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Isolation and identification of chromium reducing bacteria from tannery effluent

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ABSTRACT

Industrial wastes are capable of polluting the soil and groundwater. The tanning industry is considered as a potential environmental polluting agent through out the world. Chromium (Cr), a toxic heavy metal, is a major constituent of tannery waste, and its accumulation in soil and water causes serious environmental issues of increasing public concern in India particularly in Tamil Nadu. The present study deals with isolation and identification of chromium reducing bacteria isolated from tannery effluent collected from Crompet, Chennai, Tamil Nadu (latitude 12°57′51.8″N and longitude 80°07′58.1″E). Two chromium reducing bacteria were isolated; and the identification of the isolates were done by 16 s rRNA sequencing followed by BLAST results as *Klebsiella pneumoniae* and *Mangrovibacter yixingensis*. Furthermore the presence of chromium reductase gene in the isolated bacteria was confirmed by PCR techniques. Bioremediation using bacteria may provide an alternative or aid conventional methods of metal removal/metal recovery. The newly identified chromium reducing bacteria would be useful for the detoxification of heavy metal contaminated tannery effluent and metal recovery in mining process.

1. Introduction

Chromium pollution in the environment is due to the discharge of contaminants from industries (McGrath and Smith, 1990). As chromium and its compounds have multifarious industrial uses such as, production of refractory steel, electroplating, cleaning agents, catalytic manufacture, drilling mud and also in the production of chromic acid. They are extensively employed in leather industry especially in Tamil Nadu, India (Nriagu and Nieboer, 1988). These anthropogenic activities have led to the wide spread contamination of chromium in the environment and have increased its bioavailability and bio mobility (Kotas and Stasicka, 2000).

Chromium is one of the class one hazardous waste (Ribeiro et al., 2012) which can exist in different oxidation states. Although it can exist in nine valence states, from $^{-}2$ to $^{+}6$ (Smith et al., 2002) only the trivalent chromium Cr (III) and the hexavalent chromium Cr (VI) are ecologically important, since these are the most stable oxidation states in the natural environment. Trivalent and hexavalent chromium differ largely in physico-chemical properties as well as biological reactivity. The Cr (VI) species is extremely water-soluble and mobile in the environment, while Cr (III) species is much less water-soluble and comparatively immobile (Viamajala et al., 2004). This is one of the reason for recognising, Cr (VI) as highly toxic, carcinogenic, mutagenic and teratogenic for mammals including humans (Flores and Perez, 1999) whereas Cr (III) is considered as an essential trace element necessary for glucose, lipid and amino-acid metabolism as well as a popular dietary supplement (Viamajala et al., 2004).

Chromium (VI) is found to be one of the most common hazardous waste; hence it needs treatment before disposal. Generally it is subjected to biological, chemical and physical treatments. Bioremediation using soil bacteria is regarded as the most suitable technique since bacterial populations can show resistance to as much as 500 mg L^{-1} of Cr (VI). Chromium remediation through microorganisms is accepted as the best and economically affordable technology at present to clean-up Cr contamination (Yadav et al., 2005).

Staphylococcus aureus, Pediococcus pentosaceus and some species of *Klebsiella* are found to show resistance towards Cr. It was noted

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that some chromium resistant bacteria possessed the property of chromium reduction with the help of reductase enzymes. The chromate reductases found in chromium resistant bacteria are known to catalyse the reduction of hexavalent chromium Cr (VI) to trivalent chromium Cr (III) (Marsh and McInerney, 2001; Wani et al., 2018). This property may be due to the presence of chromate reductase gene or induced protein (Zahoor and Rehman, 2009). Therefore this study was performed in order to isolate and identify chromium reducing bacteria from tannery effluent.

2. Materials and methods

2.1. Collection of chromium contaminated tannery effluent

Chromium contaminated tannery effluent (semi solid soil) was collected from Chrompet (latitude 12°57′51.8″N and longitude 80°07′58.1″E), Tamil Nadu, India. About 50 ml of tannery effluent was collected in a sterile falcon tube from the effluent tank. The collected samples were immediately brought into the laboratory with ice box and stored at 4 °C until further use.

2.2. Isolation and enumeration of microorganisms

The microbes were isolated from tannery effluent (semi solid) by pour plating method. About 1 g of effluent was mixed with 9 ml of sterile physiological saline (0.85%) and serially diluted. From the different dilution, 1 ml of sample was pour plated with brain heart infusion agar and incubated for 24 h at 30 °C to identify the number of colonies forming unit per gram of effluent.

2.3. Pure culture of isolates

Out of various colonies obtained from serial dilution by pour plate method, unique colonies were selected based on the colony morphology. The selected colonies were separately cultured by streak plate method in Nutrient Agar plate. The colony shape, elevation, opacity, pigmentation and texture of each isolates were observed.

2.4. Chromium tolerance

The chromium tolerant bacteria were identified by selected grown bacterial colonies on Mueller Hinton Agar medium amendment with different concentration of 20, 40, 60, 80 and 100 mg/l chromium along with control (without chromium).

2.5. Isolation of genomic DNA from bacteria

Genomic DNA was isolated from bacteria by Phenol–Chloroform method. Using 0.5 ml of 100 mM Tris-HCl at pH 8.0, 0.1 ml of 10 mM EDTA at pH 8.0, 1.4 ml of 1.4 M NaCl, 0.5 ml of 1 % SDS and 0.01 ml of 0.2 % mercaptoethanol.

2.6. Detection of chromium reductase gene

Detection of chromium reductase gene in the genomic DNA of the bacteria was done by PCR amplification method using Chromium Reductase Gene (ChR) specific Primers; Forward 5′TCACGCCGGAATATAACTAC-3′ and Reverse 5′CGTACCCTGATCAA TCACTT-3′ (Patra et al., 2010). The amplified PCR products were electrophoresed along with Ready- to -Use DNA marker on 1.5% gel and photographed using GELSTAN gel documentation system.

2.7. Molecular identification of chromium reducing bacteria

For the molecular identification of chromium reducing bacteria, 16S rRNA gene of the bacteria was amplified by PCR and sequenced. Universal forward primer 27F – 5′-AGAGTTT GATCCTGGCTCAG-3′ and reverse primer 1492R - 5′-GGTTACCTTGT TACGACT-3′ for amplification of 16S rRNA gene from isolated genomic DNA. It was analysed for amplification using 1.2% agarose gel followed by electrophoresis at 70 V for 1 h using 1X TAE buffer and photographed using GELSTAN gel documentation system.

The amplified 16S rRNA gene fragment was purified and sequenced using Sanker sequencing employing the same primer used for PCR amplification. 16S rRNA gene sequences were exported into "Basic Local Alignment Search Tool" (BLAST) available from the website of National Centre for Biotechnology Information (NCBI) to identify matches with existing characterized reference sequences. The output of BLAST searches were sorted based on maximum identity with other genus or species names in GenBank records. The DNA sequence was subjected to DNA editing and Noise editing using a Bioinformatics software Bio Edit version 7.5. The phylogenetic trees were constructed based on the 16S rRNA sequence of the chromium tolerance bacteria with reference sequence in GenBank using MEGA software version 7.0 (Kumar et al., 2016). The edited DNA was submitted to the GenBank NCBI and accession numbers were obtained.

3. Results and discussion

3.1. Isolation and enumeration of microorganisms

The bacteria were isolated from tannery effluent and were studied for their colony morphology characteristics (Table 1). Colonies forming unit per ml were calculated as 1.68×10^4 CFU/ml from 10^{-1} dilution and were used to enumerate the microbial diversity in the collected tannery effluent. Similarly, Shukla et al. (2007) stated that bacteria colony count was 1.5×10^3 when it was isolated from tannery. The tannery effluent primarily consisted of chromium and protein (Saranraj et al., 2013), which makes it an ideal medium for many bacterial species to grow, in order to give a considerably high CFU/ml of 1.68×10^4 for 10^{-1} dilution in the present study. Eleven unique colonies were isolated by streaking on to a separate nutrient agar plate. Similarly, bacteria was identified from tannery effluents and their tolerance limit was more than 40 mg/L concentration of the chromium (VI) (Farag and Zaki, 2010).

3.2. Chromium tolerance

Chromium tolerance of isolated bacteria was assessed by grown on Mueller Hinton medium containing varied concentration of chromium. Eleven selected bacteria reacted in different manner to different concentrations of chromium as illustrated in Table 2 and Figs. 1a–1d. Out of the eleven bacterial strains, two bacterial strains exhibited chromium tolerance to most of the chromium concentrations. Strain 1.5 had a positive growth up to 80 mg/l chromium concentration and Strain 2.4 had a positive growth up to 100 mg/l chromium concentration. Bacterial populations has been noted to show resistance to as much as 500 mg/l Cr (VI) (Zahoor and Rehman, 2009; Ilias et al., 2011) which supports the positive growth of strain 1.5 up to 80 mg/l concentration and strain 2.4, a positive growth up to 100 mg/l. Similarly, Wani et al. (2015) reported that bacterial strains were resistance to Cr (VI). Among the bacterial strains, *Klebsiella* sp., showed highest tolerance to Chromium (VI) in agar plate dilution method. Also see Pradhan et al. (2017), Various biological techniques used to reduced toxic

Table 1
Colony morphology of isolated bacteria.

Strains	Shape	Elevation	Opacity	Pigmentation	Texture
1.1	Circular	Flat	Opaque	Yellowish	Viscid
1.2	Circular	Convex	Opaque	Whitish	Smooth
1.3	Irregular	Convex	Opaque	Whitish	Rough
1.4	Rhizoidal	Raise	Opaque	Whitish	Rough
1.5	Circular	Pulvinate	Transparent	Colourless	Smooth
1.6	Irregular	Convex	Translucent	Yellowish	Viscid
1.7	Circular	Convex	Opaque	Whitish	Smooth
2.1	Circular	Pulvinate	Translucent	Whitish	Smooth
2.2	Circular	Raise	Translucent	Whitish	Rough
2.3	Irregular	Raise	Translucent	Whitish	Rough
2.4	Irregular	Convex	Opaque	Whitish	Smooth

Table 2
Identification of chromium tolerance bacterial strains.

Strain. No:	Concentration of chromium					
	Control	20 mg/l	40 mg/l	60 mg/l	80 mg/l	100 mg/l
1.1	+	+	–	–	–	–
1.2	+	+	–	+	–	–
1.3	+	–	–	–	–	–
1.4	+	+	–	–	–	–
1.5	+	+	+	+	+	+
1.6	+	–	–	–	–	–
1.7	+	+	–	–	–	–
2.1	+	–	–	–	–	–
2.2	+	+	+	+	–	–
2.3	+	+	–	–	–	–
2.4	+	+	+	+	+	–

+ indicates- present.
– Indicates – absent.

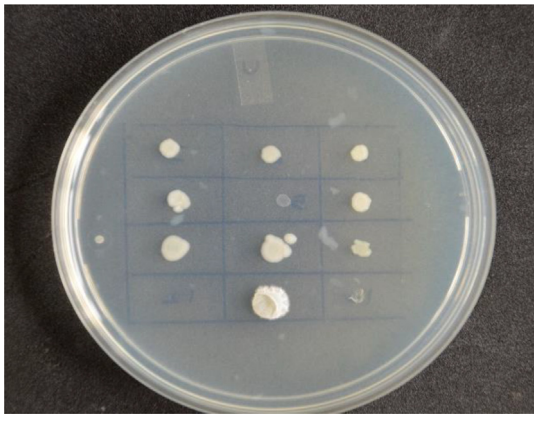

Fig. 1a. Control.

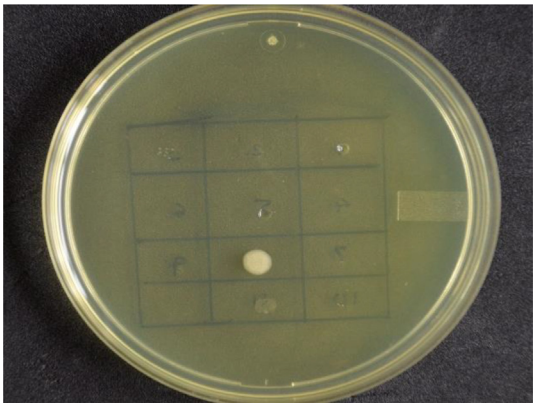

Fig. 1c. 80 mg/l.

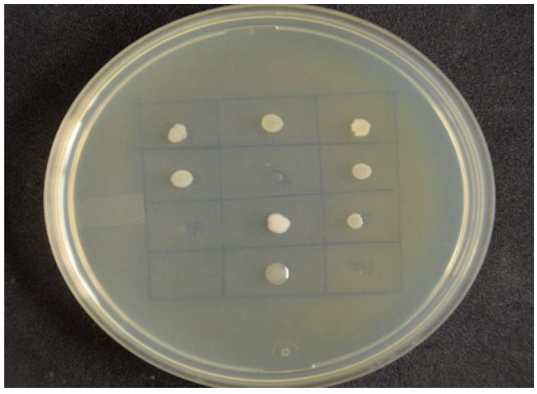

Fig. 1b. 20 mg/l.

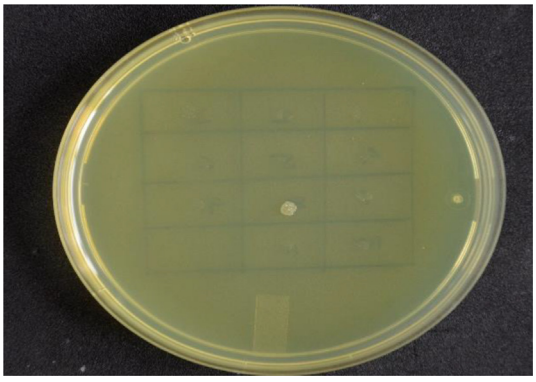

Fig. 1d. 100 ml/l.

substance from environment such as bacteria and fungi etc., the living organisms transform and remove Cr (VI) from water during the cellular metabolisms, extracellular activities, physical and chemical adsorptions on the cell surface, and photosynthesis. The bacteria *Cellulosimicrobium* sp. (KX710177) isolated from tannery effluent, survived in more than 100 mg concentration of the chromium (VI) (Bharagava and Mishra, 2018). Also, *Shewanella loihica* bacteria reduced the chromium from the waste water by extra cellular process (Wang et al., 2017).

3.3. Molecular screening for chromium reductase gene

Chromium reductase property was found in chromium resistant bacteria, which are known to catalyse the reduction reaction of Cr

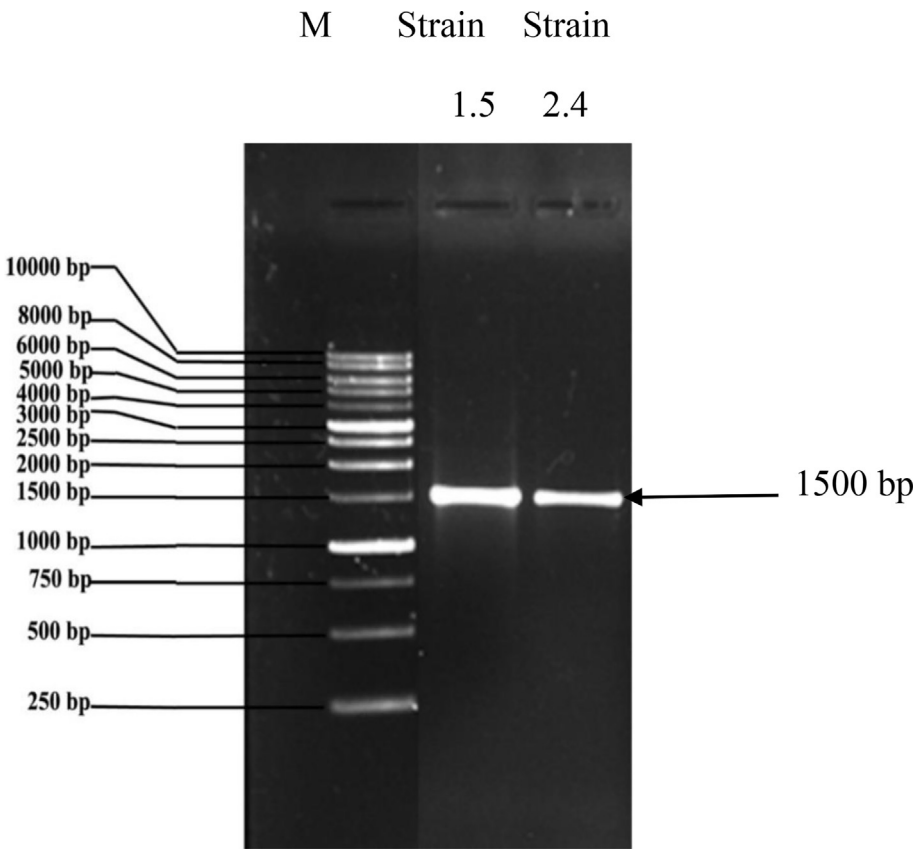

Fig. 2. PCR Product of 16S rRNA region of chromium tolerance bacteria on 1% agarose gel.

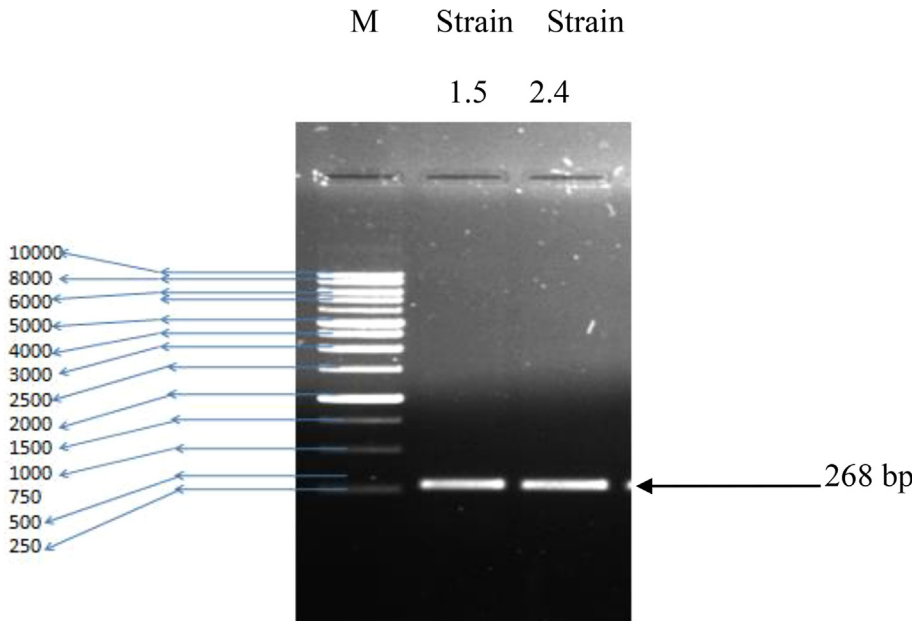

Fig. 3. PCR product of Chromium Reductase Gene on 2% agarose gel.

(VI) to Cr (III) (Deshpande et al., 2005). The PCR amplified product shows 268 bp when it was run along with a DNA ladder Fig. 2. Similarly, Patra et al. (2010) who stated that partial amplification of chromate reductase gene showed 268 bp fragment in 3 Gram positive bacteria collected from Cr polluted area. Deng et al. (2015) obtained 321 bp DNA of Chromium reductase gene. This confirms the presence of chromium reductase gene in the DNA sequences of these two bacteria reaffirming their chromium reducing property.

3.4. Molecular identification of chromium reducing bacteria

A significant advantage of this protocol is that a bacterial isolate can be identified within two or three days than conventional biochemical tests, which generally takes several weeks. Numerous previous reports suggested that 16S rRNA gene sequence analysis is superior when compared to conventional phenotypic methods in identifying bacteria (Tang et al., 2000; Bosshard et al., 2003). The PCR amplified product of 1500 bp of DNA segment shows the 16S rRNA gene amplification in the image produced by gel documentation system Fig. 3. Similar methods used to identify chromium reducing bacteria from soil samples in electroplating industry (Xiao et al., 2017). Gupta et al. (2018) stated that chromium reductase bacteria *Klebsiella* sp. was identified by 16S rRNA gene sequence and the same technique used to identify the *Cellulosimicrobium* sp. isolated from tannery wastewater (Bharagava and Mishra, 2018). In this study, the bacteria were identified as *Klebsiella pneumoniae* (Accession No: KY317923.1) and *Mangrovibacter yixingensis* (Accession No: KY321826.1) by 16S rRNA sequencing. The isolated bacteria showed 100% similarity with reference sequence retrieved from NCBI (Figs. 4a–b).

3.5. Ancestry

Various strains of *K. pneumoniae* have been recorded as heavy metal tolerant species, such as *K. pneumoniae* IFCu4, *K. pneumoniae* NCIB 418 and *K. pneumonia* CBL (Ainsworth et al., 1980; Shah et al., 2014). The cladogram of the bacterial strain *K. pneumoniae* strain

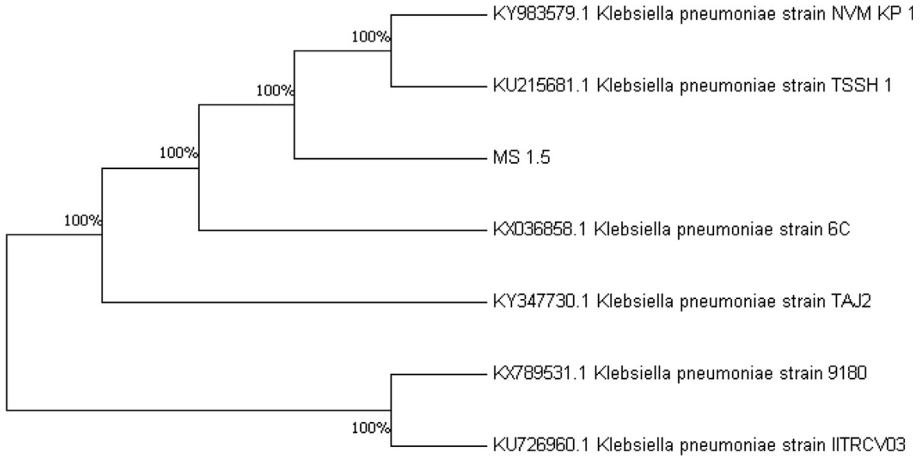

Fig. 4a. Phylogenetic tree based on the 16S rRNA gene sequence of strain 1.5 compared with closely related organisms in GenBank.

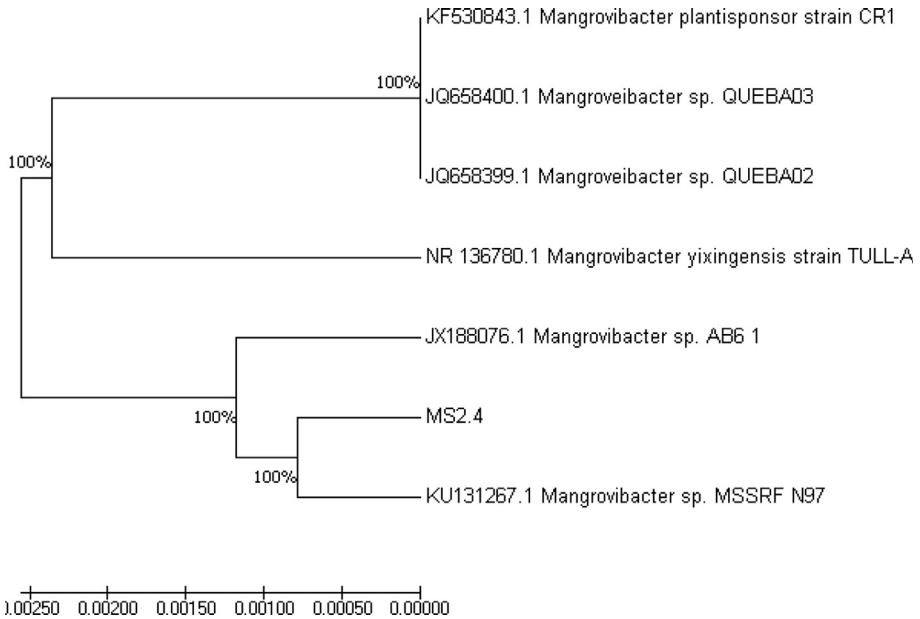

Fig. 4b. Phylogenetic tree based on the 16S rRNA gene sequence of strain 2.4compared with closely related organisms in GenBank.

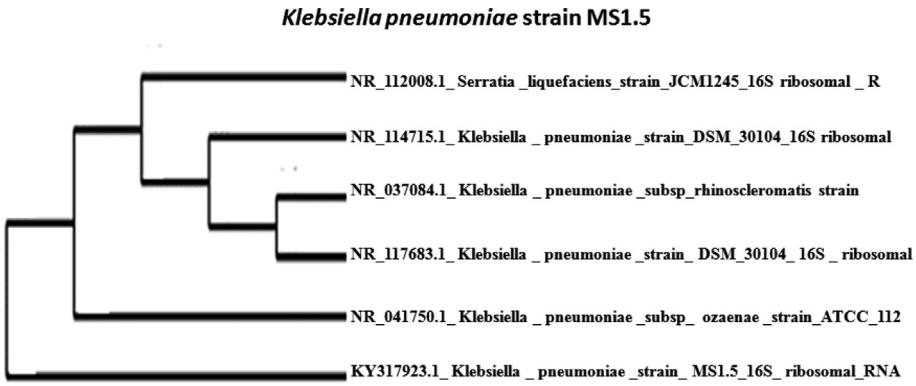

Fig. 5a. Cladogram tree of *Klebsiella pneumoniae* (Accession No: KY317923.1).

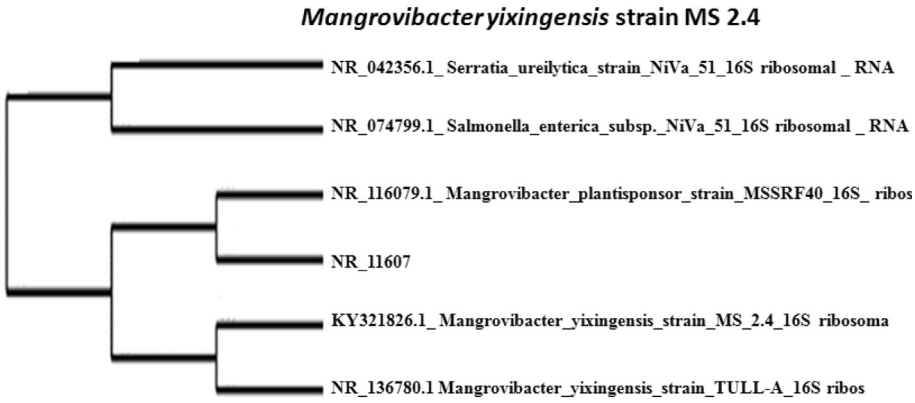

Fig. 5b. Cladogram tree of *Mangrovibacter yixingensis* (Accession No: KY317923.1).

MS 1.5 reveals that this particular bacterial strain has been originated directly from an unknown common ancestor. This unknown common ancestor has also given rise to five related strains from many intermediate strains through several genomic variations along various generations. None of these related strains show heavy metal tolerance; which may be due to the genomic variations that had occurred along various generations. The isolate *K. pneumoniae* strain MS 1.5, which is the direct progeny of the unknown common ancestor must have retained its novel characteristic of heavy metal tolerance. There is also a possibility for other heavy metal tolerant *K. pneumonia* strains originating from the same unknown common ancestoras shown in Cladogram 1 (Fig. 5a).

The *Mangrovibacter* sp. is generally considered as a nitrogen-fixing bacterium associated with the rhizosphere of mangrove-associated plants (Joseph et al., 2014). Since the first draft, genome sequence of a member of the genus *Mangrovibacter* was discovered in the year of 2014, presently, very less information is available regarding these bacteria. The cladogram of the bacterial strain *M. yixingensis* strain MS 2.4, reveals that this has been originated from an unknown common ancestor. All the four, selected related species also had originated from the same unknown common ancestor. The cladogram also reveals that *M. yixingensis* strain MS 2.4 has a sister strain called *M. yixingensis* strain TULL-A as shown in Cladogram 2 (Fig. 5b).

4. Conclusion

It can be concluded from this study that indigenous bacterial species from polluted samples and effluents use their innate ability to degrade pollutants like Chromium, which is also economically viable when compared to conventional methods. Two different bacterial species *K. pneumoniae* strain MS 1.5 and *Mangrovibacter yixingensis* strain MS 2.4 were isolated and identified through molecular techniques. They have high reducing ability for Cr (VI) upto 80 mg/l and 100 mg/l Cr respectively. As these species have significant potential to reduce the toxic hexavalent chromium, this property can be harnessed to detoxify the chromium contaminated sites. Therefore, these findings are highly relevant to the tannery industry and mining industry from the perspective of bioremediation as this can also be practiced in the metal recovery techniques in the mining industry. However, further optimization studies are required to optimize the characters of these bacteria before they can be used to reduce high concentration of chromium.

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