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Fungal weathering of asbestos in semi arid regions of India

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ABSTRACT

The science of Geomicrobiology, which deals with mineral- microbe interaction in nature contributes effectively to three important processes namely- mineral and metal bioremediation, biomining and soil mineral formation by microbes. Bioremediation one of the important process of the above, degrades or transforms hazardous contaminants to less toxic compounds. Several groups of fungi have proved highly efficient in this aspect, with asbestos being one such toxic entity in the environment on which their activity was studied. The present investigation uses the same tool as a device for detoxifying asbestos, a potent carcinogenic entity; with fungal isolates native to the asbestos mines of Rajasthan, India, being investigated for the first time. The cellular mechanism of asbestos toxicity is mainly attributed to the presence of iron in its chemical composition which catalyzes generation of free radicals leading to oxidation of biomolecules. The two dominant novel species found therein, identified as *Aspergillus tubingenesis* and *Coemansia reversa* have proved capable of actively removing iron from asbestos fibers as studied by scanning electron microscopy- electron diffraction X-ray (SEM–EDX) analysis. This probably could lead to a reduction in toxicity of asbestos, due to reduced iron concentration as reported in related studies. Many fungi are known to release iron chelating compounds, siderophores, which could be instrumental in the study. The findings related to two new fungal species being added to the list of earlier identified fungal bioremediators of asbestos, widens the prospect of using bioremediation as an effective tool for asbestos detoxification

1. Introducton

Intensification of agriculture and manufacturing industries is leading to an unending increase in the content of the wide range of xenobiotic compounds being released to the environment. The global endeavor to sustain and preserve the environment, is at present largely focused on the technique of bioremediation, which capitalizes mainly microorganisms to clean up contaminated soil and water (Strong and Burgess, 2008). The fungi are reported to possess the most varied and efficient system to degrade a huge array of organic molecules including waxes, rubber, feathers, plant polymers, insect cuticles, and animal flesh (Bennet et al., 2002, Rhodes, 2013, 2014). Fungi not only colonize the rock surface but also show effective penetration of their hyphae into the cracks and crevices of the rocks (Caneva et al., 2008; Gueidan et al., 2008), thereby bringing about their biodegradation by various biochemical activities.

One such mineral which has been shown to be a potent hazard for human health is asbestos, being listed as the third most abundant pollutant at the global scale (Air Quality Guidelines for Europe, 1987; Fubini et al., 1990; Wachowski and Domka, 2000). Asbestos fibers have been designated as a Group (I) definite carcinogen by IARC (International Agency for Research on Cancer), a part of WHO (World Health Organization), in the year 1987(Shinya Toyokuni, 2009) causing about half of the deaths from occupational cancer (Concha-Barrientos et al., 2004). Asbestos is a naturally occurring hydrated magnesium phyllosilicate mineral with many of its forms showing a considerable presence of iron. It is broadly divided into two mineralogical groups – the amphibole and the serpentine (Mossman et al., 1996). Internalization of micro to nano sized asbestos fibers suspended in air, leads to their gradual accumulation particularly on lungs and their manifestation later as lung carcinoma, pleural mesothelioma or asbestosis (Kilburn, 2000). Asbestos accumulation in lungs leads to genotoxic effects such as, causing chromosome breaks and deletions (Okayasu et al., 1999) or smaller gene mutations (Rihn et al., 2000, Unfried et al., 2002, Kristina Luus, 2007, Wei et al., 2012, Kim et al., 2013). Reactivity and toxicity of asbestos fibers has been reported to be due to the presence of iron at their surface (Fubini et al.,

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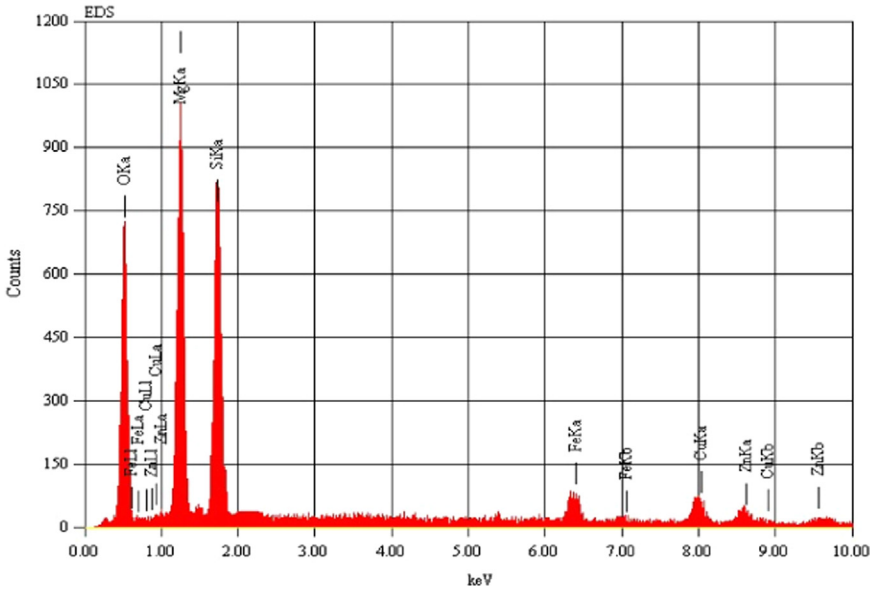

Fig. 2. EDX analysis image of processed asbestos fibers (control).

1995). In vitro experiments have reported that various chelators can extract iron from fibers modify its surface properties (Fubini et al., 1995) and even promote disruption of several sub layers (Prandi et al., 2002). Another important breakthrough was disruption of magnesia–silicate framework of asbestos by the aposymbiotic lichen forming fungus *Xanthoparmelia tinctina* (Favero-Longo et al., 2007). The fungus *Fusarium oxysporum* could effectively extract iron from chrysotile, crocidolite and amosite by siderophore secretion also (Daghino et al., 2005). Fungi contribute to the deterioration of silicate-bearing rocks and iron- and magnesium-bearing minerals (Kumar and Kumar, 1999, Hoffland et al., 2004, Gadd, 2007). While *Verticellum leptobactrum* and *Paecilomyces lilacinus* were able to extract iron from crocidolite (Daghino et al., 2006), *Verticellum leptobactrum* also removed significant amounts of magnesium from chrysotile fibers bulk (Daghino et al., 2009). The results obtained showed a general decrease of reactivity for both chrysotile and crocidolite forms of asbestos (Martino et al., 2003; Daghino et al., 2006). *Fusarium oxysporium* and *Aspergillus fumigatus* (Daghino et al., 2008), have also been shown to be potent bioremediator of asbestos. The mobilization of ferric ions by fungi is attributed to the secretion of low molecular weight iron chelating organic molecules termed as siderophores (Winkelmann, 2007).

The present investigation aims at identifying some novel potential fungal bioremediators of asbestos from the soils of Rajasthan, India which till 2010 had been the leading asbestos producer in the country and still houses a number of defunct asbestos mines, which pose a major health threat in the state. In this context we also propose to study the changes in asbestos elemental composition, in particular iron, upon fungal treatment; using scanning electron microscopy- electron diffraction X-ray analysis. A preliminary analysis of their siderophore synthesizing ability enabling iron extraction would also form a part of the proposed investigation.

2. Materials and method

2.1. Sampling, isolation and identification of fungal isolates

Asbestos rock and asbestos contaminated soil samples were collected from four abandoned mines of Rajasthan, India. Two were from the Rajsamand district and two from the Jodhpur district. As mines have been officially banned by the Government of the country, access to the mines was restricted and thus the small sample size. Fungi were isolated by a modified 'soil dilution plate' technique (Waksman, 1927; Warcup, 1950). The rock samples crushed into small pieces and the soil samples were suspended in sterile water at different concentrations ranging from 1:50 (w/v) to 1:500 (w/v). All suspensions were then vortexed for 10–15 min and plated onto potato dextrose agar plates amended with 4×10^{-5} g mL^{-1} gentamycin and 3×10^{-5} g mL^{-1} streptomycin. Three replicate plates were prepared for each sample. As a part of the preliminary identification of the fungal isolates, colony morphology was studied and colonies transferred to separate culture tubes.

2.2. Asbestos fibers and their fungal treatment

Asbestos fibers for experimental work were obtained from one of the asbestos- cement product manufacturing industry situated in Jaipur, Rajasthan. The fibers were separated from the ore by crushing, air suction, and vibrating screens, and in the process sorted into different lengths, or grades. The processed fibers so obtained, were weighed and a homogenous suspension prepared in sterile water by vortexing so as to maintain a total concentration of 2% (w/v) in the final culture flasks. The fibers were also characterized for structural and elemental composition by Scanning Electron Microscopy-Energy Dispersive X-ray (SEM–EDX) (Figs. 1 and 2).

Based on the nature of growth in asbestos supplemented media six fungal isolates were selected as the dominant species found in the rock and soil isolates and cultured on potato dextrose agar plates. Liquid cultures were then prepared, as reported in literature (Daghino et al., 2005) Culture medium used was Czapek medium supplemented with glucose (composition-glucose 20 mg/mL, NaNO3 3 mg/mL, K2HPO4 1.31 mg/ml, MgSO4 0.5 mg/ml, FeSO4 0.01 mg/ml, KCl 0.5 mg/ml, MES (4-Morpholine ethane sulfonic acid) 3.9 mg/ml) (Daghino et al., 2005).Tubular dialysis membranes bags were filled with suspension of processed asbestos fibers in distilled water, maintaining a final concentration of 2.3% w/v in the culture volume. They were tied at the ends and autoclaved with media before being inoculated with fungi in liquid culture. The fibers were incubated with the fungal isolates for 20

days, at 25 °C and pH 5.5 under shaking at 120 rpm. Control flasks were also set up under identical conditions but without any fungal inoculums being added to the liquid medium having asbestos fibers (Daghino et al., 2006). After the specified period of incubation the cultures were filtered through Graded Whatman filter papers and asbestos fibers collected. The fibers were repeatedly washed with milliQ Millipore water, dried to powder and analyzed by SEM–EDX (scanning electron microscope coupled to energy dispersive X-ray).

2.3. SEM–EDX of treated asbestos fibers

To analyze the changes in elemental composition of fungi treated asbestos fibers, the dried fibers were subjected to SEM–EDX analysis. Literature survey on the analytical techniques best suited for physic–chemical characterization of asbestos indicated scanning electron microscopy coupled with electron diffraction analysis as the most apt method in comparison to more convenient techniques of light microcopy or phase contrast optical microscopy (AIA Health and Safety Publication, 1984; Heinz et al., AMAM 2005; Dumitru and Ruse, 2005; Cecchetti, 2005). The samples were examined under Scanning Electron Microscope [SEM] using Zeiss EVO 18 Special edition model coupled to Energy dispersive Spectrum detector provided by Oxford Instruments (Oxford X-Act) for imaging and elemental analysis. Examination was done with working distance (WD) of 12.5 mm, at an acceleration voltage of 30 kV and magnifications ranging from 1,050 × to 50,000 × .The field of view for each sample were approximately 6 mm.The elemental composition obtained as respective peaks and values of weight % and atomic % were then studied.

2.4. Molecular analysis of the selected fungal species.

Nucleotide sequencing of 18 s rRNA genes of the selected species was done with the help of Zeal Biologicals, Andhra Pradesh, India. Fungal genomic DNA samples were extracted using a Insta Genetm Matrix (BIO-RAD). The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 μl reaction mixture by using a EF-Taq (SolGent, Korea) as follows- activation ofTaq polymerase at 95 °C for 2 min was followed by 35 cycles of 95 °C for 1 min, 55 °C, and 72 °C for 1 min each, finishing with a 10 minute step at 72 °C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). Each sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers (White et al., 1990). 18S rDNA region sequencing analysis primers used were-ITS1 primer (TCCGT AGGTGAACCTGCGG),ITS5 prime (GGAAGTAAAAGTCGTAACAAGG) and ITS4primer (TCCTCCGCTTATTGATATGC). Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of Seaview software (Gouy et al., 2010).

2.5. Siderophore detection

As a step further towards assessing the ability of identified isolates to reduce iron concentration in asbestos their siderophore producing ability was analyzed. The fungal cultures inoculated in Czapek medium with asbestos, after an incubation period of 20 days, under experimental conditions as mentioned above, were subjected to centrifugation at 10,000 rpm for 15 min. The cell-free culture filtrate was used for the assessment of siderophore production by the Chrome Azurol S liquid assay (Schwyn and Neilands, 1987) and modified Chrome Azurol S agar assay as given by Milagres and his associates (Milagres et al., 1999). The fungal growth media used for modified Chrome Azurol S agar assay was potato dextrose agar media.

3. Results

3.1. Fungal isolation from asbestos samples

As mentioned, the asbestos rock and soil samples were collected from four different mines (two of Rajsamand district and two from Jodhpur district), to study the dominant fungal species thriving there in. Following, the inoculation of diluted rock and soil samples of asbestos, the dominant colonies based primarily on morphology were subcultured to yield pure cultures. A total of six isolates (the most dominant one from each of the six samples- four rock samples and two soil samples) were chosen for further studies of asbestos bioremediation. The two isolates from Jodhpur mines were named J1F and J2F, the two from Rajsamand rock samples were termedUR1F and UR2F and the two dominant isolates obtained from asbestos contaminated soil around the Rajsamand mines were called US1F and US2F.

3.2. Structural and elemental analysis of asbestos

he processed asbestos fibers obtained from asbestos-cement product manufacturing industry situated in the Jaipur, Rajasthan were subjected to scanning electron microscopy (Fig. 1) and energy dispersive X-ray analysis (Fig. 2) for structural and elemental studies respectively. (Kuczumow, 2011). The processed asbestos showed a considerable amount of iron, amounting to 6.86% of the total elemental composition (Table1) and was further used as substrate for fungal activity studies.

3.3. Treatment of asbestos fibers with fungi

The fungal extraction of iron from asbestos after 20 days of incubation was assessed by SEM–EDX analysis in comparison to a control asbestos sample which was also subjected to similar parameters of incubation as the experimental samples but without any fungal inoculation. Structural studies as obtained by SEM did not show any significant variation between the control and experimental samples. But the elemental analysis which was carried out in triplicates for each sample by EDX studies (Figs. 3 and 4)

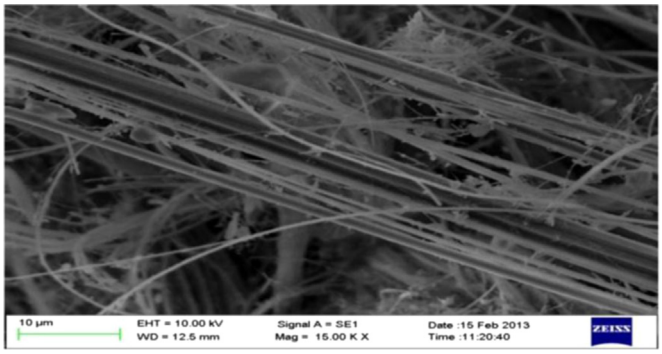

Fig. 1. Scanning electron microscope image of asbestos fibers (processed). ("With kind permission from Springer Science+Business Media: Silicon, Bacterial weathering of asbestos, vol 7, 2015, 419–431 Shabori Bhattacharya. P.J. John. Lalita Ledwani, figure number(2)").

Table 1
Comparative chart indicating weight % of elements in asbestos after fungal treatment.

S.No	Element	Weight % variation with different isolates						
		J1F	J2F	UR1F	UR2F	US1F	US2F	CONTROL
1	Carbon	3.61	———	2.93	———	———	2.44	———
2	Oxygen	31.77	28.77	27.81	31.23	37.49	38.35	29.92
3	Magnesium	27.4	30.58	26.69	29.73	28.42	27.29	26.33
4	Silicon	26.76	29.11	25.7	24.76	25.73	22.53	23.99
5	**Iron**	5.72	4.72	10.85	6.47	6.94	6.65	6.86
6	Calcium	———	———	———	1.96	1.43	1.96	———
7	Sodium	———	———	———	———	———	———	———
8	Aluminum	2.55	———	———	———	———	———	———
9	Copper	2.2	3.1	8.04	4.99	———	———	7.87
10	Zinc	———	2.17	———	———	———	———	5.02
11	Potassium	———	1.54	2.16	0.87	———	———	———
	TOTALS	100	100	100	100	100	100	100

Dotted lines indicate absence of respective elements in the samples.

brought forth two fungal isolates out of the six used, as potential extractors of iron from asbestos. The EDX spectrum displayed in digitized form has the *x*-axis representing X-ray energy as emitted by constituent elements and the *y*-axis representing the number of counts (as photons) indicating a measure of X-ray intensities. The relative intensity of an X-ray line is approximately proportional to the mass concentration of the element concerned. Thus inferences drawn from EDX spectrum, indicated the percentage of the main constituents of asbestos namely oxygen, magnesium, silica, iron on the basis of weight % and atomic % respectively. They reveal that iron concentration was less in the treated samples in comparison to control (Table 1).The weight % variation both with J1F and J2F isolates for iron was found to be statistically significant than that of control ($P < 0.05$). The results were analyzed by one-way ANOVA using GraphPad Prism 6 software.

As mentioned in the comparative chart indicating weight % of elements in asbestos after fungal treatment based on the obtained EDX data, a reduction of 16.6% by fungal isolate JIF and 31.2% by fungal isolate J2F was estimated in the iron content of treated asbestos in comparison to the control (Table 1). The other isolates did not show an equivalent appreciable result. The ratio of Mg–Si which has also been studied as a part of fungal asbestos bioremediation by Daghino et al. (2009), did not show any significant changes between control and experimental studies in the present investigation. The Fe/Si atomic ratio evaluation for the bulk, as reported in literature (Daghino et al., 2006), was also carried out for this study and showed a considerable variation only in case of samples JIF and J2F in relation to control with decreased values. As seen from the chart there was no significant variation in weight % of magnesium or silica elements after fungal treatment.

3.4. Molecular identification of the potent bioremediators

Nucleotide sequencing of 18srRNA genes of the selected fungal isolates and its phylogenetic analysis presents the following dendogram (Figs. 5 and 6) based on the similarity values of reference strains obtained from the GenBank. The two fungal strains were identified as *Aspergillus tubingensis*; CICR4 (sample J1F) and *Coemansia reversa*; NRRL1564 (sample J2F).The phylogenetic trees provide information about the inferred evolutionary relationships between a set of fungi. The length of horizontal lines (branches) represent evolutionary lineages changing over time, with the bar at the bottom of the figure which is 0.2 in the given figures (Figs. 5 and 6) providing a scale for this. The units of branch length are usually nucleotide substitutions per site. The vertical lines simply provide information about two species evolving from a common ancestor represented as a node. The numbers next to each node, in red, above, represent a measure of support for the

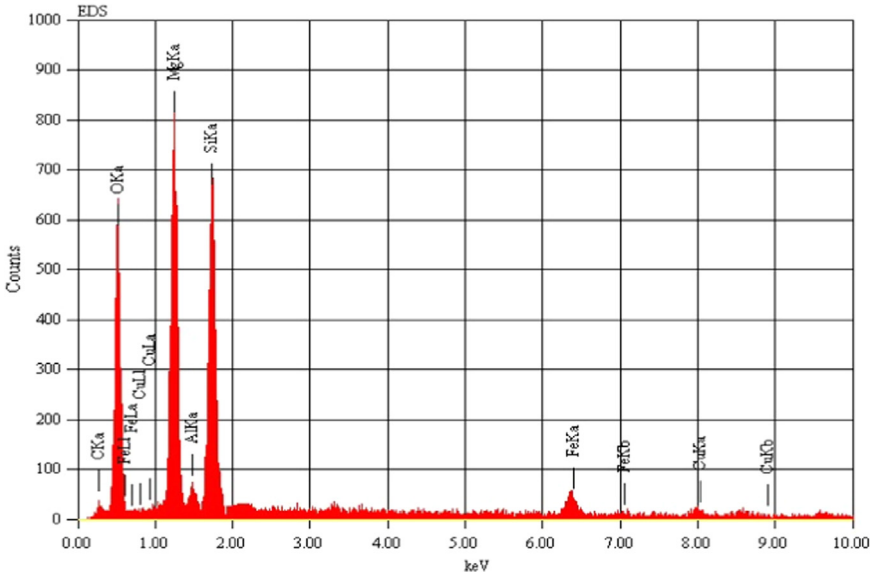

Fig. 3. EDX analysis image of processed asbestos asbestos after J1F isolate treatment.

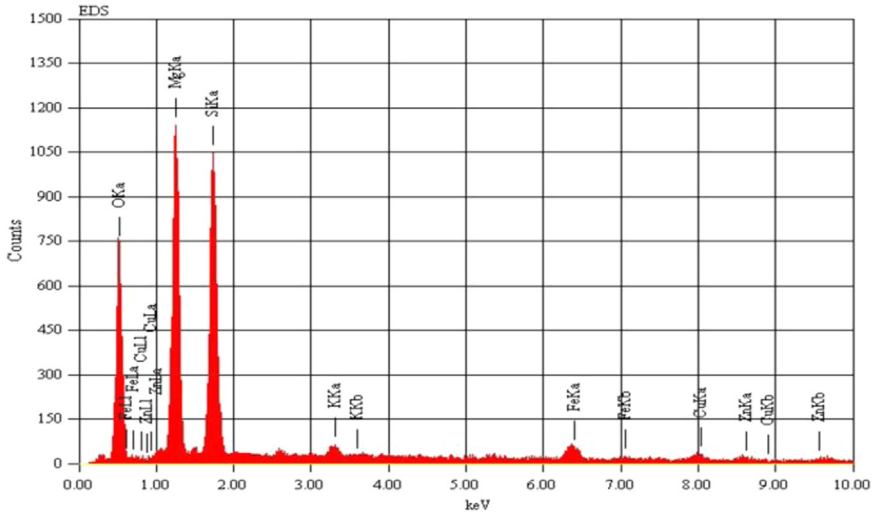

Fig. 4. EDX analysis image of processed asbestos asbestos afterJ2F isolate treatment.

node, ranging from 0 to 1 with 1 depicting maximum support to the node. As seen in Fig. 5 *Aspergillus tubingenesis* has evolved form a common ancestor as *Aspergillus niger* which are more closely related to *Penicillium* species than *Geomyces* species. Similarly from Fig. 6 it can be interpreted that *Coemansia reversa* evolved from a common ancestor as *Smittium morbosum* and they differ widely from species of *Entomophthora.*

3.5. Siderophore detection

When the identified fungal isolates were incubated on CAS-agar Petri plates for siderophore detection the results as observed were qualitatively distinct in terms of color of CAS reaction. A change from blue to purple or dark purplish-red was observed starting from the borderline of CAS agar and PDA. Responses were consistent for all replicates of both fungal species.

4. Discussion

Geomicrobiology research has focused on the role of fungi, related to their bioweathering activity and ability to modify metal ions (Gadd, 2010) since a long time. As an addition, the present experimental work brings to light the ability of different fungal strains to weather asbestos.Asbestos minerals although highly resistant to weathering, have been reported to be biodegraded by certain fungal species like *Verticellum leptobactrum, Paecilomyces lilacinus* and *Aspergillus fumigatus* by iron extraction or by disruption of the magnesium–silicate framework of asbestos (Daghino et al., 2008).Probably the first of its kind, an *in vitro* approach, was therefore chosen,to study the effects of fungi on asbestos in semiarid soils of India. Fungi isolated from asbestos contaminated soil and asbestos rocks of four abandoned defunct asbestos mines of Rajasthan, India were the candidates of study. The limited sample size could be explained due to the ban imposed on asbestos mines by the Government and strict regulations to avoid access to such locations. The fungal community isolated from the soil and rock samples as mentioned put forth two dominant isolates capable of asbestos bioremediation, namely *Aspergillus tubingenesis* and *Coemansia reversa.* Their activity was based on extraction of iron from asbestos, which has been shown in many cell free tests, to reduce the ability of the fibers to catalyse the generation of hydroxyl radicals and thereby cause a decrease in fiber-induced DNA oxidation (Daghino et al., 2006) and its carcinogenic effects. SEM–EDX analysis of fungi treated asbestos samples showed much depletion in its iron content with respect to the control with Fe/Si ratio being used as one of the measures. Compared with the 0.285 Fe/Si ratio measured for asbestos fibers exposed to the Czapek medium alone, *Aspergillus tubingenesis* induced a limited but noteworthy extraction of Fe giving a Fe/ Si ratio of 0.21 while *Coemansia reversa* reduced it further to a remarkable value of 0.16. Statistical analysis shows this to be a significant variation with respect to control ($P < 0.05$). But the ratio of Mg–Si also reported in literature as a part of fungal asbestos bioremediation (Daghino et al., 2009), did not show any significant changes in this case. On the other hand a substantial decrease in iron content of asbestos, accompanied with presence of siderophores in medium suggests that the extraction of iron from fibers is mediated by the water soluble molecules, secreted in the culture media by the respective fungi. This is in accordance with

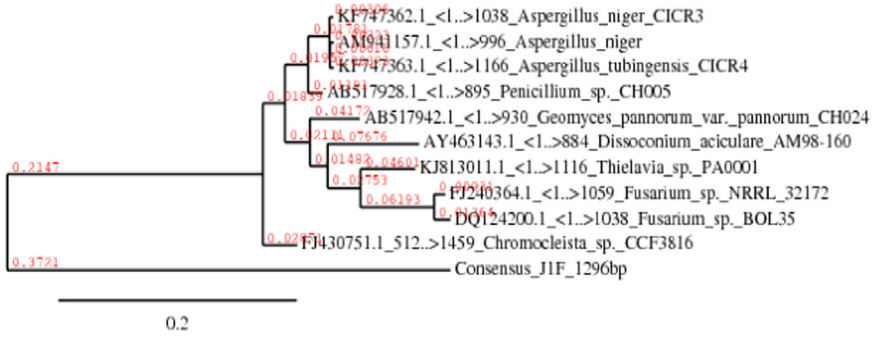

Fig. 5. Phylotree for sample J1F identified to be *Aspergillus tubingensis*; CICR4; KF747363.

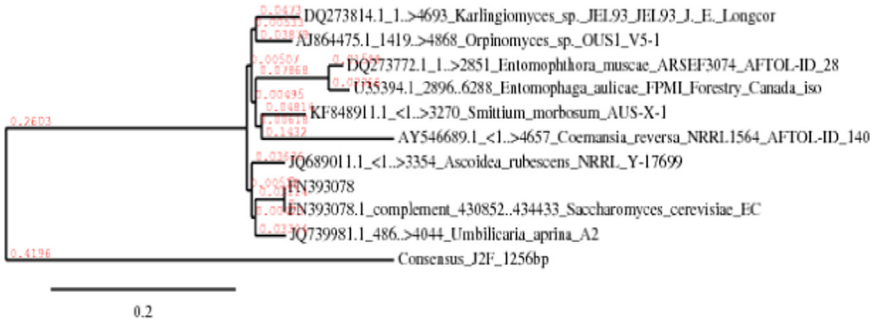

Fig. 6. Phylotree for sample J2F identified to be *Coemansia reversa*; NRRL1564; AFTOL-ID 140; AY546689.

the study that interaction of fungi with iron in the soil is based on mechanisms of solubilization and adsorption, mediated by siderophores or by specific chelators like organic acids (Daghino et al., 2008). Siderophore production has been reported for *Fusarium oxysporum* (Domsch et al., 1980), for some species of *Paecilomyces* (Vala et al., 2000) and *Aspergillus* (Machuca and Milagres, 2003), which have proved to be potent bioremediators of asbestos. Enhanced iron content of certain samples mainly URIF and US1F could possibly be due to absence of siderophores, which causes iron deposition from the medium onto the asbestos fiber surface without being simultaneously extracted from the fibers, by siderophore mediated process (Prandi et al., 2001) as seen for J1F and J2F. *Aspergillus fumigates*, *Aspergillus nidulans* and *Aspergillus niger* have been shown to be highly efficient in synthesizing siderophores as a part of high-affinity iron uptake mechanism (Franken et al., 2014). *Aspergillus fumigatus* synthesizes four different types of siderophores for capturing extracellular iron and storing it in the hyphae and conidia (Schrettl et al., 2007). *Aspergillus flavus* was shown to produce hydroxamate siderophore when tested alongside other microbial isolates obtained from rhizosphere soil (Kannahi and Senbagam, 2014). *Aspergillus nidulans* and *Aspergillus nidulans versicolor,* are also considered as important siderophore producing fungi. In the present investigation *Aspergillus tubingenesis* likewise other species of its genera indicated positive results for siderophore production, thereby highlighting promising chances of being considered a potent asbestos bioremediator.

On the other hand, a study of the contemporary literature reveals that so far about 20 species have been reported in the genus Coemansia (Hibbett et al., 2007; Kirk et al., 2008)., with only two species namely *Coemansia erecta* (Rugmini, 1956) and *Coemansia reversa* (Agnihothrudu, 1957), being reported from India till 1957. *Coemansia ceylonensis* was later introduced as the third species of Indian soil in 1965 (Prasad, 1966). Although much has not been reported about the physiology and metabolism of *Coemansia,* its ability to detoxify asbestos as seen in the present investigation by reduction in iron content and siderophore synthesizing ability, suggest that it has adapted to grow and to interact with asbestos as a substrate.

A recent finding by our group based on the search for bacterial bioremediators for the same asbestos samples led to the identification of four probable bacterial strains being endowed with the ability of asbestos bioweathering (Shabori et al., 2015), probably involving the mechanism of siderophore mediated iron chelation as seen for *Aspergillus tubingenesis* and *Coemansia reversa* in the present study. The % decrease in iron as reported for bacterial activity was higher in comparison to fungi after the consistent 20 days treatment.

Although suggested that fungi induced morphological and chemical modifications of asbestos, could be instrumental in reducing the asbestos-related environmental hazard (Fubini, 1997), bacteria too could play an important role in asbestos bioremediation. Thus a microcosm approach under field conditions with more diverse samples under prevailing atmospheric agents could be exploited in future to study asbestos weathering in abandoned mines and thereby paving a way to greener pastures.

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