Tolerance of Microorganisms in Soil Contaminated with Trace Metals: An Overview

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Dhritiman Chanda, G.D. Sharma, D.K. Jha, and Mohamed Hijri

Abstract

Trace metal (TM) pollution of soil is a worldwide problem threatening the quality of human life and a proper environment. We investigated fungal and bacterial diversity of trace metal-polluted site contaminated with paper mill effluent in India. Twelve fungal dominant isolates, viz. *Aspergillus*, *Penicillium*, *Fusarium*, *Cunninghamella*, *Simplicillium*, *Trichoderma*, *Rhizomucor*, *Cladosporium* and *Hypocrea*, were identified. Subsequent screening approach to assess their TM tolerance was performed in vitro cultures which revealed that the majority of the isolates were tolerant to Ni-, Cu-, Zn- and Cd-amended medium. The minimum inhibitory concentration (MIC) for Ni, Cu, Zn and Cd was also determined in isolated strains of *Aspergillus*, *Penicillium*, *Rhizomucor*, *Trichoderma* and *Fusarium* to study the concentration of growth against various trace metals. A total of 22 bacterial isolates was also isolated using 16S rRNA, and the dominant genera such as *Bacillus*, *Rhizobium*, *Microbacterium*, *Arthrobacter*, *Kribbella*

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P. Shukla (ed.), *Recent Advances in Applied Microbiology*,
DOI 10.1007/978-981-10-5275-0_8

and *Chitinophaga* were identified. The relative growth and LD_{-50} were also estimated against the different trace metals from concentration 0.1 to 4 mM. Thus, these fungal and bacterial isolates showed a high TM tolerance and would be a great interest for their use in bioremediation to clean up TM-polluted soil.

Keywords
Trace metal • Fungi • Bacteria • Biodiversity • Paper mill effluent • Bioremediation

8.1 Introduction

8.1.1 Microbial Diversity in Extreme Habitats

Soil harbours a variety of fungi, bacteria and other soil microorganisms. Soil microorganisms are the living component of soil organic matter and are responsible for mineralization of nutrients, decomposition and degradation or transformation of toxic compounds. Metals and metal compounds are natural constituents of all ecosystems, moving between atmosphere, hydrosphere, lithosphere and biosphere (Bargagli 2000; Wuana and Okieimen 2011). One of the challenges facing the mankind in recent times is the degradation and pollution of soil. Since soil is a vital natural resource, its degradation threatens the basic life support system. The industrial influent's sludge and solid waste are the sources of potentially harmful inorganic as well as organic contaminants. Microorganisms growing in such habitats evolved under conditions that permitted their survival and growth (Thakre and Shanware 2015). They multiplied in accordance with natural selection. For such adapted microorganisms, the conditions of these habitats are not 'extreme' but rather the normal physiological conditions for their growth in their natural habitats (Ali et al.2013; Akponah 2013; Kumar et al. 2014; Smith et al. 2015). In metal-contaminated soils, the siderophores and plant growth hormones are produced by plant-associated microbes (Pattus and Abdallah 2000; Wu et al. 2006; Schalk et al. 2011; Ullah et al. 2015). The secretion of siderophores by fungi and bacteria is dependent on several factors like soil pH, nutrient availability in soils and type and concentration of trace metals (Rajkumar et al. 2010; Sessitsch et al. 2013; Yu et al. 2014).

8.1.2 Heavy Metal Resistance in Fungi

Trace metals like Cu, Ni, Zn, Cd and Mn present in paper mill effluent can be removed by indigenous fungi isolated from effluent itself (Khan 2000, Karn and Reddy 2012). Biosorption of metal is carried out by (1) extracellular accumulation/precipitation, (2) cell surface sorption/precipitation and (3) intracellular accumulation through the cell wall of microorganisms (Volesky and Holan 1995; Valix et al. 2001; Madhaiyan et al. 2007; Ma et al. 2016). *Penicillium*, *Aspergillus*, *Trichoderma*, *Cladosporium*, etc., are found to be very useful for the removal of trace metals (Dursun 2008; Ezzourhi et al. 2009; de Lima et al. 2011). El-Morsy (2004) reported

that *Cunninghamella echinulata* biomass could be employed as a biosorbent of metal ions in waste water. De Lima et al. (2013) and Bello and Abdullahi (2016) also studied the cadmium tolerance by *Cunninghamella elegans* by the polyphosphate metabolism. *Trichoderma* sp. produces organic acids like fumaric acid, citric acid and glycolic acid which can decrease the pH in alkaline soil and thus increase the solubility of macro- and micronutrients necessary for plant growth and metabolism (Malgorzata et al. 2014; Song et al. 2015).

8.1.3 Heavy Metal Resistance of Bacteria

Heavy metals can decrease carbon mineralization, nitrogen transformation and soil enzyme activities, microbial numbers (CFU), biomass (Borjesson et al. 2012) and frequency of trace metal-resistant bacteria (Wang et al. 2007, Kanmami et al. 2012). The molecular fingerprinting techniques are also useful to study the changes in the microbial community in trace metal stress conditions (Anyanwu et al. 2011; Andrew et al. 2013). Bacterial populations negatively affected by trace metals. Bacteria are found to develop five important mechanisms to detoxify the trace metals available in contaminated soils: (1) extracellular detoxification, (2) extracellular sequestration, (3) reduced permeability, (4) intracellular sequestration and (5) export. These resistant mechanisms are encoded in bacterial plasmids and transposons due to spontaneous mutation and gene transfer (Osborn et al. 1997; Karelová et al. 2011; Cetin et al. 2012; Zhou et al. 2013). Pal et al. (2004) reported Ni-resistant genes in Gram-positive and Gram-negative bacterial isolates from Ni-rich serpentine soil. In Gram-negative bacteria, the czc-genes encode for a cation-proton antiporter (CzcABC) which is responsible for the resistance against Cd, Zn and Co metals (Nies 1995; Harriso et al. 2007; Abdelatey et al. 2011; Mindlin et al. 2016).

The trace metal tolerance by a particular group of bacteria or isolate in artificial medium supplemented with trace metal showed high tolerance level as reported by Ahmed et al. (2001), Hayat et al. (2002) and Rajbanshi (2008). Olukoya et al. (1997) isolated 228 trace metal-resistant bacteria belonging to 9 genera, and the most common genera were *Staphylococcus*, *Streptococcus* and *Bacillus* found to be resistant to cobalt, zinc, copper, nickel and mercury. Temperature is also a determined factor that affects the growth of bacteria and bioaccumulation of trace metals (Lee et al. 2011a, b). The gene expression study revealed that mercuric ion (*mer*A) and chromate (*chr*B) genes were downregulated in all the strains of bacteria, i.e. *S. aureus*, *Bacillus subtilis*, *B. cereus*, *Pseudomonas* sp. and *Bordetella* sp., when treated with Co and Cd. The expression level of genes *mer*A, *chr*B, *czc* D and *ncc* A in these bacterial strains was measured by real-time PCR method (Abou-Shanab et al. 2007). Nies (1999) and Hirak and Das (2014) compared the metal resistance physiology in 63 species of bacteria and examined the protein-level similarities and suggested that these metal-resistant bacteria can be developed into metal pollution biosensors. Long et al. (2012) described the importance efflux transporters as a metal tolerance lactic by bacteria. Braud et al. (2010) reported a low level of toxicity of trace metals like Ni, Cu, Zn, Cd and Pb in *Pseudomonas aeruginosa. Chitinophaga eiseniae* was also reported as a trace metal tolerant by Yasir et al. (2011) and Gao et al. (2012);

Stan et al. (2011) studied the significant increase of growth, abundance, genetic diversity, nodulation ability and efficacy in the diversity of *Rhizobium* sp. in the soil polluted with copper, zinc and lead. Hemida et al. (2012) and Hao et al. (2014) also discussed the potential role of legume-rhizobia symbiosis in aiding phytoremediation. Hijri et al. (2014) also studied the linkage between fungal and bacterial communities in rhizosphere in hydrocarbon-contaminated soil and their significant effect for plant productivity.

The present study was carried out to understand and evaluate the status of heavy metal-resistant fungi, bacteria and actinomycetes in the Hindustan Paper Corporation (HPC), Assam. Geographically the site is situated at longitude of 24°41′29.9″N and latitude at 92°45′25.9″E.

8.1.4 Characterization of Fungal and Metal-Resistant Bacteria Isolates

The fungal isolates were isolated and were identified to species level using colony diameter and spore measurement following references and monographs adopted by Gilman (1957) and Raper and Fennell (1965). The fungal DNA was isolated with help of nucleic acid and protein purification kit (Macherey-Nagel, USA). The fungal strains have been characterized by PCR with (forward) ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and (reverse) ITS43′TCCTCCGCTTATTGATATGC-5′ (White et al. 1990).

The isolation and purification of chromosomal DNA as well as the amplification and sequencing of partial 16S rRNA gene of potential metal-resistant bacteria isolate was carried out. Bacterial 16S rDNA sequences were amplified using the 27F Lane (1991) and 1492R Turner et al. (1999) primer sets.

The selected bacterial isolate was tested for their resistance to different trace metals by their growth in nutrient broth tubes containing various concentrations of trace metals (0.1, 0.5, 2.0, 4.0 mM). The metals selected for the present investigation included Ni, Cu, Zn and Cd. The bacterial growth was determined by measuring the optical density using spectrophotometer at 540 nm. Relative growth of the isolate was expressed as the percentage of those obtained in untreated control. Lethal dose (LD-50) was estimated for all the tested bacterial isolates (Essam et al. 2013; Anderson and Hughes 2014). DNA sequencing was performed on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA). The nucleotide sequence of bacterial isolate is thus obtained by the use of database using the ‘NCBI BLAST’ (Altschul et al. 1990).

8.1.5 Metal-Resistant Fungal Isolates

Twelve fungal strains were isolated from polluted soil contaminated with trace metals in paper mill effluents and tested with different trace metals in different concentrations. The 12 genera like *Aspergillus*, *Penicillium*, *Cladosporium*, *Cunninghamella*, *Trichoderma*, *Fusarium and Hypocrea* showed significant tolerance against various trace metals (Table 8.1). Minimum inhibitory concentration

Table 8.1 Genetic characteristics of isolated fungal strains

Sl no:	Isolated fungal strains	Accession number	Hit in NCBI database	Max indent (%)
1.	*Penicillium* sp.	KC602310	*Penicillium aculeatum*	99
2.	*Trichoderma* sp.	KC602314	*Trichoderma koningiopsis*	94
3.	*Cunninghamella* sp.	KC602315	*Cunninghamella* sp.	90
4.	*Trichoderma* sp.	KC602331	*Trichoderma harzianum*	97
5.	*Penicillium* sp.	KC602344	*Penicillium simplicissimum*	98
6.	*Rhizomucor* sp.	KC602345	*Rhizomucor variabilis*	99
7.	*Fusarium* sp.	KC602349	*Fusarium proliferatum*	99
8.	*Aspergillus* sp.	KC602350	*Aspergillus tamarii*	98
9.	*Penicillium* sp.	KC602359	*Penicillium janthinellum*	99
10.	*Aspergillus* sp.	KC602371	*Aspergillus niger*	99
11.	*Hypocrea* sp.	KC602373	*Hypocrea lixii*	95
12.	*Cladosporium* sp.	KC602374	*Cladosporium tenuissimum*	100

Table 8.2 Minimum inhibitory concentration (MIC) for tested fungal strains

		MIC (Mm)			
Fungal isolates accession numbers		Ni	Cu	Zn	Cd
Penicillium sp.	KC602310	10<mic>15	1<MIC>2.5	10<mic>15	2.5<MIC>5
Trichoderma sp.	KC602314	10<mic>15	1 < MIC>2.5	20<mic>25	15<mic>20
Cunninghamella sp.	KC602315	10<mic>15	5<MIC>10	15<mic>20	5<MIC>2.5
Trichoderma sp.	KC602331	15<mic>20	1<MIC>2.5	20<mic>25	Cont<MIC>1
Penicillium sp.	KC602344	15<mic>20	1<MIC>2.5	20<mic>25	15<mic>20
Rhizomucor sp.	KC602345	15<mic>20	15<mic>25	15<mic>25	15<mic>20
Fusarium sp.	KC602349	5<MIC>10	1<MIC>2.5	15<mic>25	Cont<MIC>1
Aspergillus sp.	KC602350	5<MIC>10	1<MIC>2.5	15<mic>25	5<MIC>10
Penicillium sp.	KC602359	15<mic>20	2.5<MIC>5	20<mic>25	1<MIC>2.5
Aspergillus sp.	KC602371	10<mic>15	1<MIC>2.5	20<mic>25	15<mic>20
Hypocrea sp.	KC602373	15<mic>20	2.5<MIC>5	15<mic>20	1<MIC>2.5
Cladosporium sp.	KC602374	15<mic>20	5<MIC>10	15<mic>20	2.5<MIC>5

(MIC) of the isolated fungal strains against the different concentration of trace metals was estimated and found that, at higher metal ion concentrations, most of the tested fungal strains were found tolerant and showed strong growth (Table 8.2).

In the presence of various concentrations of nickel, the fungal strains which were able to grow in 15–20 mM were *Trichoderma* sp., *Penicillium* sp., *Rhizomucor* sp., *Cladosporium* sp. and *Hypocrea* sp. The other tested strains like *Penicillium*, *Aspergillus* and *Cunninghamella* were also to grow in MIC of 10–15 mM (Plates 8.1 and 8.2).

In the presence of various concentrations of copper, most of the tested strains showed a very low MIC except *Cunninghamella* and *Cladosporium* where MIC range was 5–10 mM. Their mycelia became diffused compared with the control. All strains studied could not grow in higher concentrations except *Rhizomucor* sp. (KC602345) which showed the highest MIC of 15–25 mM. The white colour of the

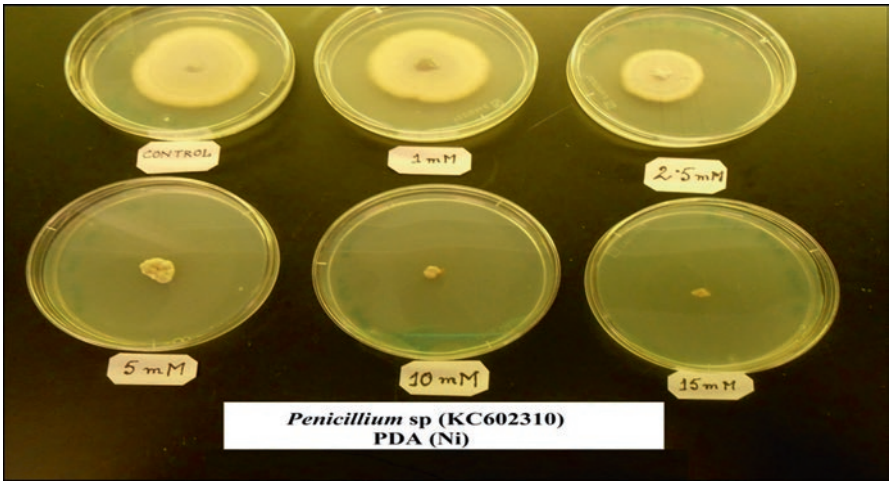

Plate 8.1 The growth of *Penicillium* sp. (KC602310*)* in nickel

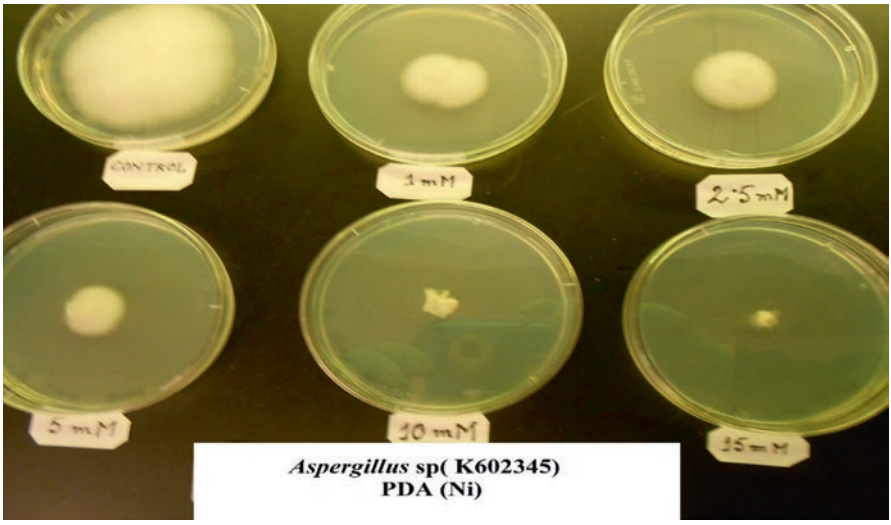

Plate 8.2 The growth of *Aspergillus* sp. (KC602345) in nickel

mycelium became blue green due to accumulation of Cu ions inside the cell wall of the tested fungi (Plate 8.3). The growth rate of fungi tested was reduced, and their conidiogenesis was also slowed down. Addition of copper sulphate to the PDA resulted in the growth of the isolated fungal strains and changed the colour and morphology of the mycelium. The mycelium of *Cladosporium* sp. (KC602374) secreted a deep brown substance (Plate 8.4), and the *Fusarium* isolate (KC602349) (Plate 8.5) secreted violet pigment due to the response to the metal stress.

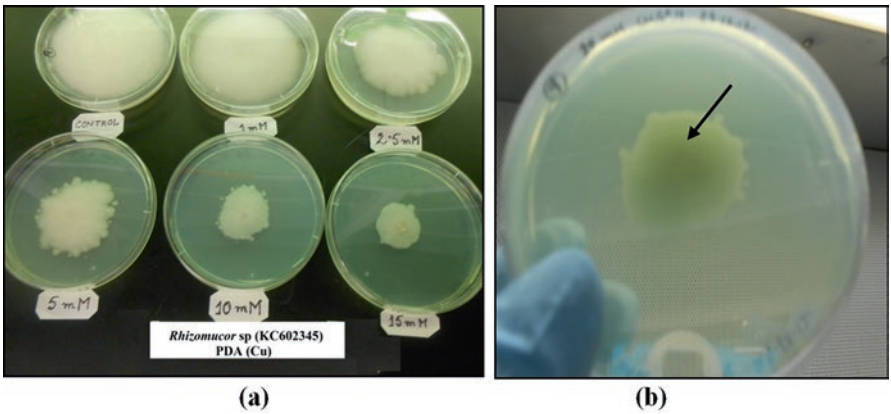

Plate 8.3 The growth of *Rhizomucor* sp. (KC602345) (**a** and **b**) in copper

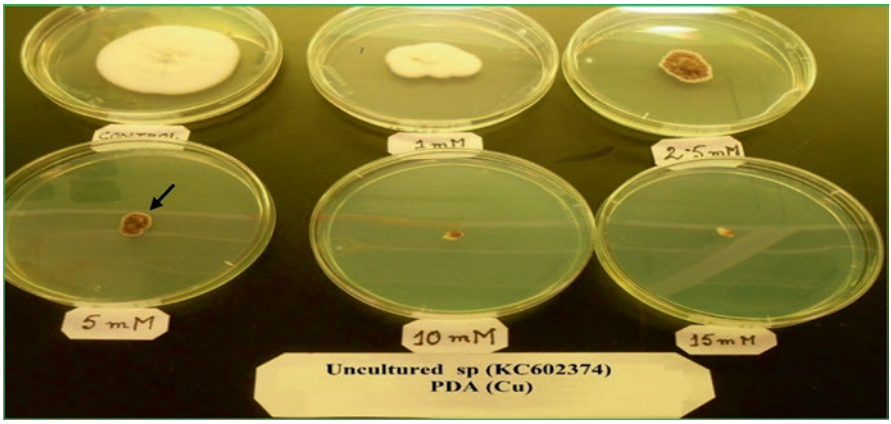

Plate 8.4 The growth of *Cladosporium* sp. (KC602374*)* in copper

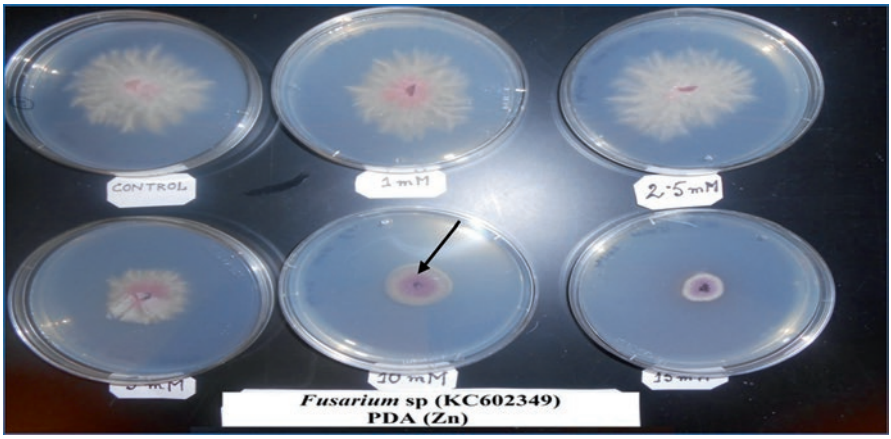

Plate 8.5 The growth of *Fusarium* sp. (KC602349) in copper

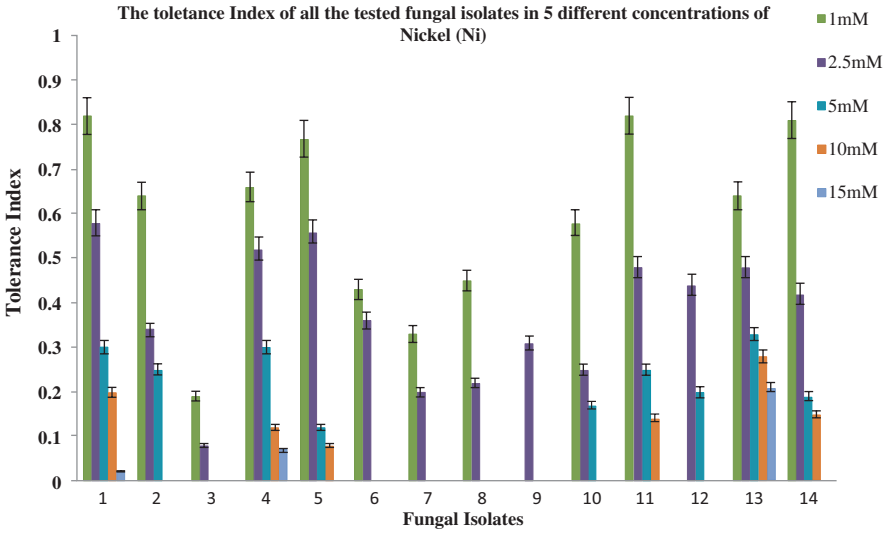

Fig. 8.1 The tolerance index of fungal strains in nickel (Ni)

In the presence of various concentrations of cadmium, the isolates *Trichoderma*, *Aspergillus* sp. and *Penicillium* sp. showed a high MIC with 15–20 mM. When the concentration of cadmium increased in the media, the absorbance of the fungal culture was found to be decreased. The most tolerant fungi which were found to grow in high concentration of the trace metals were *Penicillium* sp. (KC602310), *Trichoderma* sp. (KC602314), *Aspergillus* sp. (KC602350), *Fusarium* sp. (KC602349), *Hypocrea* sp. (KC602373), *Penicillium janthinellum* (KC602344) and *Cladosporium* (KC602374). The value of tolerance index of *Penicillium* sp. (KC602310 and KC602359), *Aspergillus* sp. (KC602350 and KC602371), *Rhizomucor* sp. (KC602345), *Fusarium* sp. (KC602349) and *Trichoderma* sp. (KC602314 and KC602331) showed a maximum value of 0.9 tested against all the metals, i.e. Ni, Cu, Zn and Cd (Figs. 8.1, 8.2, 8.3 and 8.4).

8.1.6 Identification and Characterization of Metal-Resistant Bacteria Isolates

Twenty-two bacterial isolates showed resistance to different trace metals, and the molecular characterization for these isolates was carried out (Table 8.3). The trace metals like Ni, Cu, Zn and Cd were selected in a concentration ranged from 0.1 to 4.0 mM for identification. Among the various genera, *Bacillus*, *Agromyces*, *Microbacterium*, *Arthrobacter*, *Chitinophaga*, *Rhizobium* and *Kribbella* were showing a range of 30–40% relative growth at the higher concentrations of all heavy metals tested. These bacterial isolates are capable to grow at higher concentrations of trace metals, and thus they were resistant to Ni, Cu, Zn and Cd. The species of

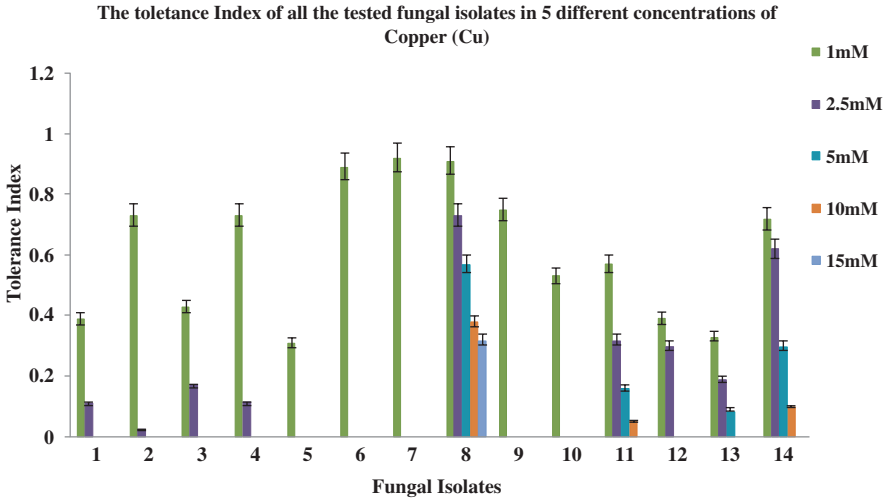

Fig. 8.2 The tolerance index of fungal strains in copper (Cu)

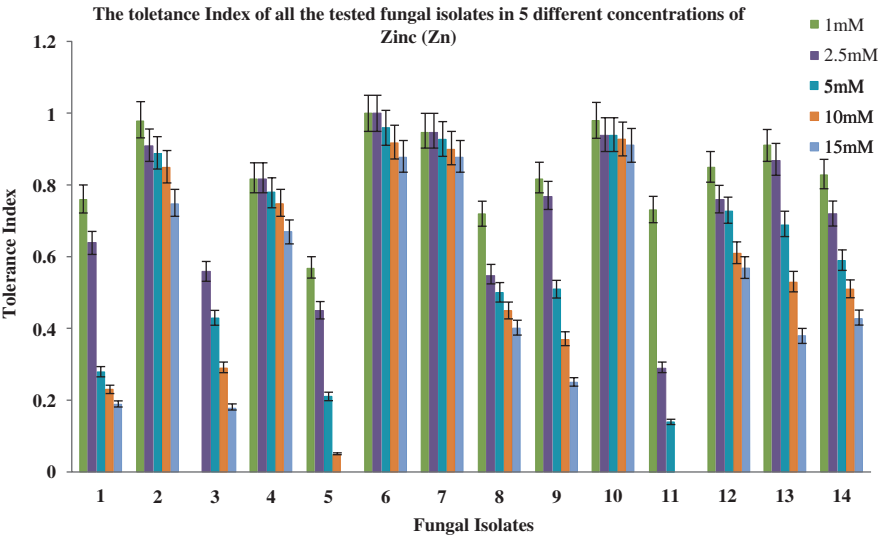

Fig. 8.3 The tolerance index of fungal strains in zinc (Zn)

Agromyces, *Bacillus*, *Chittinophaga* and *Kribbella* (isolates 1, 3, 4, 6, 10) showed significant relative growth values ranging from 40 to 70% at 2 mM and 4 mM concentrations of zinc. The species of *Rhizobium*, *Bacillus* and *Arthrobacter* showed a range of 20–60% of relative growth at 2 mM and 4 mM concentrations of nickel, copper and cadmium. The species of *Bacillus* and *Microbacterium* (isolates 18 and 19) showed a range of 20–30% of relative growth at 2 mM and 4 mM concentrations of cadmium.

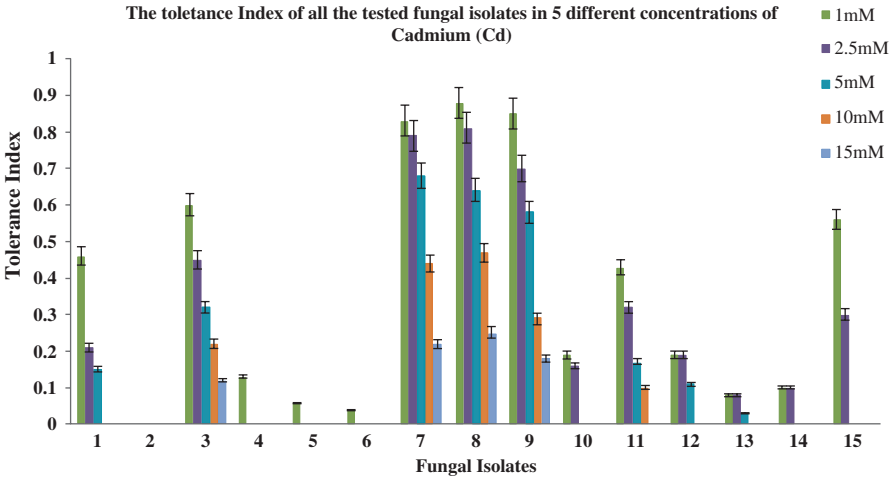

Fig. 8.4 The tolerance index of fungal strains in cadmium (Cd)

Table 8.3 List of bacterial strain tested for trace metal resistance and their accession numbers (NCBI)

Isolate no:	Name of the bacterial genus of max indent of 99%	Accession number
1.	*Agromyces* sp.	**KC602240**
2.	*Arthrobacter* sp.	**KC602245**
3.	*Bacillus cereus*	**KC602258**
4.	*Bacillus* sp.	**KC602265**
5.	*Chitinophaga* sp.	**KC602266**
6.	*Chitinophaga* sp.	**KC602269**
7.	*Rhizobium* sp.	**KC602276**
8.	*Microbacterium* sp.	**KC602277**
9.	*Bacillus* sp.	**KC602282**
10.	*Kribbella* sp.	**KC602294**
11.	*Arthrobacter* sp.	**KC602298**
12.	*Bacillus* sp.	**KC602301**
13.	*Arthrobacter oryzae*	**KC602305**
14.	*Arthrobacter nicotinovorans*	**KC602306**
15.	*Arthrobacter globiformis*	**KC602307**
16.	*Arthrobacter humicola*	**KC602308**
17.	*Arthrobacter* sp.	**KC602309**
18.	*Bacillus aryabhattai*	**KC602264**
19.	*Microbacterium* sp.	**KC602239**
20.	*Agromyces* sp.	**KC602270**
21.	*Bacillus drentensis*	**KC602283**
22.	*Bacillus* sp.	**KC602286**

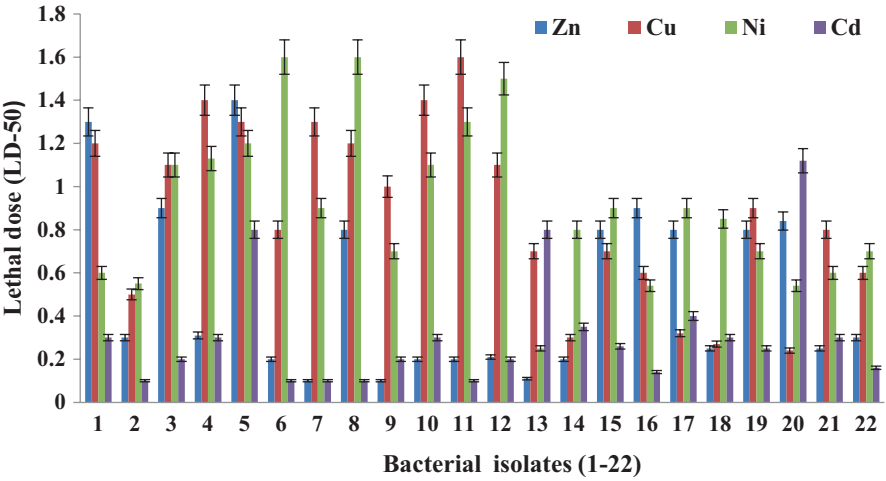

Fig. 8.5 Lethal dose (LD_{-50}) of bacteria isolates (1–22)

The isolated trace metal-resistant bacterial strains that were identified with their accession numbers are *Bacillus cereus* (KC602258), *Bacillus* sp. (KC602265), *Chitinophaga* sp. (KC602266), *Chitinophaga bacter* (KC602269), *Rhizobium* sp. (KC602276), *Microbacterium* sp. (KC602277), *Bacillus* sp. (KC602282), *Kribbella* sp. (KC602294), *Arthrobacter* sp. (KC602298), *Arthrobacter oryzae* (KC602305) and *Arthrobacter nicotinovorans* (KC602306) and were found to show positive test as tested against the 15 sugars, i.e. glucose, sucrose, xylose, maltose, rhamnose, rafffinose, cellobiose, dextrose, galactose, arabinose, lactose, sorbitol, melibiose, saccharose and trehalose. All the bacterial strains were tested for antibiotic sensitivity (Bauer 1996). Most of the isolates of *Bacillus*, *Agromyces*, *Microbacterium*, *Arthrobacter*, *Chitinophaga*, *Rhizobium*, *Brachybacterium* and *Kribbella* appeared to be inhibited by eight antibiotics and resistant to ampicillin, while *Chitinophaga* sp. (KC602269) was resistant to chloramphenicol (Adesoji et al. 2015). Among all the strains tested, the isolates (KC602240, KC602277, KC602301, KC602283 and KC602286) showed resistance to ampicillin, whereas the rest showed no inhibition. The antibiotics like streptomycin, polymyxin B, vancomycin, tetracycline, gentamicin, amikacin, ciprofloxacin and levofloxacin were found to be susceptible to all the 22 tested strains.

Lethal dose (LD-50) was estimated for all the tested bacterial isolates. The species of *Arthrobacter*, *Chitinophaga*, *Kribbella*, *Microbacterium*, *Bacillus*, *Agromyces* and *Rhizobium* showed a significant range of LD-50 values (0.2–1.8) tested against zinc, (0.3–1.6) for copper, (0.6–1.5) for nickel and (0.1–0.8) for cadmium. The highest LD-50 value of 1.8 was showed by the *Chitinophaga* sp. (KC602266), while the highest LD-50 value of 1.6 was showed by *Chitinophaga bacter* sp. (KC602269) and *Microbacterium* sp. (KC602277) against Ni. The highest LD-50 value of 0.8 was showed by the *Chitinophaga* sp. (KC602266), *Arthrobacter* (KC602303) and *Agromyces* (KC602270) against the metal Cd (Fig. 8.5).

The fungal and bacterial sequences were analysed by the Basic Local Alignment Tool (BLAST) for finding the closest homologous sequences. These sequences

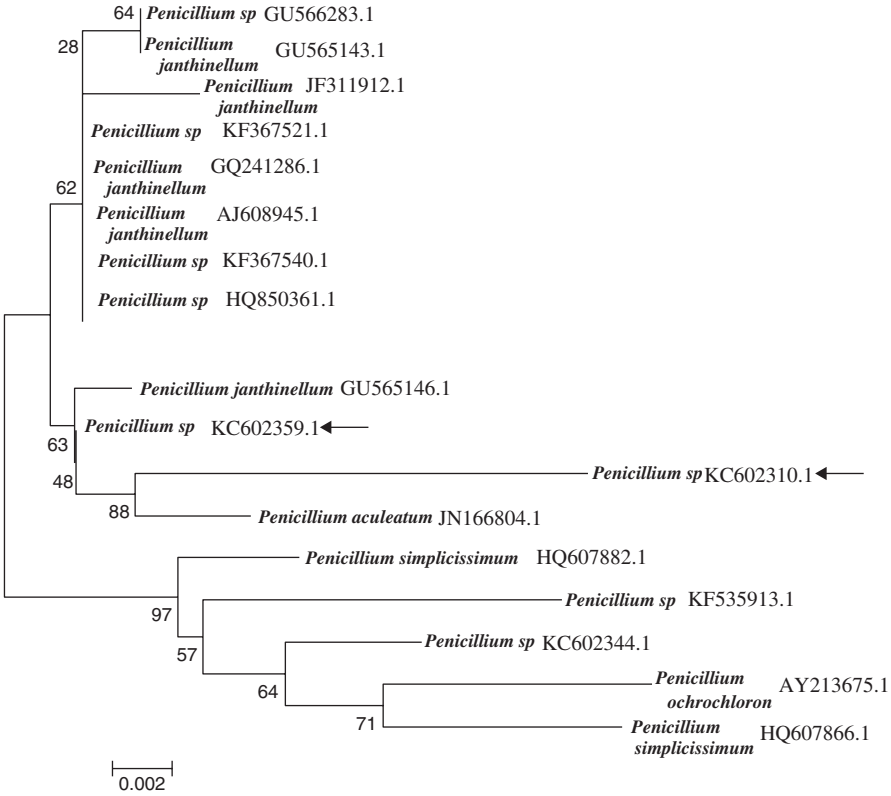

Fig. 8.6 Phylogenetic tree of *Penicillium* sp. (KC602310 and KC602359)

were saved in a fasta format (*.fasta or *.txt) and aligned with CLUSTAL X2. The output of CLUSTAL (i.e. *.aln file) was saved for the output of MEGA version 5. Phylogenetic and molecular evolutionary analysis was carried out by MEGA version 5 (Tamura et al. 2011). A distance matrix was made based on nucleotide sequence homology, and neighbour joining (NJ) consensus trees were obtained using Kimura-2 parameter substitution model (MEGA 5) (Saitou and Nie, 1987). The bootstrap values above 50% and the genetic distance scale are shown for the relationship of the isolated fungal (Figs. 8.6, 8.7, 8.8, 8.9 and 8.10) and bacterial strains (Figs. 8.11, 8.12, 8.13, 8.14, 8.15, 8.16 and 8.17) with their closely related neighbouring species.

The present experimental findings revealed the effects of trace metals on microbial diversity, i.e. fungi, bacteria and actinomycetes, in the polluted site of Hindustan Paper Corporation (HPC) paper mill. The diversity and abundance of soil microorganisms were found to be affected by naturally occurring environmental variables, including soil types, soil pH, moisture content and natural availability. Carson et al. (2010) and Stefanowicz et al. (2010) also reported that the soil microorganisms are affected positively by environmental factors. All the isolated strains of fungi,

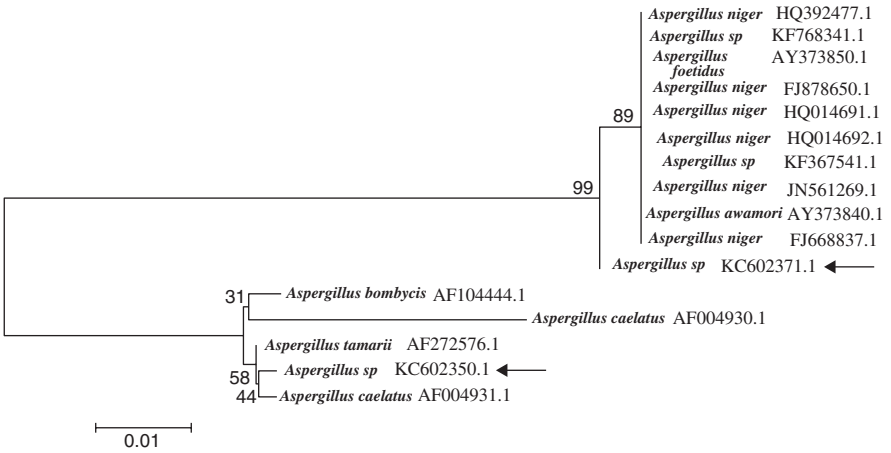

Fig. 8.7 Phylogenetic tree of *Aspergillus* sp. (KC602350 and KC602371)

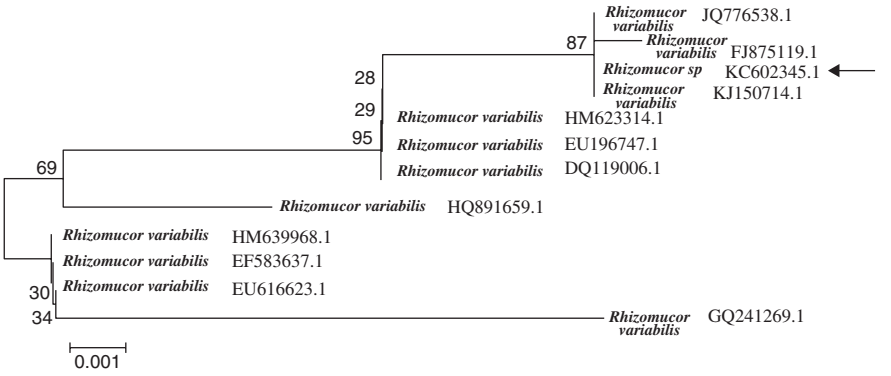

Fig. 8.8 Phylogenetic tree of of *Rhizomucor* sp. (KC602345)

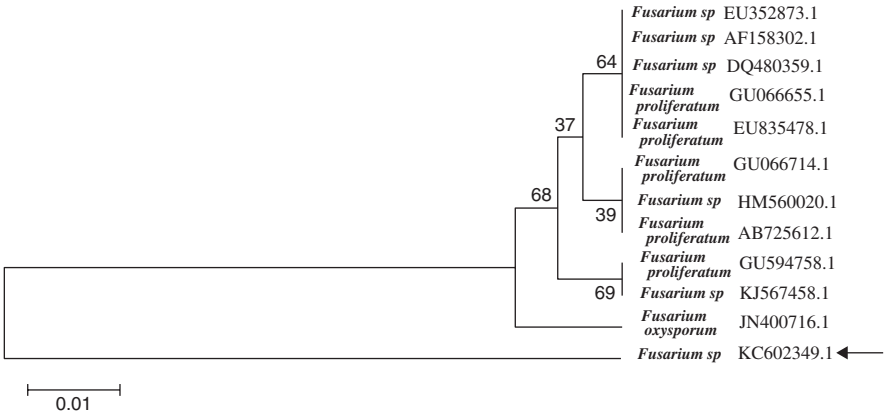

Fig. 8.9 Phylogenetic tree of *Fusarium* sp. (KC602349)

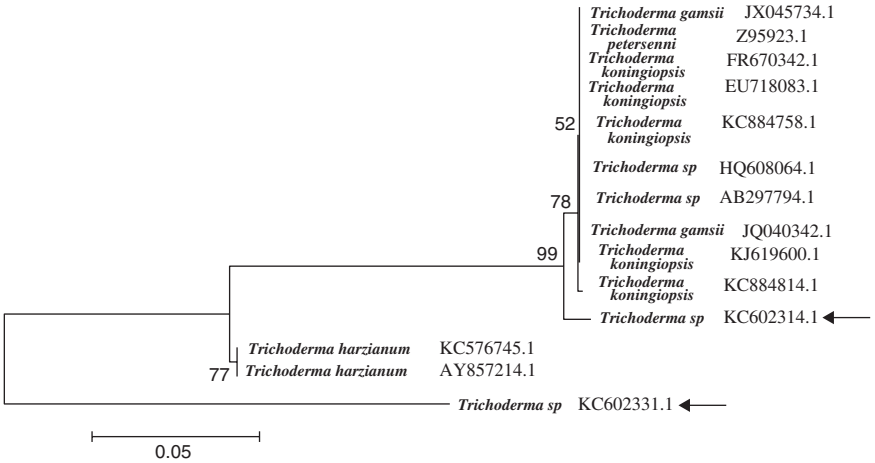

Fig. 8.10 Phylogenetic tree of *Trichoderma* sp. (KC602314 and KC602331)

bacteria and actinomycetes were found to be resistant to various trace metals at higher concentrations. Similar observations were observed by Freitas et al. (2009) and Appenroth (2010). Soil microbial populations were found to multiply even under metal-contaminated soil which in turn maintains the diversity of fungi and bacteria (Chen et al. 2014). The resistance of the selected strains to Cr^{6+}, Pb^{2+}, Zn^{2+} and Cu^{2+} was determined by the dilution method to calculate the tolerance index for all the tested fungi. *Penicillium* sp. (KC602310), *Trichoderma* sp. (KC602314), *Aspergillus* sp. (KC602350), *Fusarium* sp. (KC602349), *Hypocrea* sp. (KC602373), *Penicillium janthinellum* (KC602344) and *Cladosporium* (KC602374) were reported for their great importance in removal of trace metals from contaminated site. Some deuteromycetes have been reported by Ghorbani et al. (2007) and Zafar et al. (2007). Metals such as copper and zinc are essential to bioactivities; however, they tend to show toxicity after a certain level.

The fungal strains which were able to grow in 15–20 mM were *Trichoderma* sp., *Penicillium* sp., *Rhizomucor* sp., *Cladosporium* sp. *and Hypocrea* sp. The other tested strains like *Penicillium*, *Aspergillus* and *Cunninghamella* were also to grow in MIC of 10–15 mM (Table 8.2). Rao et al. (2005) and Sun and Shah (2007) also observed that with the increasing metal concentration of trace metals, the fungi *Aspergillus niger* and *Cunninghamella echinulata* can increase the rate of metal removal by saturation adsorbent concentrations by increasing mobilization of metal ions (Burford et al. 2003, Thippeswamy et al. 2012, 2014). *Penicillium* and *Aspergillus* showed a higher metal tolerance against nickel. Similar effects were also observed by Shivkumar et al. (2011) who discussed the high tolerance and bioaccumulation ability in *Penicillium* sp. and *Rhizopus* sp. against the various trace metal like Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+} and Pb^{2+}. The growth of all fungi tested was decreased after addition of copper in high concentration in comparison with zinc, nickel and cadmium. All strains studied could not grow in higher concentrations except *Rhizomucor* sp. (KC602345) which showed the highest MIC of 15–25 mM. Van and Christov (2002) and Tripathi et al. (2007)

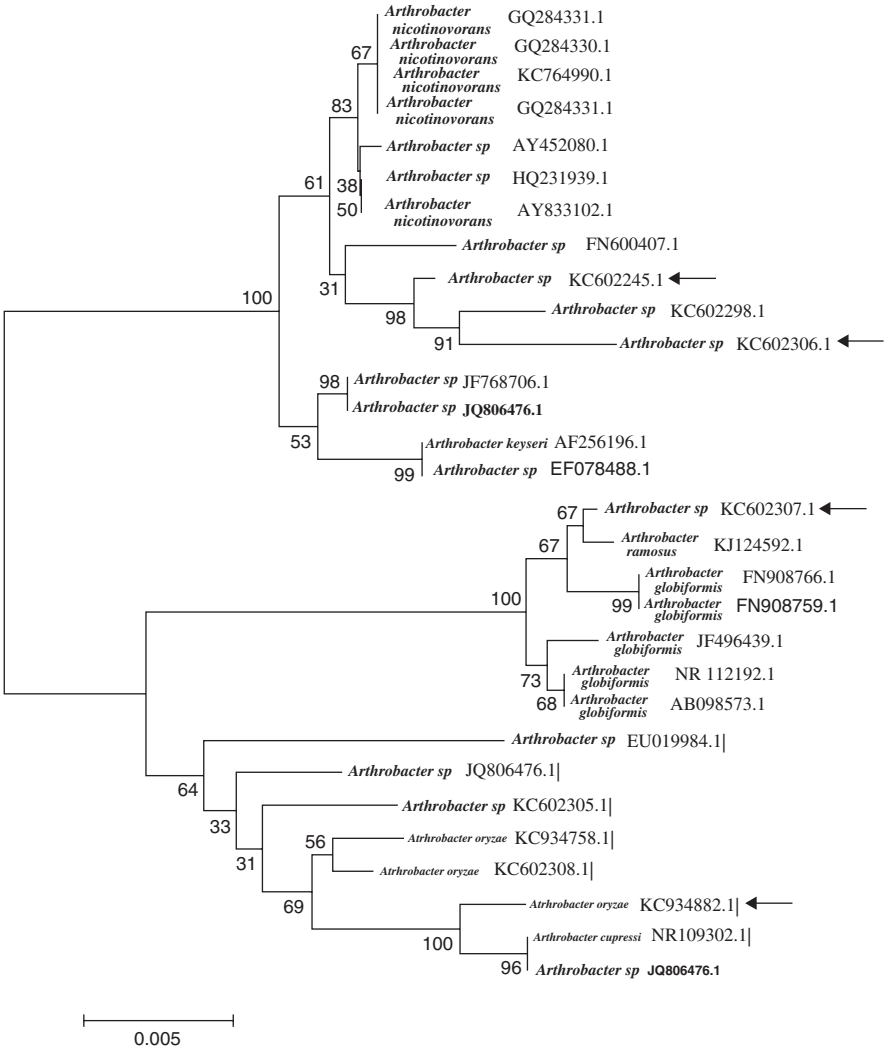

Fig. 8.11 Phylogenetic tree of *Arthrobacter* sp.

also observed that *Rhizomucor pusillus* adsorption capacity was isolated from effluent plant. Rouhollahi et al. (2014) studied the nickel biosorption capacity of *Rhizomucor pusillus* by enzymatic and alkali treatments. The white colour of the mycelium became blue green due to accumulation of Cu ions inside the cell wall of the tested fungi. Copper tolerance in fungi ascribed to diverse mechanisms also described by Cervantes and Gutierrez (1994). The most of the tested strains showed a very low MIC except *Cunninghamella* and *Cladosporium* where MIC range was 5–10 mM. The morphology of strains was highly affected by the presence of Cu. Their mycelia became diffused compared with the control. The growth rate of fungi tested was reduced, and their conidiogenesis was also slowed down. In *Cladosporium* sp. (KC602374), the

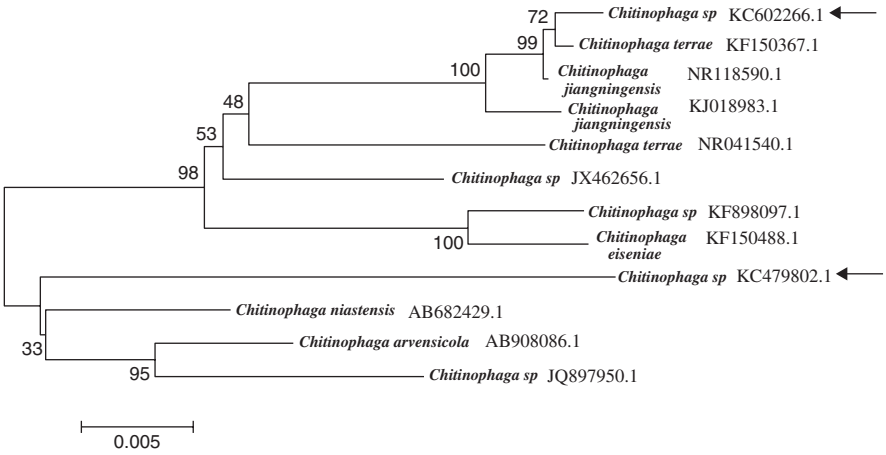

Fig. 8.12 Phylogenetic tree of *Chitinophaga* sp. (KC602266)

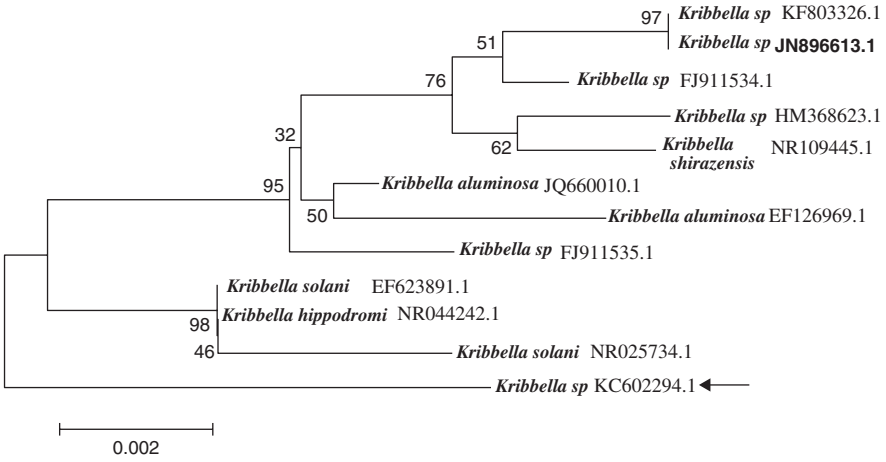

Fig. 8.13 Phylogenetic tree of *Kribbella* sp. (KC602294)

mycelia changed into deep brown colour in the high concentration of Cu. The tolerance of the tested fungi to high copper concentrations could be related to metallothioneins and other thiol compounds which may be promising detoxifying agents for copper as reported by Malik (2004) and Dusrun (2008). Similar biosorption mechanisms were also reported by Juliana et al. (2013) who discussed the biomass of *Cladosporium* as an efficient biosorbent of copper.

The fungal colour and morphology were both affected by high Zn concentrations in *Fusarium* sp. as the mycelium changed to violet pigment which is probably due to the stress imposed by the Zn. The zinc MIC was in the range 20–25 mM,

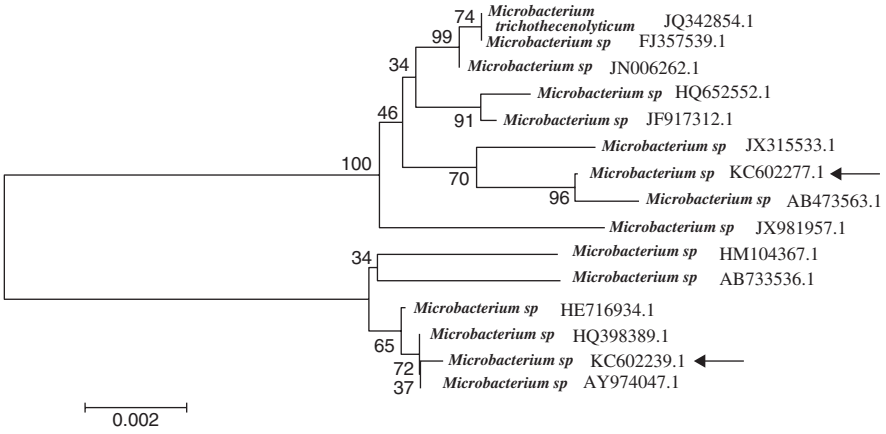

Fig. 8.14 Phylogenetic tree of *Microbacterium* sp. (KC602277 and KC602239)

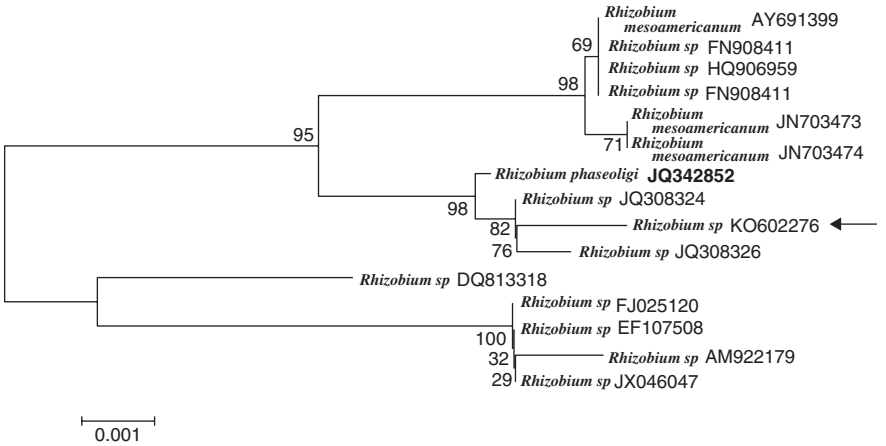

Fig. 8.15 Phylogenetic tree of *Rhizobium* sp. (KC602276)

15–20 mM, 10–15 mM and 5–10 mM for the *Fusarium* sp. Biosorption of various trace metals by *Fusarium* sp. was also reported earlier by Sen (2011), Zhang et al. (2012) and Verma et al. (2016).

The isolates *Trichoderma*, *Aspergillus niger*, *Cunninghamella* sp. and *Penicillium* sp. showed a high MIC with 15–20 mM in Cd-amended media. DeLima et al. (2011, 2013) also reported a higher potential of cadmium tolerance in the fungi *Trichoderma harzianum* and *Cunninghamella elegans.* The dominant genus of fungi identified and characterized were *Aspergillus*, *Penicillium*, *Fusarium*, *Cunninghamella*, *Trichoderma*, *Rhizomucor*, *Cladosporium* and *Hypocrea* by PCR with (forward) ITS1 and (reverse) ITS4 from the polluted soil. This may be due to the processes of valence transformation, active uptake, complexation, crystallization and biosorption of trace metals to the fungal cell walls (Jaeckel et al. 2005; Willie et al. 2007;

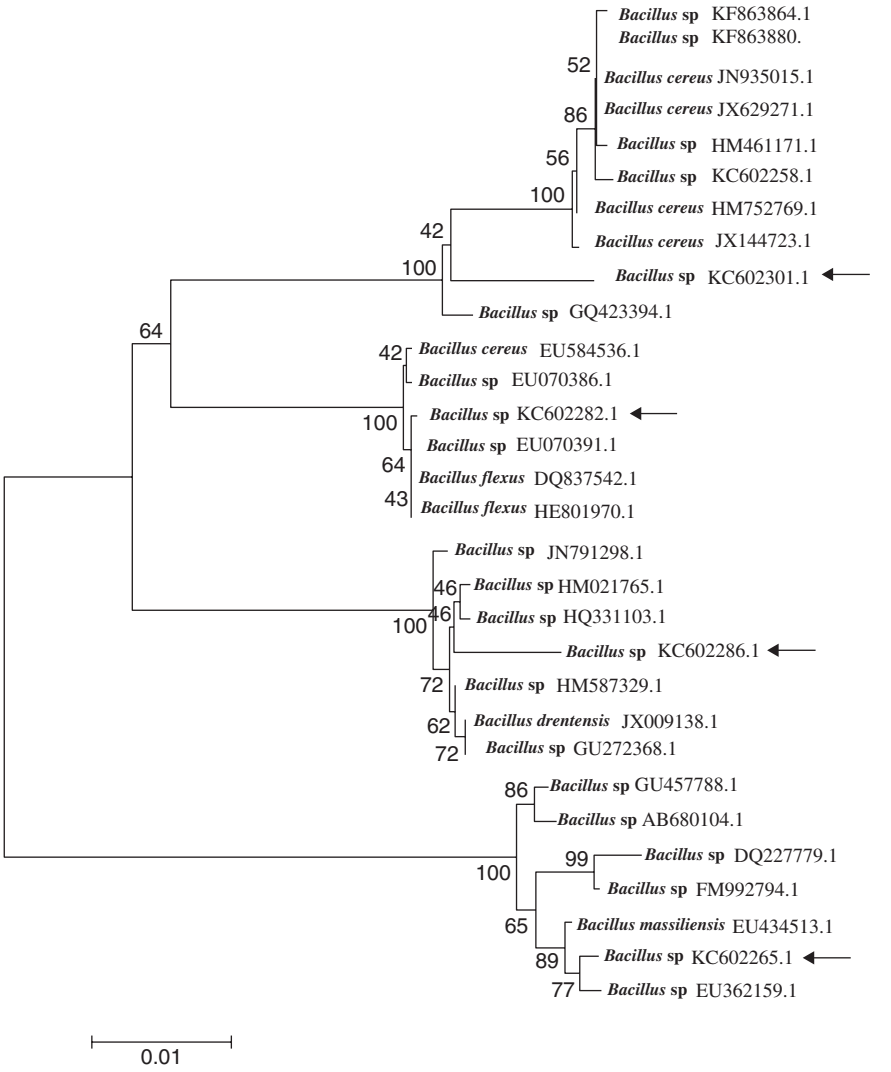

Fig. 8.16 Phylogenetic tree of *Bacillus* sp.

Palanivel et al. 2010; Anahid et al. 2011; Iram et al. 2012; Do Carmo et al. 2013; Rhodes 2013; Akhtar et al. 2013). Yazdani et al. (2009) and Malgorzata et al. (2014) found the application of *Trichoderma* sp. on various plant and found that this fungus has positive effects on increasing the biomass, soil parameters (C, N and P) and solubility of trace metals in soil, thereby enhancing phytoextraction in the plants. Copper tolerance of various *Trichoderma* sp. is also studied by Petrovic et al. (2014). Teng et al. (2015) also studied the phytoremediation in Cd-contaminated soil by *Trichoderma reesei* FS10-C strain.

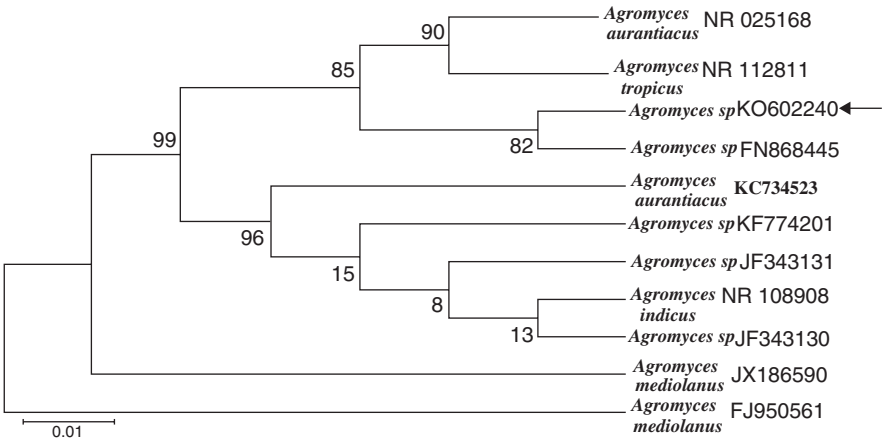

Fig. 8.17 Phylogenetic tree of *Agromyces* sp. (KC602240)

A total of 22 bacterial isolates exhibited resistance to different trace metals. These bacterial isolates were capable to grow at higher concentrations of trace metals and showing different degree of resistance to Ni, Cu, Zn and Cd. The major bacterial genera were identified as *Arthrobacter*, *Brachybacterium*, *Chitinophaga*, *Kribbella*, *Microbacterium*, *Bacillus*, *Agromyces* and *Rhizobium.* The resistance of these bacterial strains towards trace metal could be a result of the interaction between the metals and amphoteric groups such as the carboxyl and phosphoryl groups. In the present study, Gram-positive bacteria showed a major group for absorption capacity than the Gram-negative isolates as tested against different trace metals as Gram-positive bacteria have high chemisorption sites (Tunali et al. 2006, Long et al. 2012). The glycoproteins present on the outer site of Gram-positive bacterial cells have more potential binding sites than the Gram-negative bacteria having an outer layer of lipopolysaccharide (LPS), phospholipids and proteins (Gupta et al. 2012, 2016; Issazadeh et al. 2013).

The isolates of *Bacillus*, *Agromyces*, *Microbacterium*, *Arthrobacter*, *Chitinophaga*, *Rhizobium*, *Actinobacterium* and *Kribbella* showed positive activity towards urease, nitrate, H_2S production, citrate utilization, methyl red, malonate utilization, oxidase production, starch amylase and catalase activity. The Gram-positive isolates found to be positive against catalase and negative against oxidase activity were identified as *Brachybacterium*, *Agromyces*, *Arthrobacter*, *Kribbella* and *Microbacterium*. Similar observations were reported by different workers for these Gram-positive strains of same bacterial strain: *Agromyces* sp. (Chen et al. 2011; Thawai et al. 2011), *Arthrobacter* sp. (Elanvogvan et al. 2010; Rosales et al. 2012; Santa et al. 2013; Sahoo et al. 2014; Swer et al. 2016), *Chitinophaga* sp. (Gao et al., 2015), *Kribbella* sp. (Clara et al. 2008) and *Microbacterium* sp. (Mondani et al. 2012; Brown et al. 2012; Tappe et al.2013).

The strains of *Microbacterium* sp. showed a positive catalase activity and negative oxidase and H_2S production. Piccirillo et al. (2013) also observed similar

biochemical activities in *Microbacterium oxydans* to be tolerant against Zn (II) and Cd (II). The isolated strains of *Brachybacterium* sp. were observed negative for catalase and oxidase activities and positive against starch hydrolysis and found to have relative growth range of 40–80% on higher concentrations of Zn, Cu, Ni and Cd at 2 mM and 4 mM (Wang et al. 2009; Park et al. 2011). Various strains of *Arthrobacter* sp. were isolated and found to be resistant against different trace metals (Paris and Blondeau 1999; Bafana et al. 2010; Inga 2013). The genus *Kribbella* sp. was isolated and showed 40–90% of relative growth on different concentrations of trace metals. Biochemical tests showed positive against catalase activity, nitrate reduction and H_2S production and negative against oxidase production. Similar chemotaxonomic characteristics were reported earlier by Carlsohn et al. (2007) who also reported the greater accumulation capacity of *Kribbella aluminosa* against the metal Pb, Fe, Zn and Cu when grown in medium with 200 ppm of Pb, Fe and Zn and 100 ppm of Cu.

Bacillus sp. was found to be resistant with a relative growth of 30–40% on higher concentrations of all the trace metals. The isolates were found to be positive against nitrate reduction, citrate utilization, oxidase production, starch amylase, methyl red test and catalase activity. Similar biochemical activities and multi-tolerance and bioremediation of trace metals in *Bacillus* strains were observed earlier by various workers (Rathnayake et al. 2009; Elsilk et al. 2014). *Chitinophaga* sp. was negative against oxidase, catalase and starch amylase tests. The similar biochemical characteristics were observed in *Chitinophaga* sp. by Lee et al. (2009) and Wang et al. (2014). *Rhizobium* sp. is a Gram-negative, aerobic, non-endospore-forming rods, showed positive results against nitrate and catalase test and negative against oxidase, indole, VP test and urease test (Kuykendall et al. 2005; Grison et al. 2015). *Rhizobium* sp. was found to grow with 20–40 % of relative growth of copper and nickel at higher concentrations. The resistance of *Rhizobium* towards trace metals can produce huge amount of extracellular polysaccharide (EPS) and lipopolysaccharide (LPS), which can attach most of the metals extracellularly, acting a first-defence barrier against trace metal stress (Mohamed et al. 2012; Mandal and Bhattacharyya 2012). Our results were supported by Reeve et al. (2002), Hemida et al. (2012) and Hao et al. (2014) who also observed that *Rhizobium* played a very important role of legume-rhizobia symbiosis in aiding phytoremediation of polluted site contaminated with trace metals (Mergeay et al. 2003; Piotrowska-Seget et al. 2005; Zhang et al. 2011; Aafi et al. 2012; Rajkumar et al. 2012; Yang et al. 2012; Adel et al. 2014).

8.2 Conclusion

The present study focused on the effect of trace metal on the diversity of microorganisms (fungi, bacteria, actinomycetes) in the Hindustan Paper Corporation (HPC), Cachar. The most tolerant fungi grown in high concentration of the trace metals were identified as *Penicillium* sp. (KC602310), *Trichoderma* sp. (KC602314),

Aspergillus sp. (KC602350), *Fusarium* sp. (KC602349), *Hypocrea* sp. (KC602373), *Penicillium janthinellum* (KC602344) and *Cladosporium* sp. (KC602374).

The most tolerant bacteria grown in high concentration of the trace metals were identified as *Bacillus cereus* (KC602258), *Bacillus* sp. (KC602265), *Chitinophaga* sp. (KC602266), *Chitinophaga bacter* (KC602269), *Rhizobium* sp. (KC602276), *Microbacterium* sp. (KC602277), *Bacillus* sp. (KC602282), *Kribbella* sp. (KC602294), *Arthrobacter* sp. (KC602298), *Arthrobacter oryzae* (KC602305) and *Arthrobacter nicotinovorans* (KC602306).

From the results of the present investigation, it can be concluded that biotic and abiotic stress in trace metal-polluted soil of the paper mill greatly influenced the enzyme activity, composition and function of the indigenous microorganisms (fungi, bacteria, actinomycetes). The current study clearly showed that the native dominant resistant indigenous fungal, bacterial isolates can be used as a biosensor to assess the trace metal toxicity in the polluted environment. Thus, future research may be proposed for further advances in microbial genetics by studying the mechanism of metal-microbe-plant interactions and their potential use as metal-resistant microbial inoculants in microbial-assisted phytoremediation.

8.2.1 Future Prospective

With the increased demand of paper, the treatment of effluents emerges as most pressing problem in environmental protection. The current study clearly showed that the native dominant resistant indigenous fungal, bacterial isolates can be used as a biosensor to assess the heavy metal toxicity in the polluted environment contaminated with paper mill effluents. A further understanding of metal-microbe-plant interactions will increase our knowledge to design microbial-assisted phytoremediation in the trace metal-contaminated sites.

Acknowledgements The authors are grateful to the Commonwealth Scholarship grant, Canada, for carrying out the present work in the Department of Biological Science, University De Montreal, Montreal, and Genome Quebec Innovation Centre, Canada, for getting the accession numbers of all isolated trace metal-tolerant microbial strains.

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