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Characterization of Chromium Degrading Fungal Isolates Using ITS Gene Sequencing

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ABSTRACT

Due to various anthropogenic activities the level of chromium is increasing at an alarming rate around the globe. To tackle with this problem, various microorganisms were reported, to play an important role with their potential to utilize chromium as a carbon source. In our previous study we reported such chromium degrading fungal isolates collected from the chromium dumping sites. In the present study, an attempt was made to characterize most efficient chromium degrading fungal isolates using both morphological and molecular tools (ITS gene sequencing).

Key words *Chromium degrading fungi, Chromium dumping sites, morphological keys, ITS gene sequencing*

Identification of an organism is very important after knowing their importance and applications for future use. In the literature there are three different levels for the identification of any unknown micro-organism *viz a viz* morphological level, biochemical level and at the molecular level (Aneja 2003). For fungal identification morphological studies have been used since earlier. Recent emphasis is however on molecular identification involving certain specific sequences. The sequences from the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA are commonly used for the identification of fungi (Kõljalg et al. 2005); (Naumann et al. 2007); (Nilsson et al. 2008). Internal transcribed spacer (ITS) refers to a piece of non-functional RNA situated between structural rRNA on a common precursor transcript. When read from 5' to 3', this polycistronicr RNA precursor transcript contains the 5' external transcribed sequence 5' ETS, 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3'ETS. During rRNA maturation, ETS and ITS pieces are excised and as non-functional maturation by-products rapidly degraded. Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because it is easy to amplify even from small quantities of DNA (due to the high copy number of rRNA genes), and has a high degree of variation even between closely related species. This can be explained by the relatively low evolutionary pressure acting on such non-functional sequences.

The ITS region is the most widely sequenced DNA region in molecular ecology of fungi (Peay et al. 2008) and has been recommended as the universal fungal barcode sequence (Schoch et al. 2012). It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions.

In continuation to our previous report (Butt et al. 2017) where we had reported the chromium degrading fungal isolates. In the present study, an attempt was made to characterize the most efficient chromium degrading fungal isolates at both morphological (macroscopic and microscopic and molecular level (ITS gene sequencing).

MATERIALS AND METHODS

The Most efficient chromium degrading fungal isolates reported in our previous study (Butt et al. 2017) were characterized, both at morphological and molecular level.

Characterization of chromium degrading fungal isolates at morphological level:

At morphological level, the most efficient chromium degrading fungal isolates were characterized at both macroscopic and microscopic level using referred identifications keys.

At macroscopic level:

The chromium degrading fungal isolates were cultivated on potato dextrose agar plates at corresponding isolated temperature for 7 days. The following morphological characteristics were evaluated: colony growth (length and width), presence or absence of aerial mycelium, colony color, presence of wrinkles and furrows, pigment production etc.

At microscopic study:

Microscopic observation of spore and their arrangement is very important in the classification of fungi. Fungal spores were cultivated on potato dextrose medium. The germination and growth of mycelium was observed daily under a light microscope. The microscopic examination was made by observing needle mount preparations and slide cultures after staining with lactophenol cotton blue. All microscopic identification was carried out by using CH20i microscope (Olympus) and photographs of each fungal strain were taken by means of MIGS (Magnous Image Projection System).

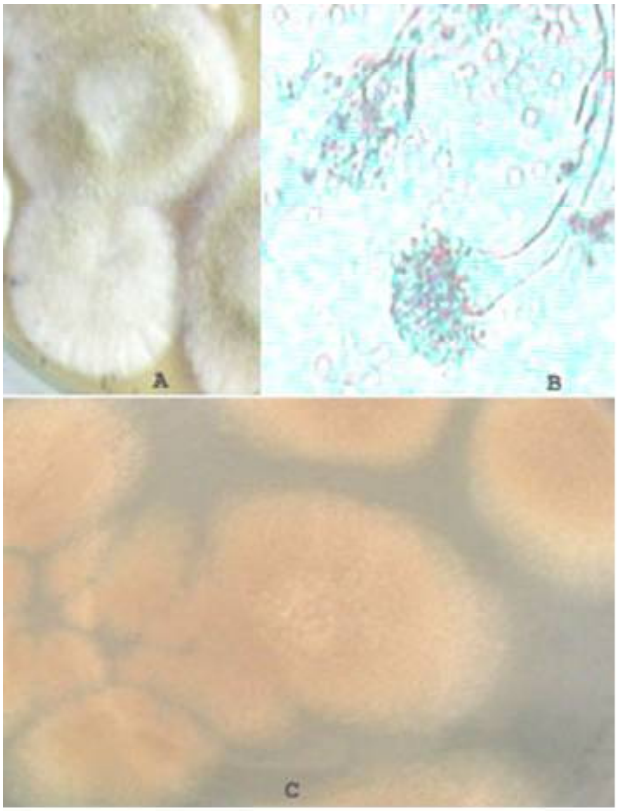

Fig. 1. Microphotograph of colony morphology of F1md under magnification power 40X: A. colony structure; B. conidiophores structure; C. old colony.

Characterization of chromium degrading fungal isolates at molecular level:

At molecular level the most efficient chromium degrading fungal isolates (BPF-4) was identified based on ITS gene sequencing tool.

Extraction of genomic DNA:

The genomic DNA of the most efficient chromium degrading fungal isolates (BPF-4) was using fungal genomic DNA extraction kit (Bhat Biotech Pvt. Ltd., Bangalore) as per the instruction manual. The quality and quantity of the DNA was checked using 0.8% agarose gel electrophoresis using lambda DNA as a marker.

Quantification of the DNA:

The quality and quantity of DNA was confirmed by comparing with that of the lambda (λ) phage DNA (50ng/µl) as a marker separated on 0.8% Agarose gel. The concentration of DNA was also measured using Nanodrop (NanoDrop 2000 Spectrophotometer, Thermo Scientific, United States). All DNA solutions were diluted to uniform concentration of 10ng/µl for PCR amplification of ITS gene.

PCR Amplification of ITS gene:

A 25 µl PCR reaction was performed with 1.5mM MgCl2, 0.5 mM of each dNTP, 1XTaq buffer, 1U/µl Taq DNA polymerase, 0.5pM of each primer primer(ITS1: 5' TCCGTAGGTGAACCTGCGG 3'; ITS4: 5' TCCTCCGCTTATTGATATGC3' (Esteve-Zarzoso et al. 1999) and 50 ng DNA template. The PCR (Veriti, gradient thermocyler cycler, Applied Biosystem, USA) was programmed at initial denaturation 95°C 5 min, 35 cycles with denaturation at 94°C 1 min, annealing 55°C 2 min, extension 72°C 2 min followed by final extension at 72°C 10 min. The PCR products were separated on 1.5%

Agarose gel (Invitrogen) against negative control and 100 plus DNA ladder (GeneRular, Fermantas) to check amplification of the gene. The gel documentation was done using gel doc unit (Alpha Imager, Germany).

Elution and sequencing of the amplified bands:

After electrophoresis (1.2% Agarose gel) all the genes were eluted using gel elution kit (GeneMark, Taiwan) using the instruction manual. 2 µl of the eluted DNA was checked on 1.2% Agarose gel for quality against the 100 plus bp DNA ladder (Gene Ruler) as marker After quantification using Nanodrop, the good quality eluted DNA along with primers was given to commercial lab for sequencing Identification and molecular phylogeny of the chromium degrading fungal isolate:

The chromatograms of the sequences were viewed using Chromas Lite software (Chromas, 2012). MEGA6 software (Tamura et al. 2013) was used to curate the

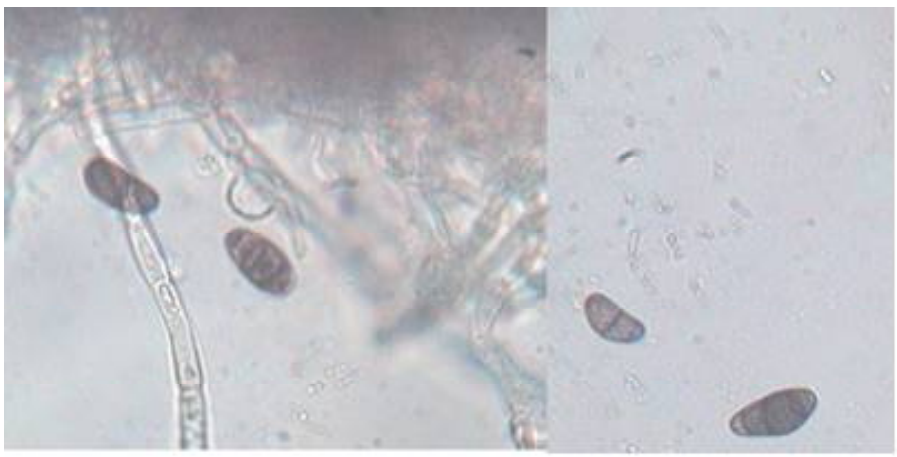

Fig. 2. Microphotograph of F_1md (*Curvularia lunata)* mycelial and conidial structure, under magnification power 40X

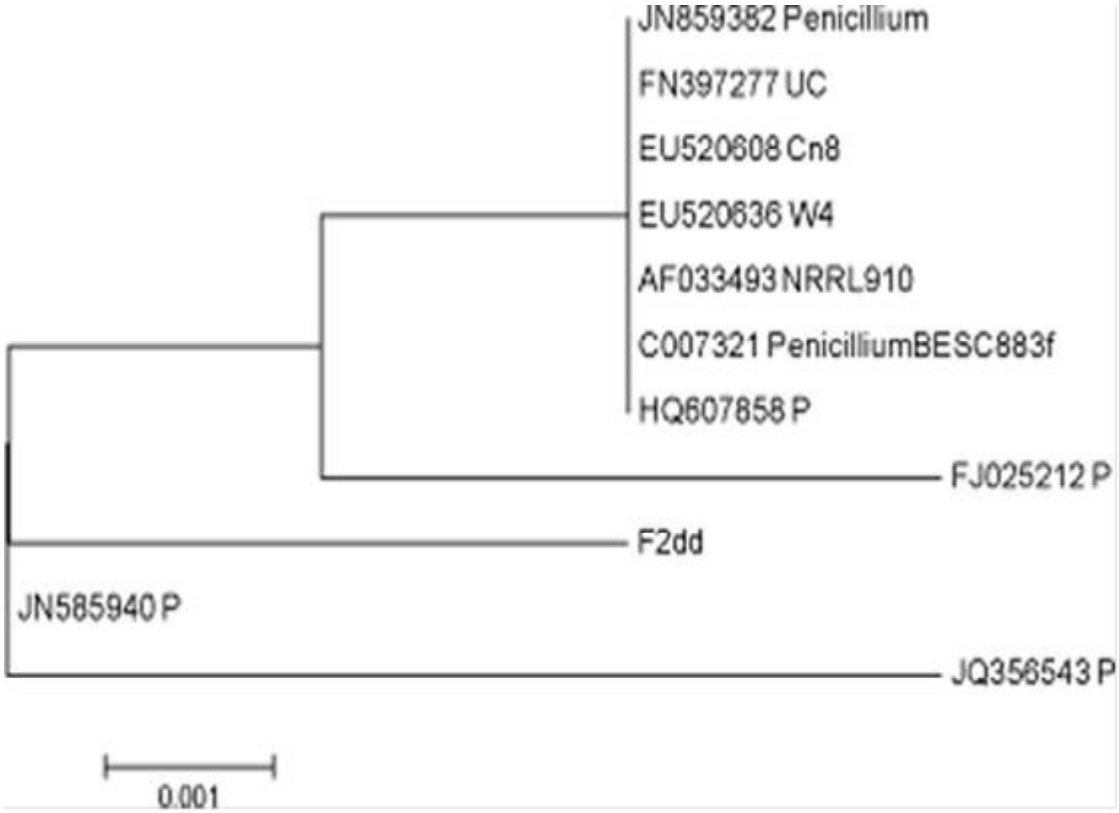

Fig. 3. Phylogenetic relationship based on ITS gene sequence between F2dd and ten closely related texa retrieved from Genebank database

sequences.

The sequences were aligned using clustal W and muscle tool of the MEGA6 software (Tamura et al. 2013). All the curated sequences were compared to the non-redundant NCBI database by using BLASTN, with the default settings used to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbors was aligned using CLUSTAL W2 for multiple alignments with the default settings. The multiple-alignment file was then used to create a neighbor-joining phylogram with CLUSTAL W2.

RESULTS AND DISCUSSION

Characterization of chromium degrading fungal isolates at morphological level:

At macroscopic level:

F_1md colonies on potato dextrose agar at 25°C were initially creamish, sometimes with light green center, gradually becoming beige to buff to cinnamon (Fig. 1A). Reverse is colorless or very light yellow. Moderate to rapid growth rate, colonies become finely granular with conidial production and brown in colour when grew old (Fig. 1C).

In case of F_2md fungal isolate, dark shiny velvety-black colony with fluffy surface and dark bluish-black reverses of the colonywas reported. The older colony becomes compact and fleshy.

Where as in F2dd isolate, the colony was compact or dense, center somewhat raised, sulcate, velutinous, margin entire and white in colour. The old colony showed greenish sectors. The growth rate of colony was moderate.

At microscopic level:

Hyphae of F_1md were septate and hyaline. Conidial heads were biseriate (containing metula that support phialides) and columnar. Conidiophores were smooth-walled and hyaline, 70 to 300µm long, terminating in mostly globose vesicles. The conidiophores head was short, columnar and uniseraite with ovate vesicle. Conidia are small (2-2.5 µm), globose, smooth and brown. Globose, sessile, hyaline accessory conidia (2-6 µm) frequently produced on submerged hyphae (Fig. 1B). On the bases of macro and microscopic characters the isolate was identified as *Aspergillus terries* (Barnett and Hunter 1972); (Gilman 1957).

The F_2md mycelia were found to beseptate, dematiaceous producing brown conidiophores. The conidia were curved slightly, distinctly, transversely septate, with an expanded third cell from the pore end of the conidium. The conidia were septate from edge to edge of the conidial wall (Fig. 2). The isolate was thus identified as *Curvularia lunata* (Gilman 1957).

Whereas the mycelium of F2dd isolate was thin-walled; penicilli mostly biverticillate, often monoverticillate; metulae in verticils of 2-3, roughened, phialidesampulliform, in verticils of 5-10, smooth to finely roughened, mostly short; conidia subspheroidal, less often ellipsoidal, finely roughened, thin walled, borne in loose, disordered, entangled chains.

Characterization of chromium degrading fungal isolates at molecular level:

Phylogenetic analysis of the most efficient chromium degrading fungal isolate strain F_2dd:

The ITS sequences of ten closely related taxa were retrieved from GeneBank and evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.02129630 is shown (next to the branches). The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 540 positions in the final data set. Evolutionary analyses were conducted in MEGA6. The dendrogram is shown in Fig.3. F_2dd was identified as the species of *Penicillium*; most likely *P. canescense*.

In literature maximum microbes which were reported to degrade chromium were from domain bacteria. Only few fungal isolates e.g. *Aspergilli* (Gouda 2000) with potential to degrade Cr (VI) were reported.

In the present study we documented the efficient chromium degrading fungi at both morphological and molecular level, and the isolate F_2dd was identified as the species of *Penicillium*; most likely *P. canescense*.

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