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Characterization of Cadmium and Lead Heavy Metals Resistant Fungal Isolates from Ghawar oil field, Saudi Arabia

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Contamination of soil with heavy metals is considered the main concern around the world due to dramatic increases in agricultural, industrial, and oil extraction activities. Highly potentials were recorded for fungi, which reflected heavy metal resistant patterns among microflora. Our investigation was carried out for isolating, identifying, and evaluating Cr and Pb resistant capabilities for fungal strains. By using morphological characterization, seventeen fungal strains which reflected resistance against Cr and Pb were identified as Aspergillus, ***Rhizomucor, Neosartorya*****, Penicilliumand Rhizomucor , Aspergillus , *Penicillium* , *Cunninghamella* and *Fusarium* for the second site. Among nine microorganisms isolated from the first site which reflected resistant against Cr, Aspergillus flavus reflected highly resistant against all Cr concentrations. Also, Aspergillus Ochraceus and Penicillium oxalicum were resistant to all Cr concentrations except the highest (750 ppm). Only Aspergillus flavus which was isolated from the first site reflected distingushable resistance to all Pb concentrations. Also, *A. terreus* showed resistant patterns against Cr at 50, 75, 150, and 300 ppm of Pb. At the second site, *Aspergillus terreus*, *Penicillium aurantiogriseum* and Aspergillus wentii showed resistant patterns against all Led concentrations. Four selected fungi which reflected resistance against all concentrations of Cr and Pb were identified via 18S rDNA molecular marker and assigned to GeneBank as follows: *Aspergillus flavus* (OP113794), *Aspergillus terreus*(OP113795), *Penicillium aurantiogriseum* (OP113796) and *Aspergillus wentii* (OP113797).**

Keywords: Fungal isolates, oil extraction, heavy metals resistant, morphological characterization, 18S rDNA.

Rapid industrial development and the dramatic increase in heavy metal addition revealed soil heavy metal contamination as a major issue[1]. Metalliferous mining and mining, metal smelting, metallurgical industry activities, metal corrosion, and petroleum exploration are the primary methods of introducing heavy metals into the environment.

Pressure on the ecosystem due to adding heavy metals like cadmium (Cd), arsenic andAsbestos (As) and lead (Pb) are toxic to plants, animals, and humans[2-3]. Naturally, interactions between metal concentration and availability, as well as medium nature, metal type, and microflora composition, influenced microbial response to heavy metals[4].

Bioaccumulation and biosorption are considered the main strata for the removal of heavy metals by different microorganisms through

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uptaking heavy metal ions passively (biosorption) or actively (bioaccumulation). Furthermore, microorganisms introduce varied processes that are dedicated to cleaning up or removing metal from the environment, like permeability barrier blockage, sequestration by intracellular and extracellular, detoxification by enzyme activities, and cellular targets reduction sensitivity to metal ions[5-6].

Resistant microbial populations cause continuous exposure to heavy metals which belong to the genus *Corynebacterium, Bacillus, and Ralstonia* (7-9). Also, heavy metal leaching by fungi is considered a promising biological strategy based on the induction of weak organic acids by fungi, which could remove heavy metals by forming water soluble complexes[10- 11]. *Cd and Cr biosorption activities were demonstrated by Aspergillus sp. and Rhizopus sp.*Thus, filamentous fungi are considered the most commonly applied fungi for heavy metal leaching[12].

This investigation was carried out to isolate and identify the most nominated resistant fungal strains against heavy metals from of Ghawar oil field in Al-Ahsa Governorate. These fungal isolates may develop heavy metal resistance mechanisms, making them suitable for use as bioremediation tools.

MATERIALS AND METHODS

For fungal culturing and colony morphological studies, potato dextrose agar (PDA) media, Czapek yeast extract agar (CYA) and Sabouraud dextrose agar (SDA) media with 0.05% chloramphenicol were used[13-14].

Sampling

Heavy metal polluted soils were sampled from subsoil (020 cm) of Ghawar oil field in Al-Ahsa Governorate, Eastern Province, Saudi Arabia., 25.43oN, 49.62oE), Saudi Arabia for various locations from May 1 to 20, 2022 (Fig. 1).Sterile bags were used for keeping samples in sterile, which were directly transported to the laboratory and stored at 4 °C until further use. After air drying for two weeks, a 1.5 mm polyethylene sieve was employed for soil sieving and dried in an oven at 75 °C. Different physicochemical parameters such as pH, EC and heavy metal contents were evaluated and analysed in triplicate. The Polarized Zeeman Atomic Absorption Spectrophotometer ZA3000 Series (HITACHI, Japan) was used to evaluate the total metal contents of soil samples.

Fungi that can withstand heavy metalsIsolation

The serial dilution method[15] was applied to isolate nominated heavy metal resistant fungal isolates. To achieve this goal, 1000-ppm stock solutions of Cr (NO3)3 and Pb(NO3) were used to prepare 50, 75, 150, 300, 500, and 750 ppm concentrations. Then, sterilised 50, 75, 150, 300, 500, and 750 ppm concentrations were added to potato dextrose agar (PDA) sterilised media petri dishes. As follows, the spread plate protocol was used for isolating resistant fungal heavy metals. After serial dilution with saline solution, polluted soil samples were poured onto potato dextrose agar (PDA) media plates supplemented with 50, 75, 150, 300, 500, and 750 ppm of Cr(NO3) and Pb(NO3). For comparing the growth rate of fungi on different concentrations of heavy metals , normal growth was performed via streaking of fungal isolates on normal SDA media and used as a control after 96 h of incubation.

Evaluation of Cr and Pb were recorded through one-way ANOVA followed by post-hoc multiple comparisons by Duncan's method with P 0.05 as the significant level.

Microscopic examination

CYA, SDA and PDA plates supplemented with 0.5 gL-1 chloramphenicol were used for incubating growing fungal hyphae (2 days old) at 30°C for 7 days and observed for colony morphology under these conditions.

Fluorescence Microscope (MSC-F201, Bioevopeak) In the end, varied colonies were purified and preserved on the PDA plates and stored at -80°C.

Molecular Characterization

Fungi isolates were identified using 18S rRNA. To achieve this goal, total fungal DNA was extracted and purified using the E.Z.N.A.® Fungal DNA Mini Kit (D3390-01, Omega BIO-TEK, USA) according to manufacturer protocol. DreamTaq Green PCR Master Mix (2X)(K1081, Thermo Fisher, USA) was used for specific gene amplification according to manufacturer protocol through a Creacon (Holland, Inc) Polymerase Chain Reaction (PCR) system cycler. The reaction consists of 1 min of denaturation for 1 min at 95ºC, annealing for 45 sec at 55ºC, and extension for

1 min 30 sec at 72oC. A final extension at 72ºC was performed for 5 min (16). 1.0 % agarose was applied for visulations. Specific DNA fragments were eluted from the agarose gel. The E.Z.N.A.® Gel Extraction Kit (D2500-01, Omega BIO-TEK, USA) was used to purify the PCR products. Sequence analysis was employed using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp. Korea). Gel documentation system (Geldoc-it, UVP, England) was applied for data analysis using Totallab analysis software, ww.totallab.com, (Ver.1.0.1).

Phyllogenetic tree construction

The obtained sequences were aligned on the NCBI website (http://www.ncbi.nlm.nih.gov/webcite) using BLAST to confirm their identity. Genetic distances and MultiAlignments were computed by the Pairwise Distance method using ClusteralW software analysis (www.ClusteralW.com). The nucleotide sequences were also compared to highly similar isolate sequences from the GenBank database.

RESULTS

Soil characterization from two studied sites

As shown by Table (1), heavy metal concentration, electrical conductivity (EC), and pH values reflected significant variation between the two sites. The first site was superior for Cr and Pb concentrations and EC values compared with the second sites. pH values showed acidic and alkaline prosperities for the first and second sites, respectively.

Morphological analysis of fungi

Growing fungal isolates on PDA, CYA, MEA, and Sabouraud dextrose agar (SDA) media were selected for morphological studies. Seventeen heavy metal resistant fungi were identified in heavy metal soil samples. After incubation for 24

Fig. 1. Ghawar oil field in Al-Ahsa Governorate, Eastern Province, Saudi Arabia. (https://www.researchgate.net/figure/Map-showing-the-location-of-the-Ghawar-oil-field-and-Zagros-tectonic-belt-in-the-Arabian_fig6_222517325)

h at 30°C, fungal colonial morphology, colour, texture, shape, and diameter were used for initial characterization. Moreover, septation in mycelium, distinguishable reproductive structures, conidia shape and structure were applied for macroscopic examination. Based on previous morphological features, nine and eight fungal isolates from the first and second sites respectively showed resistant patterns against Cr and led and were identified as **Aspergillus,** *Rhizomucor, Neosartorya*, Penicilliumand Rhizomucor , Aspergillus , *Penicillium* , *Cunninghamella* and *Fusarium* for the second site.

Resistance to heavy metals in fungal strains

Among seventeen heavy metal-resistant fungal isolates

As shown by (tables 2 and 3), identified microorganisms from two sites reflected varied resistant patterns against Cr and lead. Among nine microorganisms isolated from the first site which reflected resistant against Cr, Aspergillus flavus reflected highly resistant against all Cr concentrations. Also, Aspergillus Ochraceus and Penicillium oxalicum were resistant to all Cr concentrations except the highest (750 ppm). Furthermore, Penicillium aurantiogriseum, Rhizomucor pusillus and A. tubingensis showed resistant patterns only against Cr at 50, 75, 150, and 300 ppm. *Aspergillus terreus*, *Penicillium aurantiogriseum* and Aspergillus wentii which were isolated from the second site, reflected noticeable resistance to all Cr concentrations.

Only Aspergillus flavus, which was isolated from the first site, reflected distinguishable resistance to all Pb concentrations. Also, *A. terreus* showed resistant patterns against Cr at 50, 75, 150, and 300 ppm of Pb.

Molecular Identification for Heavy Metal Resistance of Fungi

Using 18S rDNA as a molecular identification marker, four selected fungi which reflected resistance against all concentrations of Cr and Pb were identified and assigned to Genebank as follows: *Aspergillus flavus* (OP113794), *Aspergillus terreus*(OP113795), *Penicillium aurantiogriseum* (OP113796), and *Aspergillus wentii* (OP113797). As shown by (Table 4), the phyllogenetic tree represented genetic similarity among nominated isolates and high similarity.

DISCUSSION

Heavy metal pollution is the main concern for researchers due to its spreading influence in developing countries[17].

Variation of pH values among two sites refers to ions H+ adsorbed replacement of soil absorbing complexes by soil solution, which shows the direct influence of accumulated heavy metals on general soil features. According to findings by Smith (1996) who indicated that heavy metal bioavailability was affected by pH, accumulated heavy metals in our experiment could be explained in the light of decreasing heavy metal mobility as a direct influence of high pH values due to hydroxides, carbonate precipitation, or insoluble organic complex formation.

In accordance of many studies, potato dextrose agar (PDA) and Czapk yeast extract agar (CYA) were used as selective media for morphological fungal identification[18].

Different fungal genera were frequently found in heavily metal-contaminated environments[19]. The identified specific fungal genus that exhibited high heavy metal resistance could be explained by the following findings.They indicated that R. pusillus is considered the most common species which isolated from contaminated sites and products[20].

In agreements with[21] findings. Morphological characterization was used successfully to identify fungal strains, particularly

Table 1. metals concentration, Electrical conductivity (EC) and pH form different to sites.

	heavy metals concentration		pH	Electrical conductivity (EC) ms-cm-1
	Chromium(Cr) mg.kg-1	Lead(Pb) mg.kg-1		
First site	2877.9	2278.3	8.4	2.21
Second site	2689.2	2127.9	7.9	1.33

Table 2. Characterized microorganisms from two sites with different resistant level against Cr

Site	Microorganism	ControlCr concentrations (ppm)						
			50	75	150	300	500	750
1	Penicillium aurantiogriseum	11.4 ± 3.2	10.4±0.5	9.1±1.2	7.7±0.7	5.3±0.9	-	-
	Rhizomucor pusillus	12.4±0.1	10.2±0.6	9.9±0.4	7.4±1.7	5.3±0.4	-	-
	Aspergillusochraceus	11.6±0.8	10.9±0.8	83±0.4	7.2±0.5	5.8±0.5	2.5±0.4	
	Penicilliumoxalicum	10.7±0.6	9.9±0.4	7.3±0.1	5.8±0.7	3.6±1.1	2.1±1.1	-
	Neosartorya hiratsukae	11.4±0.9	10.5±0.3	9.2±0.8	7.7±0.3	-	-	-
	Aspergillus flavus	13.7±0.2	11.3±1.9	10.5±1.1	8.3±0.8	6.7±0.7	3.6±0.5	0.9±0.7
	Penicillium	10.7±1.1	8.3±1.4	6.1±0.8	4.4±0.5	-	-	-
	A. terreus	8.4±0.9	7.6±0.8	5.9±0.8	3.7±0.5	-	-	-
	A. tubingensis	9.7±0.9	8.2±0.5	6.4±0.4	5.8±1.1	2.4±0.2	-	-
2	*R. pusillus*	10.4±0.7	9.4±0.3	7.9±0.6	5.2±0.2	-	-	-
	Aspergillus terreus	11.3±0.2	10.2±0.8	9.4±0.9	7.8±0.1	5.2±1.4	3.8±0.2	1.2±0.7
	Penicilliumaurantiogriseum	12.3±0.1	11.5±0.9	9.4±0.1	7.3±0.4	5.8±0.2	3.3±0.5	1.1±1.1
	Aspergillus wentii	11.5±1.1	10.1±1.2	9.3±0.5	7.9±0.5	5.5±0.7	2.4±0.5	0.8±0.8
	Aspergillus tubingensis	12.4±0.8	11.3±0.4	9.3±0.6	7.3±0.5	-	-	-
	Aspergillus terreus	11.4±1.5	9.3±0.2	5.3±1.2	3.2±0.7	-	-	-
	Cunninghamella	10.3±0.8	9.1±0.7	7.4±1.4	5.2±1.1	2.8±0.5	-	-
	Fusarium	8.2±0.6	6.8±0.5	4.2±0.9	1.5±0.9	-	-	-

Data are expressed with grams as fresh weight, ± standard deviation of triplicates.

Table 3. Characterized microorganisms from two sites with different resistant level against Lead

Site	Microorganism	Control	(Pb) concentrations (ppm)					
			50	75	150	300	500	750
1	Penicillium aurantiogriseum	10.3±0.2	9.3±0.9	6.3±0.3	3.4±0.3	-	-	-
	Rhizomucor pusillus	9.1±0.6	7.4±0.9	5.3±0.1	-	-	-	-
	Aspergillusochraceus	12.3±0.9	10.4±0.9	8.3±01.1	6.5±0.2	-	-	-
	Penicilliumoxalicum	11.8±0.4	9.3±0.2	7.7±01.1	4.2±089	-	-	-
	Neosartorya hiratsukae	10.5±1.1	9.3±0.3	7.4±0.1	5.9±0.5	-	-	-
	Aspergillus flavus	11.2±0.2	10.5±0.5	9.3±0.9	7.4±0.w	5.2±0.2	2.2±0.4	1.6±0.2
	Penicillium	10.6±0.9	9.3±0.5	7.4±0.1	6.3±0.2	4.2±0.3	-	-
	A. terreus	11.5	10.1±0.2	9.3±0.9	6.4±0.1	3.5±0.5	-	-
	A. tubingensis	10.5±0.1	9.5±0.4	7.4±0.2	4.4±1.4	-	-	-
2	*R. pusillus*	11.4±0.1	10.5±0.9	8.3±0.6	5.2±0.1	3.6±0.5	-	-
	Aspergillus terreus	10.3±0.9	9.2±0.1	8.4±0.6	7.1±0.9	5.8±0.1	3.3±0.9	1.2±0.1
	Penicilliumaurantiogriseum	12.4±0.9	11.3±0.2	10.4	8.9±0.2	6.3±0.9	3.3±0.3	0.8±0.1
	Aspergillus wentii	13.2±1.2	11.3±0.5	9.3±01.1	8.1±0.9	7.4±0.1	2.2±0.5	1.1±0.6
	Aspergillus tubingensis	12.3±0.4	10.3±0.1	8.8±0.5	6.2±0.5	3.8±0.2	-	-
	Aspergillus terreus	11.7±0.7	10.5±0.2	8.3±1.1	4.2±01.1	-	-	-
	Cunninghamella	11.9±0.1	10.2±0.4	8.2±0.3	3.8±0.2	-	-	-
	Fusarium	12.7±0.4	10.2±01.1	9.6±0.7	7.2±0.3	4.7±0.1	-	-

Data are expressed with grams as fresh weight, ± standard deviation of triplicates.

Table 4. Identified microorganisms from two sites

Identified microorganism	Accession number	Identy %
Aspergillus flavus	OP113794	99.5
Aspergillus terreus	OP113795	98.6
Penicillium aurantiogriseum	OP113796	98.2
Aspergillus wentii	OP113797	100

for the Aspergillus genus, which is commonly considered fungus in the environment due to its high spore spreading.

Many studies have shown that microorganisms can remove heavy metals with high performance and at a lower cost than traditional methods [22]. Our obtained results for detecting biosorption activity against heavy metals for different fungal isolates were in agreement with many studies which indicated similar activity for the same fungal isolates[23,24]. Moreover, chrysogenum, Aspergillus nidulans, Aspergillus flavus, Rhizopus arrhizus, and Trichoderma viride isolates were characterised by distinguishable Pb and Cr resistant activity[25].

The use of fungal biomass for such purposes has been hindered due to problems such as small particle size, poor mechanical strength, low density, and rigidity. More support was added to our findings for using *Aspergillus* as a heavy metal removal agent[26, 27,28]. They indicated that Aspergillus niger could be used successfully for removing lead and cadmium ions, especially from wastewater. In accordance with our results for molecular deification of heavy metal removal fungi, A. terreus and A. tubingensis were identified respectively with unique heavy metal resistant activity[29,30,31].

Many advantageous were detected for applying fungi with heavy metal resistance due to penetrate contaminated soil by hyphae to remove heavy metals. Furthermore, more attention was needed for heavy metals bioremediation and biodegradation processes. Finally, conducted investigations for fungi tolerance heavy metals indicated huge potentials for applied fungi as heavy metals remover.

CONCLUSION

In this investigation, fungi with significant lead and chromium resistant patterns were isolated from highly heavy metal contaminated soils. For the second site, seventeen identified heavy metal fungal strains that reflected resistance to Cr and led were identified as Aspergillus, Rhizomucor, Neosartorya, Penicillium, Cunninghamella, and Fusarium based on morphological features.Based on the 18S rDNA molecular identification marker, Aspergillus flavus which was isolated from first and second sites, was highly resistant to all Cr and Pb concentrations. From the second site, *Aspergillus terreus*, *Penicillium aurantiogriseum* and Aspergillus wentii showed resistant patterns against all Led concentrations. Our obtained results could be reflected by the application of fungi as heavy metal removal for highly contaminated soils.

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