al., 2014). The conditions for the PCR amplification were as follows: initial denaturation at 94°C for 10 min, denaturation at 94°C for 1 min, annealing temperature at 53°C for 1 min, extension at 72°C for 2 min, final extension at 72°C for 10 min. PCR was run for 32 cycles and PCR cycler was GeneAmp PCR System 2400 made by Perkin–Elmer. PCR products were separated by agarose gel electrophoresis and recovered by using UNIQ column DNA gel recovery kits (BIOASIA, Shanghai). The recovered PCR products were ligated into pGEM-T easy vector and transformed into competent cells of *E. coli* JM109. The transformant were selected on plates with ampicillin. The plasmids in the transformant cells were extracted using the methods as described by Sambrook *et al*. (1989). The ITS fragments inserted on the vector were sequenced by Shanghai Sangon Company.

Phylogenetic analysis

The sequences obtained above (F1 WF, KM977883.1; F2 YBF, KM977884.1; F3 LG, KM977885.1; F4 YF, KM977886.1; F5 BF, KM977888.1; F6 DG, KM977889.1 were aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST), for comparison with currently available sequences. The sequences which showed over 98% or above resemblance or similarities with currently accessible sequences were regarded as to be the same species. Furthermore, multiple alignments were performed using Clustal X 1.83, and MEGA 4.0 is used for the constructing phylogenetic tree (Tamura *et al.*, 2007).

RESULTS

Characterization of soil samples taken from Weifang Red Star Industry

Soil characterization is given in Table I including its Electrical conductivity, Humus content which ranges 3.15%–19.9% and total metal contents in soil measured by AAS. The pH ranged from 6.9-9.24 indicating acidic, neutral and slightly alkaline (Table I).

Table I.- Characteristics of soil sample.

	Chromium (Cr)	**Lead (Pb)**
Metal concentration	3014.3 $mg.kg^{-1}$	26385.44 $mg.kg^{-1}$
pH	9.24	6.9
Electrical conductivity	3.08 $ms{\cdot}cm^{-1}$	1.16 $ms.cm^{-1}$
Moisture content	3.15%	5.48%

Morphological characteristics of fungi

The morphological characteristics of the fungal isolates were studied by growing on PDA, CYA, MEA and Sabouraud dextrose agar (SDA) media. Six resistant fungal isolates were identified in soil sample. The colonies of the fungal isolates were studied after 24 h of incubation at 30°C and also within 2-3 days. The overall morphologies of colony and cells physiological of the fungal strain F1 and F2 were identical (Table II).

Based on microscopic investigations demonstrated in results, it was proved that fungal isolates F3, F4, F5 and F6 were *A. flavus, A. terreus, A. tubingensis, N. hiratsukae*, respectively.

Molecular identification

Total genomic DNA from the all fungal isolates were extracted and a PCR product of 541 bp band of 18S rDNA gene was obtained by using gDNA as template according to the method mentioned in Material and Methods.

Phylogenetic analysis

Phylogenetic analysis of the 18S rDNA sequences for similarities between ITS of the fungal isolates and those in the NCBI database showed that many were phylogenetically related to different fungal genera (Fig.1). The topology of the phylograms confirmed that the fungal isolates used in this study were assigned to; *Rhizomucor pusillus* F1 WF (KM977883.1), *Rhizomucor pusillus* F2 YBF (KM977884.1), *Aspergillus flavus* F3 LG (KM977885.1), *Aspergillus terreus* F4 YF (KM977886.1), *Aspergillus tubingensis* F5 BF (KM977888.1) and *Neosartorya hiratsukae* F6 DG (KM977889.1).

DISCUSSION

The difference in pH of the soils (Table I) highlight the displacement of the ions H^+ adsorbed on the exchange sites of the absorbing complex from soil towards the soil solution. The pH is one of the factors which influence the bioavailability and the transport of heavy metal in the soil and according to Smith (1996), heavy metal mobility decreases with increasing soil pH due to precipitation of hydroxides, carbonates or formation of insoluble organic complexes. Heavy metals are generally more mobile at pH <7 than at pH >7. The amount of heavy metals mobilized in soil environment is a function of pH, properties of metals, redox conditions, soil chemistry, organic matter content, clay content, cation exchange capacity and other soil properties.

Broad-spectrum antibiotics are more effective in preventing bacterial growth and less harmful to fungal

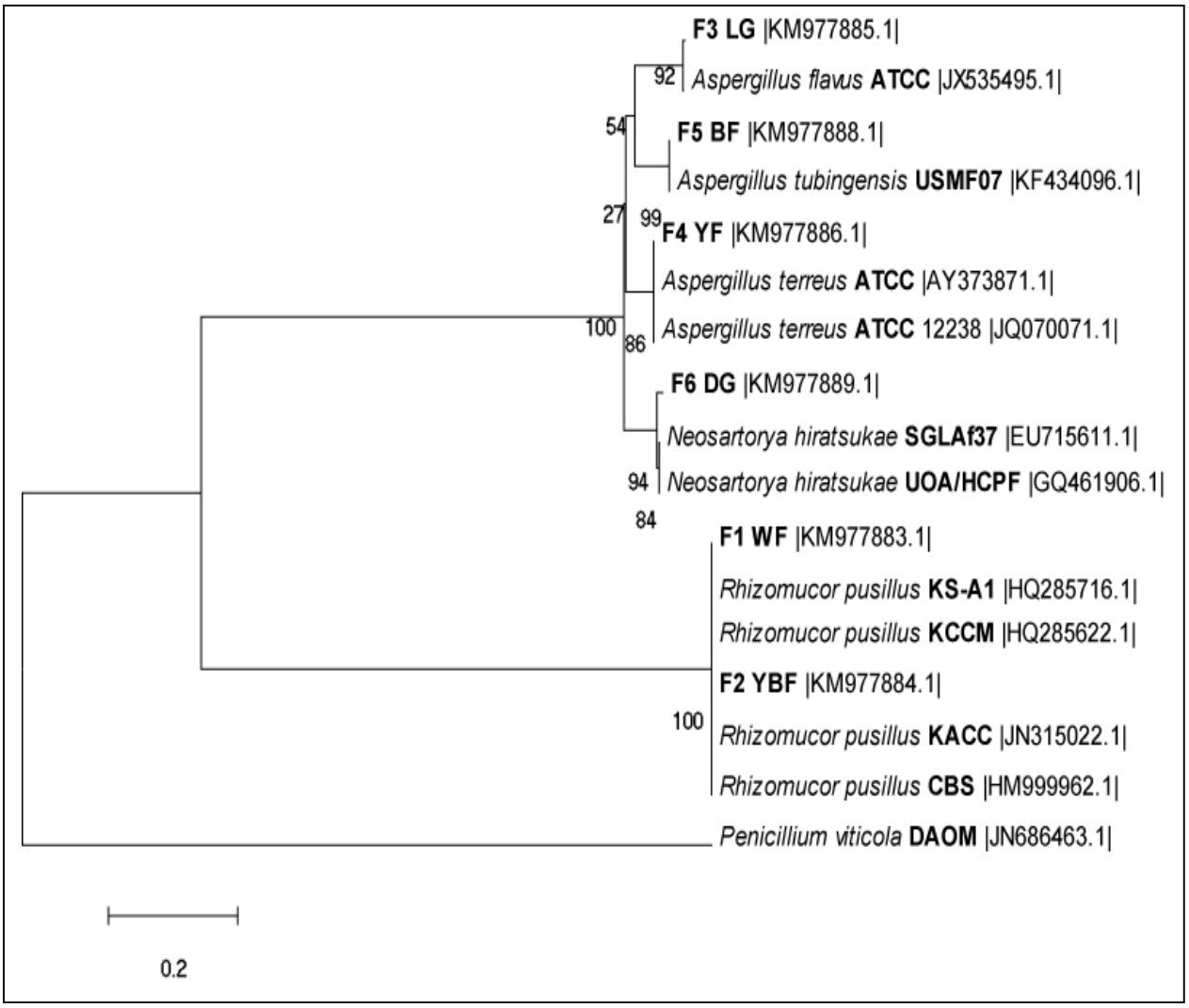

Fig. 1. Phylogenetic tree of the fungal isolates with closest relatives based on a maximum parsimony analysis of ITS sequences. While, Penicillium viticola DAOM was used as out group.

cells (Kiran *et al.,* 2014; Lass-Florl., 2012; Houbraken *et al.,* 2011). Thus, the growth medium was supplemented with broad-spectrum antibiotics that is; Chloramphenicol, in order to prevent heavily contaminated with bacteria (Del *et al.*, 1999; Xu *et al.,* 2009). The Potato dextrose agar (PDA), malt extract agar (MEA) and Czapk yeast extract agar (CYA) has been recommended for the selective isolation and phenotypical; characterization from different environmental sources (Mossel *et al.,* 1970). Resistant fungal strains belonging to different genus have been found frequently in heavy metal contaminated environments (Ren *et al.,* 2009; Chai *et al.,* 2009).

In current study six fungal strains that is; F1, F2, F3, F4, F5 and F6 which belongs to five different genera were isolated. The morphological features of all the seven strains were also determined. In 1978, the genus *Rhizomucor* was established for Mucorlike fungi forming stolons and rudimentary rhizoids and expressing thermophilism (Ribes *et al.,* 2002). Molecular phylogenetic studies based on nuclear small and large subunit rRNA gene sequences support the distinction of *R. pusillus* from Mucor species. Rhizomucor is commonly found contaminating air, soil and organic matter. The *R. pusillus* is the most common species seen and has been detected in a variety of food items, including grains, seeds, nuts and beans (Kiran *et al.,* 2014). The morphological characteristics of fungal

Table II.- Morphology characteristics of the fungal samples F1-F6.

Fungal sample	Culture medium	Morphological feature(s)	Fungus Identified	References
F1	Czapek yeast agar (CYA) Peptone dextrose agar (PDA)	Flocculent, low aerial tufts to grey Velvety, hyphae were irregular and nonseptate which were hyaline to pale brown in color	*R. pusillus*	Kiran *et al.*, 2014; Domsch *et al.*, 1980; Ellis, 1997
F2	Czapek yeast agar (CYA) Peptone dextrose agar (PDA)	Flocculent, low aerial tufts to grey Velvety, Yellow brown to grayish brown, hyphae were irregular and nonseptate which were hyaline to pale brown in color	*R. pusillus*	Kiran *et al.*, 2014; Domsch *et al.*, 1980; Ellis, 1997
F3	Czapek yeast extract agar (CYA)	velvety/powdery at first, turning to smoky-green, reverse white to tan. microscopic morphology of F3 shows abundant conidial structures, with large conidia heads and mycelium submerged, white above, and reserve light yellow in color	*A. flavus*	George *et al.*, 2010; Iheanacho *et al.*, 2014
F4	Czapek yeast extract agar (CYA)	showed rapid growth with smooth-like walls and yellowish to cinnamon brown gets darker as it ages Conidial heads in compact columns; stipes smooth walled, variable in shape, spherical to domelike. Conidia smooth walled, globose to subglobose	*A. terreus*	Lass-Florl *et al.*, 2012; Anderson *et al.*, 1980
F5	Czapek yeast extract agar (CYA)	rapid growth with 56-57 mm colonies diameter having radial villus white mycelia at the edge of each colony in early culture stages in 3~5 days After seven days, the colony became pitchy to black with white head of mycelium and pale in the reverse side, Conidia pale brown to reddish brown in mass, globose to subglobose, Sporangia have many spores	*A. tubingensis*	Houbraken *et al.*, 2011
F6	Czapek yeast extract agar (CYA)	rapid growth and reached colonies diameters to 28 and 42 mm after 7 days grayish-yellowish green	*N. hiratsukae*	Samson *et al.*, 2007; Konstantinos *et al*., 2010).

isolates F1 and F2 demonstrates that it belongs to *R. pusillus* because of their similar distinguishing features. While, the *Aspergillus* genus was found more commonly fungus in the environments, due to their wide distribution of spores (Henry *et al.*, 2014).

In this similarity, the F1 and F2 fungal isolates which were classified as *R. pusillus* strains by morphological characterization, showed 99% maximum identification, which correlated with study conducted by Kiran *et al.* (2014). While, the F3 strain identified as *A. flavus*, as it showed a similarity with 99 % maximum similarities, alike result was also shown by George *et al.*, (2010). Furthermore, the NCBI blasted ITS sequence result of F4 showed its similarities 99% with *A. terreus* (Lass-Florl, 2012). While, the fungal isolates F5 and F6 were classified or identified as *A. tubingensis* and *N. hiratsukae* respectively, with a 98 % and 99% similarity, which correlated with finding of Houbraken *et al.* (2011) and Samson *et al.* (2007). The phylogenetic association of all the resistant fungal isolates with other fungal strains

present on NCBI is shown in Figure 1. In natural remediation of metal fungi are present in aquatic sediments, terrestrial habitats and water surfaces and play a significant part. It is advantageous to use fungi for heavy metal resistance because their hyphae can penetrate contaminated soil, to reach heavy metals. Despite the abundance of such fungi in contaminated niches, indigenous fungi in particular have received little attention in bioremediation and biodegradation studies. Additionally, studies conducted with different strains of fungi, have demonstrated their ability to tolerate heavy metals and could be potentially interesting for the development of economically feasible processes for pollutant transformation.

CONCLUSION

There were total six fungal strains isolated from the heavy metal contaminated soil. These six isolates were the most tolerant to high level concentration of heavy metals including Pb (26385.44 $mg.kg^{-1}$a and Cr (3014.3 $mg.kg^{-1}$). Furthermore, phenotypic and genotypic characterization exposed that, the fungal strains isolated belong to different fungal genera. Thus, this study reveals that heavy metal contaminated soil of industries might be considered as a precious natural source of the resistant filamentous fungi.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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