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Detection and Characterization of a Multi-drug and Multi-metal Resistant Enterobacterium *Pantoea* sp. from Tannery Wastewater after Secondary Treatment Process

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Abstract

In this study, an enterobacterium was isolated from treated tannery wastewater and characterized as gram-negative, rod shaped, motile, and lactose fermenting bacterium. Further, based on the 16S rRNA gene sequence analysis, the bacterium was identified as *Pantoea* sp. with accession number KJ576899. The antibiotic and heavy metal resistant property of isolated bacterium was investigated by the disk diffusion method on Muller-Hinton and nutrient agar medium amended with the increasing concentrations of various toxic metal ions, respectively. Results showed that the isolated bacterium was sensitive for amikacin, ampicillin, cefepime, cetriaxone, chloramphenicol, levofloxacin, meropenem, nalidixic acid, piperacillin and tobramycin, resistant for aztreonam, carbenicillin, cefotaxime, ceftazidime, ciprofloxacin, cotrimoxazole, imepenam, moxifloxacin, streptomycin and tetracycline, but intermediate for amoxicillin and gentamicin. In addition, the bacterium also showed the Minimum Inhibitory Concentration (MIC) values of 250, 500, 160, 190, 600, 700, 500, 380, 600 and 350 μg ml^{-1} for Cu, Cr, Cd, Co, Zn, Fe, Ni, Pb, Mo and As, respectively with a plasmid DNA of 33.5 kb. This multi-drug and multi-metal resistant bacterium can be used as a potential agent for the bioremediation of metal contaminated sites.

1. Introduction

Tannery industries are one of the major sources of soil and water pollution mainly causing chromium pollution in environment. In India, there are more than 2,500 tanneries and most of them (nearly 80%) are engaged in chrome tanning process (Chandra *et al*., 2011). During the tanning process, chromium salt is used to convert hides into leather and the wastewater generated contains huge amount of organic matter, phenolics, tannins and toxic metals mainly chromium (Chandra *et al*., 2011). This wastewater containing a variety of toxic pollutants when discharged into the environment causes serious soil and water pollution along with serious health hazards to humans, animals as well as plants (Ramteke *et al*., 2010; Flores *et al*., 2012).

The organic pollutants present in tannery wastewater do not degrade much during the treatment process carried out at Effluent Treatment Plant (ETP) in industries as various authors have reported the presence of numerous recalcitrant organic pollutants and pathogenic microbes in wastewater discharged from industries even after the secondary treatment process (El-Lathy *et al*., 2009; Ramteke *et al*., 2010; Chandra *et al*., 2011). The organic pollutants remained in tannery wastewater after the secondary treatment process provide chance to a variety of pathogenic and non-pathogenic microbes to flourish and contaminate the aquatic environments, whereas toxic metals induce genotoxic and mutagenic changes in bacterial communities making them resistant against a wide spectrum of antibiotics and toxic metals (Filali *et al*. 2000; Malik and Jaiswal, 2000; Viti *et al*., 2003).

In literature, sewage contamination is reported as a major source of pathogenic bacteria in water resources, but few authors also suggested that besides sewage contamination, the wastewaters discharged from different industries such as distillery, pulp paper mills and tannery industries etc. also act as a good source of nutrients and support the growth of pathogenic microbes in receiving water bodies (El-Lathy *et al*., 2009; Ramteke *et al*., 2010; Chandra *et al*., 2011). However, the detail information about the pathogenic microbes that remained in tannery wastewater even after the secondary treatment process is not available so far.

Hence, this study was aimed to isolate and characterize the multi-drug and multi-metal resistant enterobacteria from tannery wastewater after the secondary treatment process, so that the treatment processes can be improved /modified accordingly for the adequate treatment of tannery wastewater for its safe disposal into the environment.

2. Materials and Methods

2.1. Collection of tannery wastewater after secondary treatment process

The treated tannery wastewater sample from aeration lagoon-II was collected in sterilized conical flasks (capacity 2 lit) from the Common Effluent Treatment Plant (CETP) of tannery industries located at Unnao (U.P.), India. The collected effluent sample was brought to laboratory, maintained at 4ºC and used for the isolation of pathogenic enterobacteria.

2.2. Isolation and characterization of enterobacteria

2.2.1. Isolation of enterobacteria

For the isolation of enterobacterium, the collected tannery wastewater sample was serially diluted to the desired concentration and spreaded on Violet Red Bile Glucose Agar (VRBGA) plates containing yeast extract: 3.0; pancreatic digest of gelatin: 7.0; bile salt no. 03; glucose: 10.0; sodium chloride: 5.0; neutral red: 0.03; crystal violet: 0.002; and agar: 15.0 g l^{-1} followed by incubation at 35ºC for 48 h. The colonies appeared on VRBGA medium were picked up and investigated for purity as well as morphological characteristics under the microscopic observation.

2.2.2. Characterization and identification of isolated enterobacterium

2.2.2.1. Biochemical characterization

The isolated enterobacterium was characterized morphologically and biochemically as per the standard procedures of Cowan and Steel's Manual for the identification of medical bacteria (Barrow and Feltham, 1993).

2.2.3. Genomic DNA preparation and PCR amplification of 16S rRNA gene

The total genomic DNA was extracted from the overnight grown culture of isolated enterobacterium following the alkaline lysis method (Kapley *et al*., 2001). About 2 µl DNA was used to amplify the 16S rRNA gene using the forward and reverse primers 27F (5'-AGA GTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), respectively (Deletoile *et al*., 2009). The reaction mixture contained 2 µl of template DNA, 1X PCR buffer, 200 µM of each dNTP, 3.0 mM $MgCl_2$, 25 pmol of primer, and 2.5 units of Amplitaq DNA polymerase (Merk, Biosciences, India) in a final volume of 50 µl. The thermocycling reactions were carried out by using Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA) as: 35 cycles of denaturation at 94°C for 1 min, followed by annealing at 45°C for 1 min and extension at 72°C for 2 min. The PCR products were gel purified using gel extraction kit (Merk, Biosciences, India), and sequenced by using 27F primer. The partial sequences obtained were subjected to BLAST analysis using the online option available at www.ncbi.nlm.nih.gov/BLAST (Altschul *et al*., 1997) to get the closest neighbor of the isolated enterobacterium.

2.2.4. Construction of phylogenetic tree and nucleotide sequence accession number

The phylogenetic tree was constructed by the neighbor-joining method using MEGA version 4.0 software (Tamura *et al*., 2007) and five enterobacteria spp., and five *Pantoea* sp., having closest relationship with the isolated enterobacterium. The 16S rRNA gene sequences of the closest enterobacteria and *Pantoea* sp. were downloaded from the GenBank and used in tree construction. Further, the nucleotide sequences of the isolated enterobacterium were also deposited in the GenBank public database under the accession number KJ576899 for public domain.

2.3. Antibiotic resistance property of bacterial isolate

The antibiotic susceptibility testing of isolated enterobacterium was done by the disk diffusion method on Muller-Hinton agar medium against the following antibiotics: Amikacin (30 mcg), amoxicillin (8 mcg), ampicillin (10 mcg), aztreonam (30 mcg), carbenicillin (100 mcg), cefepime (30 mcg), cefotaxime (30 mcg), ceftazidime (30 mcg), cetriaxone (30 mcg), chloramphenicol (30 mcg), ciprofloxacin (5 mcg), cotrimoxazole (25 mcg), gentamicin (10 mcg), imepenam (10 mcg), levofloxacin (5 mcg), meropenem (10 mcg), moxifloxacin (5 mcg), nalidixic acid (30 mcg), piperacillin (100 mcg), streptomycin (10 mcg), tetracycline (30 mcg), and tobramycin (10 mcg) (HiMedia, Mumbai) (Karbasizaed *et al*., 2003; Jain *et al*., 2009). The plates were swabbed with a faintly opalescent culture, and then the antibiotic disks were applied and incubated at 32°C for 24 h (Karbasizaed *et al*., 2003; Jain *et al*., 2009). The inhibition zone was

measured after 24 h of incubation period and the isolated enterobacterium was classified as resistant, intermediate, or sensitive based on zone size as per the standard antibiotic disc sensitivity testing method (DIFCO, 1984).

2.4. Determination of minimum inhibitory concentration (MIC) of various toxic metals for the isolated enterobacterium

The minimum inhibitory concentration (MIC) of Cu, Cr, Cd, Co, Zn, Fe, Ni, Pb, Mo and As for the isolated enterobacterium was determined in nutrient broth amended with the increasing concentrations (0-1000 µg ml^{-1}) of Cu, Cr, Cd, Co, Zn, Fe, Ni, Pb, Mo and As. The stock solutions of the analytical grade salts of $CuSO_4$, $K_2Cr_2O_7$, $CdCl_2$, $CoCl_2$, $ZnSO_4$, $FeCl_3$, $NiCl_2$, $PbNO_3$, Na_2MoO_4 and $NaAsO_2$ for Cu^{2+}, Cr^{6+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Fe^{3+}, Ni^{2+}, Pb^{3+}, Mo^{6+} and As^{3+} ions, respectively were prepared in Millipore water and autoclaved. The experiment was performed in 50 ml tubes containing 20 ml of autoclaved nutrient broth supplemented with the increasing concentrations (0-1000 µg ml^{-1}) of Cu, Cr, Cd, Co, Zn, Fe, Ni, Pb, Mo and As and 100 µl of bacterial culture followed by incubation for 48 h at 35ºC and 125 rpm in shaking incubator (Coral *et al*., 2005; Zolgharnein *et al*., 2007; Jain *et al*., 2009). The bacterial growth was monitored by measuring the optical density at 600 nm (Dynamica, Australia). The tubes containing 20 ml of autoclaved nutrient broth supplemented with the increasing concentrations (0-1000 µg ml^{-1}) of above metal ions without bacterial culture served as control. The MIC of different toxic metals for the isolated enterobacterium was designated as the minimum concentration of metal ions at which no growth of bacterium was observed.

2.5. Plasmid profiling and curing experiment

For plasmid profiling, the plasmid DNA from isolated enterobacterium was isolated following the alkaline lyses method (Marcelo *et al*., 1998; Chin *et al*., 2005). The isolated plasmid DNA was precipitated with chilled isopropanol followed by two washes with 70% ethanol and finally dissolved in TE. The molecular weight of plasmid DNA was determined by agarose gel electrophoresis on 0.6% agarose and using supermix DNA ladder as marker standard (Merck, Biosciences, India). Further, the curing of plasmid was performed by exposing the overnight grown bacterial cultures to elevated temperatures at 37°C and 1% sodium dodecyl sulphate (SDS) (Marcelo *et al*., 1998; Chin *et al*., 2005). These cultures were again subjected to plasmid isolation and visualization in 0.6% agarose gels with 0.5 mg ml^{-1} of ethidium bromide solution.

3. Results and Discussion

3.1. Characteristics of the isolated pathogenic enterobacterium

The microscopic observation of isolated enterobacterium has revealed that it was gram-negative, rod-shaped and motile. On VRBGA plates, the colonies of bacterium were pink colored, whereas on MacConkey agar plates, it appeared as smooth, punctuate, convex, glistening, and lactose fermenting bacterium. In addition, based on the biochemical reactions, this bacterium was found to be a catalase positive, oxidase negative with many other biochemical reactions as shown in Table 1.

Table 1: Biochemical characteristics of the isolated enterobacterium

Characteristics	**Isolated enterobacterium**
Gram reaction	–Ve
Shape	Rod
Motility	+
Catalase	+
Oxidase	–
Yellow pigment production	+
Casein hydrolysis	
Starch hydrolysis	
Gelatin hydrolysis	+
Gas production from D-glucose	–
Indole production	–
Methyl-red test	–
V-P Test	+
Citrate utilization	+
H_2S production	–
Urease activity	–
Nitrate reduction	+
Arginine dihydrolase	–
Lysine decarboxylase	–
Ornithine decarboxylase	–
Phenylalanine deaminase	+
Acid Production	
D-glucose	+
D-fructose	+
D-xylose	+
D-galactose	+
D-cellobiose	–
D-mannose	+
L-rhamnose	+
L-arabinose	+

Characteristics	Isolated enterobacterium
Inositol	+
Melibiose	–
Lactose	–
Maltose	+
Sucrose	+
Trehalose	+
D-mannitol	+
D-sorbitol	–
Starch	–
D-glucose	+

+=Positive, – =Negative; V-P=Voges-Proskauer

Further, the PCR amplified 1403 bp long 16S rRNA gene (Fig. 1) sequences of isolated enterobacterium have shown the closest relatedness (100%) with that of *Pantoea* sp. XJ3 (GU140078) (Fig. 2). The phylogenetic tree (Fig. 2) constructed by using five different *Pantoea* sp. and five different enterobacteria spp., also showed the closest relationship with the isolated enterobacterium. Hence, based on the 16S rRNA gene sequence similarity with *Pantoea* sp. XJ3 (GU140078), the isolated enterobacterium was identified as *Pantoea* sp. with accession number KJ576899.

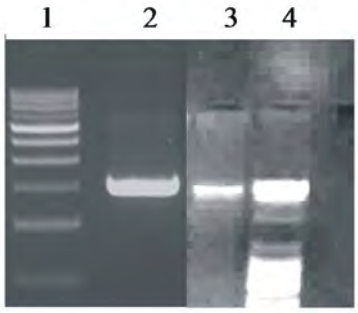

Fig. 1: PCR amplification of 16S rRNA gene and plasmid profiling of isolated enterobacterium (RMB1); Lane 1: 500 bp Marker Ladder; Lane 2: PCR amplified 16S rRNA gene; Lane 3: Plasmid DNA; Lane 4: Supermix DNA Marker Ladder

3.2. Antibiotic susceptibility of isolated enterobacterium

The results revealed that the isolated enterobacterium was sensitive for amikacin, ampicillin, cefepime, cetriaxone, chloramphenicol, levofloxacin, meropenem, nalidixic acid, piperacillin and tobramycin, resistant for aztreonam, carbenicillin, cefotaxime, ceftazidime, ciprofloxacin, cotrimoxazole, imepenam, moxifloxacin, streptomycin and tetracycline, and intermediate for amoxicillin and gentamicin as shown in Table 2. This indicates that tannery wastewater is organically enriched medium and supports the fast growth and spreading of multi-drug and multi-metal resistant microbes in aquatic environments.

3.3. Minimum inhibitory concentration (MIC) of tested metal ions for the isolated enterobacterium

The isolated enterobacterium also showed a wide range of MIC values for the tested metals ranging from 250, 500, 160, 190, 600, 700, 500, 380, 600 and 350 µg ml^{-1} for Cu, Cr, Cd, Co, Zn, Fe, Ni, Pb, Mo and As, respectively (Table 3). The resistance for toxic metals in bacteria probably reflects the degree of environmental contamination with toxic metals and may be directly related to the exposure of bacterial cells with the toxic metals (Dhakephalkar and Chopade, 1994). However, the unpolluted environments may also harbour metal

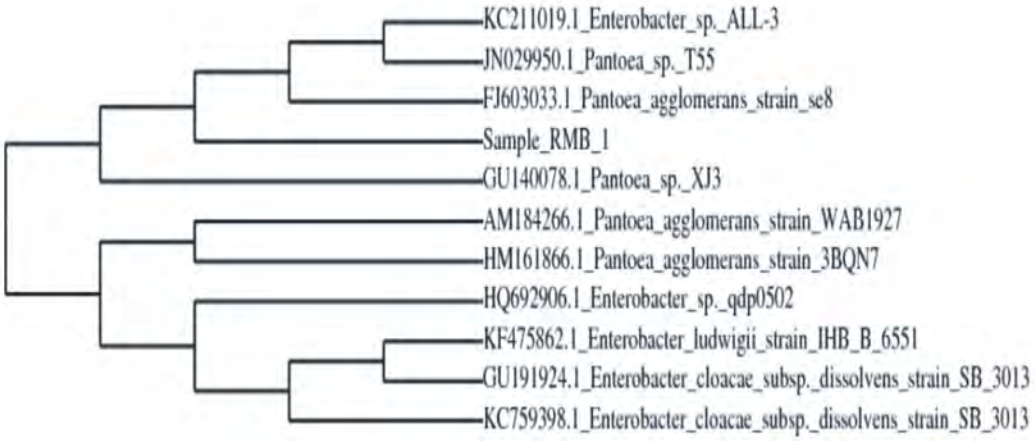

Fig. 2: Neighbor-joining tree showing the phylogenetic position of isolated enterobacterium (RMB1) with closest related species based on 16S rRNA gene sequences. The GenBank accession number for each bacterium used in the analysis is shown in parenthesis before the species name

Table 2: Antibiotic susceptibility pattern of isolated enterobacterium (RMB1)

Antibiotics used in study	Susceptibility pattern of isolated enterobacterium (RMB1)
Amikacin	S
Amoxicillin	I
Ampicillin	S
Aztreonam	R
Carbenicillin	R
Cefepime	S
Cefotaxime	R
Ceftazidime	R
Cetriaxone	S
Chloramphenicol	S
Ciprofloxacin	R
Cotrimoxazole	R
Gentamicin	I
Imepenam	R
Levofloxacin	S
Meropenem	S
Moxifloxacin	R
Nalidixic acid	S
Piperacillin	S
Streptomycin	R
Tetracycline	R
Tobramycin	S

S=Sensitive, R=Resistant, I=Intermediate

resistant organisms or organisms that readily adapt to high concentrations of toxic metals. Malik and Jaiswal (2000) have suggested that the incidence of a high metal resistant population resulted from increasing environmental pollution. They also reported that plasmid-bearing strains are more in polluted sites than unpolluted sites. However, the bacterial resistance to heavy metals is an important consideration when bacteria are to be introduced into soils for enhancing the bioremediation of metal contaminated sites. Although some heavy metals are required in low concentrations for normal metabolic activities. But, at elevated levels, these metals act as carcinogenic, mutagenic or teratogenic agents (Feuerpfeil *et al*., 1999).

Table 3: Minimum inhibitory concentration (MIC) of different metal ions for the isolated enterobacterium (RMB1)

Metal ions used in study	MIC (μg ml^{-1}) of metal ions for the isolated RMB1
Cu	250
Cr	500
Cd	160
Co	190
Zn	600
Fe	700
Ni	500
Pb	380
Mo	600
As	350

3.4. Plasmid profile of isolated pathogenic enterobacterium

The isolated enterobacterium has shown a good degree of resistance for the tested antibiotics and metal ions. It is now well established that in majority of cases the multi-drug and multi-metal resistance property of microbes is generally reside on the extra chromosomal DNA molecule, which is called as plasmid (Collard *et al*., 1994; Marcelo *et al*., 1998; Coral *et al*., 2005). The isolated enterobacterium was also found to have a plasmid DNA of size 33.5 kb (Fig. 1). Our findings are strongly supported by the previous findings of various authors, who have also studied the plasmid mediated antibiotic and heavy metal resistance property of bacterial strains isolated from the metal contaminated environments (Karbasizaed *et al*., 2003; Coral *et al*., 2005; Zolgharnein *et al*., 2007). Further, it was also observed that during plasmid curing experiment, the isolated bacterium showed neither antibiotic nor metal resistant property. It clearly indicates that the antibiotic and heavy metal resistant properties observed in isolated bacterium were coupled with plasmid DNA.

4. Conclusions

Based on the results obtained in this study, it was concluded that the contamination of soil and water resources with industrial wastewaters containing toxic metals and a variety of recalcitrant organic pollutants (ROPs) are the major sources of multi-drug and multi-metal resistant pathogenic microbes in environment. The results also confirmed that the isolated enterobacterium was *Pantoea* sp. with accession number KJ576899 and have multi-drug and multi-metal resistant property against different tested antibiotics and heavy metals. This bacterium also showed the presence of a plasmid DNA of size 33.5 kb. This plasmid may carry the genes conferring various phenotypic characters to bacterium, including toxin, hormone production and virulence factors contributing to the pathogenecity and host specificity, resistance to antibiotics and heavy metals and survival in adverse conditions, catabolism of amino and organic acids, carbohydrates and inorganic ions, colonization and dissemination in wide range of ecological niches.

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