International Journal of Bioassays
ISSN: 2278-778X
CODEN: IJBNHY
OPEN ACCESS

Original Research Article

ISOLATION AND CHARACTERIZATION OF HEAVY METAL RESISTANT *CELLULOSIMICROBIUM* SP. FROM PAPER MILL POLLUTED SOIL

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Received for publication: December 11, 2014; **Accepted:** December 21, 2014

Abstract: A Gram-positive, rod-shaped, yellow-pigmented bacterium was isolated from the polluted soil of paper mill contaminated with various heavy metals. The strain was tested for its resistance to different heavy metals (Ni, Cu, Zn and Cd) by the growth in nutrient broth tubes containing various concentrations of (0.1, 0.5, 2.0, 4.0 mM). The relative growths (%) at 2mM concentration were observed as Ni (23.52%)>Zn (20.58%)>Cu (19.20%) > Cd (15.81 %). The heavy metal resistant in the isolated strain was found to be Ni>Zn>Cu>Cd at higher concentrations. The strain showed positive activity towards urease, nitrate, citrate utilization, methyl red, Malonate utilization, starch amylase and showed negative activity against ONPG, H_2S production, phynylalanine, Lysine utilization, Voges Proskauer's (VP) test, catalase and oxidase activity. The strain was found susceptible to various antibiotics Vancomycin, Streptomycin, Rifamycin Amikacin and Ciprofloxacin. In silico study was conducted to understand the major evolutionary relationship among the different strains of *Cellulosimicrobium* species of nucleotide sequence of 16s ribosomal RNA with the isolated strain (KC602297) of *Cellulosimicrobium* sp. obtained from Gene Bank.

Key words: Heavy metal, Gram (+) positive bacteria, In silico, 16SrRNA, Gene Bank.

INTRODUCTION

Microorganisms play a very important role in cleaning up the metal contaminated soil by variety of mechanisms. i.e., accumulation and complexation of the metal ions inside the cell, efflux of metal ions outside the cell (Gadd, 1992; Spain and Alm, 2003). Heavy metals are released in to soils from industrial operations such as mining, manufacturing of alkaline storage batteries, combustion of fossil fuel (Nies, 2004; Kumar *et al.,* 2011). Paper pulp industries are the sixth largest effluent generating industries of the world (Ugurlu *et al.,* 2007). The paper mill effluents are classified as carcinogenic and mutagenic compounds and are found to contain approximately 700 organic and inorganic compounds (Karrash *et al.,* 2006). Low concentrations of heavy metals like Ni, Cu, Zn, Cd, Pb, Co and Cd are essential for many cellular and metabolic processes of bacteria but at higher concentrations these become cytotoxic to the bacterial populations thus affecting the growth, morphology, metabolic activities (Wuertz and Mergeay, 1997; Abou-Shanab *et al.,* 2007; Karelova *et al.,* 2011). Bacteria are found to develop various types of resistance mechanism by extracellular detoxification, extracellular sequestration, and intracellular sequestration. These resistant mechanisms are encoded by chromosomal genes which are located on bacterial plasmid spontaneous mutation and gene transfer (Osborn *et al.,* 1997; Nandi, 2004). Bacterial siderophores play an important role in heavy metal tolerance for protecting bacteria against heavy metal toxicity (Schalk *et al.,* 2011). Gram positive bacteria showed higher expression levels *czc*D and *ncc*A genes and found to tolerant to various heavy metals (Rathnayake *et al.,* 2009; Abdelatey *et al.,* 2011).

The genus *Cellulosimicrobium* is characterized by Gram-positive, yellow-pigmented, non-motile and rod-shaped and mainly comprises three species *C. cellulans* (Schumann *et al.,* 2001), *C. funkei* (Brown *et al.,* 2006) and *C. terreum* (Yoon *et al.,* 2007). *Cellulosimicrobium* sp. was found to resistant towards hexavalent chromium in contaminated soil through rhizosphere colonization (Chatterjee *et al.,* 2009). Song and Wei (2010) observed that the *Cellulosimicrobium* sp. are able to produce the hydrolytic enzymes xylanase and cellulose from carbon source and these enzymes can be further used in paper industry, laundries and agriculture. Sharma *et al.,* (2014) also isolated *Cellulosimicrobium* sp. strain MM from Arsenic rich microbial mats of a Himalayan hot spring.

MATERIALS AND METHODS

Description of the study area

The present study was conducted at Panchgram, Hailakandi, Assam, adjoining the Cachar Paper Mill an unit of Hidustan Paper Corporation,(HPC) limited a Government of India undertaking. Geographically the site is situated at longitude of 24°41'29.9″N and latitude at 92°45'25.9″E with an altitude of about 36 m above MSL.

Isolation and chracterization of bacterial isolate

For bacterial isolation, soil sample (1g) was suspended in 10ml of distilled water and serial dilutions

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were spread on starch-caesin agar. (1% soluble starch, 0.03% casein, 0.2% KNO_3, 0.2% NaCl, 0.2% KH_2PO_4, 0.002% $CaCO_3$, 0.005% $MgSO_4.7H_2O$, 0.001% $FeSO_4$, $7H_2O$, 1.8% agar and pH 7.2). Inoculated plates were incubated at30°C for 7 days. The isolate was preserved in a 20% (v/v) glycerol suspension at -30°C.

Cultural and morphological features of the bacteria isolate

The cultural and morphological features falls under the phenotypic characterization, which were studied by adopting standard methods (Rath and Subramanyam, 1998). Different colony features such as configuration, elevation, margin, texture, consistency etc. were noted down by using a hand lens. Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene was carried out as described by Greg, (2010).

Morphologiacal and Biochemical characterization of bacterial isolate

Gram staining, a differential staining technique was done by Gram Stains kit (Himedia K001). For biochemical characterization, the isolate was tested for ONPG, Lysine utilization, ornithine utilization, urease activity, Phenylalanine deamination, nitrate reduction, H_2S production, citrate utilization, Voges-Proskauer test, Methyl red test, Indole production, Malonate utilization, Oxidase production, Starch amylase test, Catalase activity etc. and fermentation of fifteen different sugars. Identification of the bacterial isolate was carried out according to Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 1994). Fermentative degradation of various carbohydrate source (Glucose, sucrose, xylose, maltose, rhamnose, raffinose, cellubiose, dextrose, gallactose, arabinose, lactose, sorbitol, melibiose, saccarose and trehalose) an indicator (phenol red) and pH-7.3 was carried out to observe the acid production.

Determination of antibiotic resistance

The isolate was tested for antibiotic sensitivity according to Kirby-Bauer disc diffusion method (Bauer *et al.,* 1966) to 12 antibiotics. Discs containing the following antibiotics were used: Penicillin G (10units), Polymyxin B (300units), Streptomycin (10mcg), Vancomycin (30mcg), Tetracycline (30mcg), Gentamycin (120mcg), Rifamycin (5mcg), Amikacin (30mcg), Ampicillin (10mcg), Chloramphenicol (30mcg), Ciprofloxacin (10mcg) and Levofloxacin (10mcg).

Evaluation of metal resistant bacteria

The bacterial isolate was tested for their resistance to different heavy metals by their growth in nutrient broth tubes containing various concentrations of heavy metals (0.1, 0.5, 2.0 and 4.0mM). The metals selected for the present investigation included Ni, Cu, Zn and Cd. These tubes were inoculated with freshly grown culture of the isolate and incubated at 30± 2.0°C for 48h. The bacterial growth was determined by measuring the optical density using spectrophotometer at 540nm. Relative growth of the isolate was expressed as the percentage of those obtained in untreated control.

Identification of metal resistant bacteria

The isolation and purification of chromosomal DNA as well as the amplification and sequencing of partial 16S rRNA gene of potential metal resistant bacterial isolate was carried out. The nucleotide sequence of bacterial isolate thus obtained was compared for sequence similarity level with the reference species of bacteria contained in genomic database using the "NCBI BLAST" (Altschul *et al.,* 1990).

Genotypic characterization of isolated bacterial strain

Phylogenetic and molecular evolutionary analyses of the isolate was conducted using software MEGA version 5.0 (Tamura *et al.,* 2011) package. The 16S rRNA gene sequences of the potential metal resistant bacterial isolate was aligned using the CLUSTALW program (Thompson *et al.,* 1994) against corresponding nucleotide sequences retrieved from Genbank database. A phylogenetic tree was constructed using the neighbor-joining (NJ) method (Tamura *et al.,* 2004) and by NCBI on-line service which showed the relationships with their closely related neighboring species. The sequence of the isolated strain in this study was deposited and accession number (KC602297) was obtained from Gene Bank.

RESULTS AND DISCUSSION

The isolated strain of *Cellulosimicrobium* sp. was showed resistant to higher concentrations of Ni, Cu, Zn and Cd. This strain was capable to grow at higher concentrations of heavy metals. The relative growths (%) of *Cellulosimicrobium* sp. (KC602297) at 2mM concentration was found to be Ni (23.52%) >Zn (20.58%) > Cu (19.20%) > Cd (15.81%). At higher concentrations of heavy metals (4mM), the relative growths (%) of *Cellulosimicrobium* sp. (KC602297) was found to be Ni (10.22%) > Zn (8.51%) > Cu (8.25 %) > Cd (5.27%). (Table 1). Thus, the heavy metal resistant of the strain was found to be Ni>Zn>Cu>Cd.

Table 1: Relative growth (%) of bacterial isolate in nutrient broth containing different heavy metals.

Metal tested	Incuabation period	Heavy metal concentration (mM)	Relative growth (%) of isolated bacterial strain (KC602297)
Ni	48	0.1	97.28
		0.5	65.79
		2.0	23.52
		4.0	10.22
Cu	48	0.1	78.25
		0.5	45.87
		2.0	19.20
		4.0	8.25
Zn	48	0.1	75.15
		0.5	49.89
		2.0	20.58
		4.0	8.51
Cd	48	0.1	89.26
		0.5	32.67
		2.0	15.81
		4.0	5.27

Each value represents average of duplicates

Table 2: Morphological, biochemical characteristics and production of acids from carbohydrates by the isolated bacterial strain

SL No.	Morphological tests	KC602297	Carbohydrates	Production of acids
1.	Gram staining	+		
2.	Colony morphology	Rods	Glucose	+
3.	Motility	-		
Biochemical test				
4.	ONPG	-	Sucrose	+
5.	Lysine utilization	-	Xylose	+
6.	Ornithine utilization	+	Maltose	+
7.	Urease	+	Rhamnose	+
8.	Phenylalanine deamination	-	Raffinose	+
9.	Nitrate reduction	+	Cellubiose	+
10.	H_2S production	-	Dextrose	+
11.	Citrate Utilization	+	Gallactose	+
12.	Voges Proskauer's	-	Arabinose	-
13.	Methyl red	+	Lactose	+
14.	Indole	-	Sorbitol	+
15.	Malonate utilization	+	Melibiose	-
16.	Oxidase production	-	Saccarose	+
17.	Starch amylase	+	Trehalose	+
18.	Catalase	-		

(+) = positive; (-) = negative.

The isolated strain of *Cellulosimicrobium* sp. showed positive activity towards urease, nitrate, citrate utilization, methyl red, Malonate utilization, starch amylase and showed negative activity against ONPG, H_2S production, phynylalanine, Lysine utilization and Voges Proskauer's (VP) test, catalase and oxidase activity (Table 2). The similar biochemical characteristics observed in *Cellulosimicrobium* sp. was observed by various workers (Yoon *et al.,* 2007; Gabani *et al.,* 2012). The isolated strain showed positive for the production of acids against various sugars tested, i.e., Glucose, Sucrose, Xylose, Maltose, Rhamnose, Rafffinose, Cellubiose, Dextrose, Gallactose, Lactose, Sorbitol, Saccarose, Trehalose and negative against Melibiose, Arabinose, (Table 2). The strain (KC602297) was appeared to be most susceptible being inhibited by majority of antibiotics, intermediate to antibiotics Penicillin and Chloramphenicol and showed no inhibition against antibiotic Ampicillin (Table 3).

The isolated bacterial strain of *Cellulosimicrobium* sp. was found to be resistant towards different heavy metals. This may be due to the Gram positive bacteria, has very high potentials as chemosorption sites (Tunali *et al.,* 2006; Gupta *et al.,* 2012). The resistance towards heavy metals may be a result of the interaction between the metals and amphoteric groups such as the carboxyl and phosphoryl groups that occur within the constituent polymers of isolated gram positive bacterial cell walls which act as an open ion exchange resin. The isolated strain of *Cellulosimicrobium* sp. shows positive against urease, nitrate, citrate utilization, methyl red, Malonate utilization, starch amylase. Our results are in conformity with the results of Yoon *et al.,* (2007) and Sharma *et al.,* (2014) who observed the similar chemotaxonomic characteristics of some novel species of *Cellulosimicrobium* isolated from soil. Our results also supports the findings by Chatterjee *et al.,* (2009) who also reported that the inoculation of Cr-resistant bacteria *Cellulosimicrobium cellulans* decreased Cr uptake by 56% in green chilli grown in Cr (VI) contaminated soil. Similar metal resistant mechanism was also reported in *Cellulosimicrobium* sp. by Sharma *et al.,* (2014) which was able to grow at Arsenic rich microbial mats of a Himalayan hot spring. This may be due to the metal ions transport across the cell membrane, biosorption to cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reaction of the isolated strain (Nasrazadani *et al.,* 2011; Adel *et al.,* 2014). Multiple heavy metal resistance determinants for Ni, Cu, Hg, Zn, Cd, Co, Cr and Pb have been reported from different gram positive bacterial plasmids by various workers (Berg *et al.,* 2005; Dantas *et al.,* 2008; Li *et al.,* 2009; Dong *et al.,* 2011; Gao *et al.,* 2012; Hemala *et al.,* 2014). 16srRNA gene phylogenetic analysis suggested that the isolated *Cellulosimicrobium* strain from polluted soil of Paper Mill is closely related to *Cellulosimicrobium funkei* (Figure 1). According to the present study, it can be interpreted that the isolated strain of *Cellulosimicrobium* sp. plays a very important role in the successful survival and growth of plants in contaminated soils of paper mill by alleviating the metal toxicity and supplying the plant with nutrients.

Table 3: Antibiotic sensitivity profile by the isolated bacterial isolate

S. No.	Antibiotics disc (conc.)	KC602297
1.	Penicillin G (10 units)	12I
2.	Polymyxin B (300 units)	13(S)
3.	Streptomycin (10mcg)	20(S)
4.	Vancomycin (30 mcg)	25(S)
5.	Tetracycline (30mcg)	30(S)
6.	Gentamycine (10 mcg)	29(S)
7.	Rifamycin (30 mcg)	21(S)
8.	Amikacin (30mcg)	22(S)
9.	Ampicillin (10 mcg)	NI
10.	Chloramphenicol (30 mcg)	22(I)
11.	Ciprofloxacin(10mcg)	24(S)
12.	Levofloxacin(10mcg)	23(S)

NI = No Inhibition; Diameter of disc; R = Resistant; I = Intermediate; S = Susceptible.

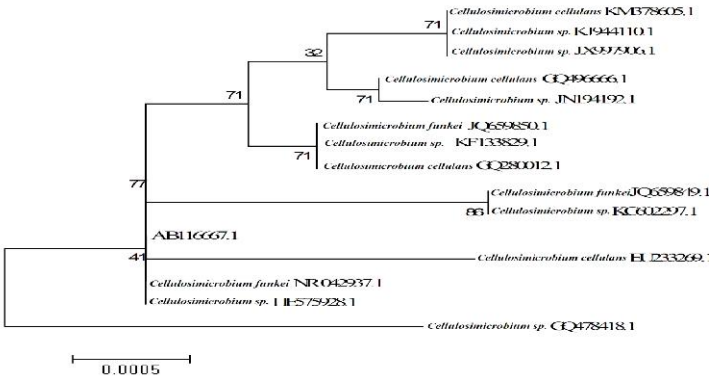

Figure 1: Neighbour-joining tree of 16SrRNA gene sequences from isolate of *Cellulosimicrobium* sp. (KC602297) with 16SrRNA of other bacteria obtained from gene bank. The Kimura two-parameter substitution model was used and the nodes are supported by 1,000 bootstrap replications. Bootstrap values above 50% and the genetic distance scale are shown (Mega 5.0 version).

CONCLUSION

From the present study, it can be concluded that the isolated strain of *Cellulosimicrobium* sp. is able to tolerate higher concentrations of heavy metals. On the basis of phenotypic and genotypic features, the isolated strain of *Cellulosimicrobium* sp. (KC602297) can be termed as heavy metal tolerant strain that might be utilized as potential bioremediation agent of paper mill polluted soil contaminated with various heavy metals.

ACKNOWLEDGEMENTS

The authors are grateful to the common wealth scholarship grant, Canada for carrying out the present work in the Department of Biological Science, University De Montreal, Montreal, Canada for providing the laboratory facilities.

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Cite this article as:
Dhritiman Chanda, Sharma GD, Jha DK, Hijri M and F Al-Otaibi. ISOLATION AND CHARACTERIZATION OF HEAVY METAL RESISTANT CELLULOSIMICROBIUM SP. FROM PAPER MILL POLLUTED SOIL. International Journal of Bioassays, 2015, 4 (01): 3648-3653.

Source of support: Common Wealth Scholarship Grant, Canada
Conflict of interest: None Declared