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Review Article

A REVIEW ON DIVERSITY OF CHROMIUM RESISTANT BACTERIAL STRAINS ISOLATED FROM TANNERY INDUSTRY

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Abstract- Chromium is a naturally occurring element found in rocks, animals, plants, soil, and in volcanic dust and gases. Chromium is present in the environment in several different forms. Most leather is chrome-tanned. All wastes containing chromium are considered hazardous by the U.S. Environmental Protection Agency. These irregularities have had a disastrous economic, social and health impacts. Chrome liquor is generally used for tanning purpose. When chrome liquor is discharged with effluents into the environment, they contain chrome salts in excess of the maximum permissible limits. In biological system, enrichment of chromium-resistant bacteria is formed by sludge deposition from such effluents.

The present study shows the diversity of chromium resistance bacteria/strains isolated from tannery effluents in past decades by researchers. Our literature review found a high chromium tolerance among isolated bacteria ranging from 10µg/ml-45000µg/ml. CMBL Crl3 exhibited the highest resistance to chromium. Isolates were screened and characterized with biochemical and 16S rRNA based sequencing methods. There are few reports are available for characterization by using 16S rDNA sequencing methods, but 16S rDNA sequencing has played a significant role in the accurate identification of bacterial isolates (its amplicon product shows highly and less conserved region but in case of 16S rRNA amplicon shows only highly conserved stretches in bacteria) and particularly important in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria and culture-negative infections. Identification of microbes related technology might provide an alternative or addition to conventional method of metal removal or metal recovery. The identified chromium resistant bacteria would be useful for bioremediation of heavy metal contaminated tannery effluent. In transferring this technology from laboratory to a large-scale application, better understanding of all these aspects is necessary. Hence, this developing biotechnological method that encompasses fields from genetic engineering to reactor engineering demands focused research in these directions, which may lead to implementation of this technology on a larger scale and drive it toward being the most opted-for technology.

Keywords- Chromium, Chrome liquor, Tannery effluents, CMBL Crl3, 16S rRNA, 16S rDNA.

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Introduction

Chromium

Atomic Number of Chromium (Cr) is 24, atomic mass 51.9961 a.m.u and electronic configuration (ground state) [Ar] $3d^5$ $4s^1$. It is a transition element. Chromium exists in nine valence states ranging from -2 to +6, but mainly occurs as Cr^{2+} in the divalent oxy anion chromate state and Cr^{3+} in trivalent cationstate. Basic compounds are divalent chromium, amphoteric compounds are trivalent chromium and hexavalent chromium compounds are as acidic. Distinctive hexavalent chromium compounds are formed by the acid anhydride (CrO_3), the acid chloride (CrO_2Cl_2), and a wide variety of metal chromates ($MCrO_4$) and Potassium dichromate ($K_2Cr_2O_7$) compounds.

Chromium toxicity

Hexavalent chromium Cr(VI) compounds are usually more harmless than trivalent chromium compounds due to strong oxidizing power and the higher rate for the transport in cell membrane [1]. Likewise, Cr (III) is highly insoluble and less toxic [2]. The solubility of Cr (III) compounds is limited by the formation of several oxides and hydroxide species [1]. However, noxious, carcinogenic and teratogenic compounds are formed at higher concentration [3]. The intake of hexavalent chromium causes death. Work related to chromium compounds have been shown to cause bronchial asthma, lung and nasal cancer, nasal and skin ulcer, and allergic reactions in the skin [1]. Easily soluble chromate anions are responsible for cellular permeability barrier over rise [3]. The heavy metals oxyanions disturb the metabolism of the structurally related non-metals in the living cells [4].

Economic impacts: Due to the utilization of contaminated irrigation water, yield of wheat, paddy and bar seem (local animal feed plants) has reduced by 50 percent. The economy of these villages was sustained on floriculture, mainly Rose farming, but these roses stink. The size was also very small. The flower yield has dipped by 60 percent. Vegetables grown in these villages could not be sold in the city even at very low rates. This decrease in output strips the basic earning of farmers.

Health: Glue-making units that use the waste (flesh) and other by-products of tanneries on the outskirts of most villages have provoked the problem. Besides, the affected villages do not have primary health centers.

Chromium released by industries

Chromium occurs mainly as a result of human activities through production of waste water in metals melting, electroplating, and tanning, metallurgy and dyestuff industries. After exemption, various chemical species of chromium occurs, such as metallic chromium {Cr (0)}, trivalent chromium {Cr(III)}, and hexavalent chromium

{Cr(VI))}[5]. Chromium(VI) is found as CrO_4^{2-} , $HCrO^{4-}$ or $Cr_2O_7^{2-}$, depending on the pH of the medium.

The leather sector is infamous for contaminating effluents by releasing chromium. The polluting nature of tanneries is apparent from the notorious smell that distinguishes tanneries and tannery zones. While local population are conscious of the air pollution uniformly local authorities also, if not more concerned about tanneries liquid effluents it tendency to be elevate inorganic and organic suspended solids content accompanied by susceptibility for high oxygen demand and holding potentially toxic metal salt residues. Treatment technologies, in effect, reduce pollutants in the liquid and convert the contaminates into semi-solid or solid forms. Threat is being transferred from receiving waters to receiving soil. Because sludge can effect on the quality of soil and groundwater chromium enters in food chain. These irregularities have had a disastrous economic, social and health impacts. The contribution of biochemical and biotechnological methods to identify and characterize natural bacterial community isolated from contaminated environment, and the potential exploitation of chromium-resistant bacterial strains in bioremediation provides an effort to remove heavy metals from tannery effluent in the environment.

Chromium (Cr) is a transition metal, which is the major cause of environmental pollution. It enters into the environment through industrial waste like leather tanning, metallurgical and metal finishing, textiles and ceramics, pigments, wood preservatives, photographic and sensitizer manufacturing, etc. [6,7]. The hexavalent form of Cr is very toxic, carcinogenic and mutagenic in humans as well as animals whereas the trivalent form is less of a problem due to is lower toxicity, ability to readily precipitate and form less chromium hydroxide. The deposition of metallic chromium on materials imparts a refractory nature on such materials thus depiction them resistant to microbial attack and flexible over extended periods [8]. A consequence of industrial and manufacturing activities discharges more than 170,000 tons of Cr waste into the environment annually [9]. Its presence in different concentration in the agricultural soils, fertilizers and wastewater for irrigation, effects on growth of chlorophyll and mineral nutrients also [10]. Increase of world population has resulted in the pollution of the environment. The main factors responsible for pollution and other type of environmental degradation in any community are combined effects of pollution increase, effluents and technology [11]. In the environment, chromium occurs mainly in both trivalent and hexavalent forms. Trivalent chromium (Cr^{3+}) compounds are less toxic comparatively hexavalent chromium (Cr^{6+}) compounds [12]. Its rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acid, it is the motive for appearing toxicity [13]. Four chromium-resistant bacteria have been isolated from tannery effluent collected from Burgelarab, Alexandria, Egypt. These isolate dissimilar degrees of chromate reduction beneath aerobic conditions. Based on 16S rDNA gene sequence analysis [14] numerous physical and chemical methods subsist to eliminate heavy metals such as chromium from the environments.

Biological treatment

Bioremediation is the use of microorganisms to break down toxic and hazardous compounds in the environment [15]. The two main biological treatment processes are available, first the adsorption of Cr(VI) onto microbial cells, and second, reduction of Cr(VI) to Cr(III) by enzymatic reaction or indirectly by reducing compounds produced by micro-organisms [16, 17]. It has been confirmed that bioremediation is a cost-effective and favorable to chemical and physical methods of managing wastes and environmental pollutants. Modern methods of bioremediation are emerging based on advances in molecular biology and process engineering. Newly developed rapid-screening assays can recognize organisms capable of degrading specific wastes and new gene-probe methods can establish their abundance at specific sites. In bioremediation (in situ), bio filters and bioreactors, new tools and techniques are contributing to the rapid growth of this field [18]. Microorganisms have the competency to accommodate a diversity of pollutants in both organic and inorganic, it is important to appreciate from the beginning that microorganisms cannot destroy metals. However, microorganisms can influence a metal's mobility in the environment by modifying their chemical and/or physical characteristics [19]. In addition, bioremediation rising the concentration and radioactivity of materials to avoid toxicity or to recover metals for reuses. Major benefits, microbes easily biodegrades to organic chemicals; pollutant degradation & waste-site clean-up operations can be enriched by this natural process [18].

Metals and microorganisms

Heavy metals discharges through wastewater are dangerous to the environment and their consequences on biological system are very rigorous. It has been reported that microorganisms become adapted to food chain by the acquisition of specific resistance systems [20]. Due to the higher concentration of heavy metals, microorganism becomes receptive.

Mechanisms of metal resistance by bacteria

Four resistance mechanisms of bacterial heavy metals are recognized. Maintenance of toxic ion out of cell by changing a membrane transport arrangement involved in primary cellular growth is the first mechanism. The intracellular or extracellular sequestration by specific mineral-ion binding components (analogous to metallothioneins of eukaryotes and the phytochelatins of plants, but generally at the level of the cell wall in bacteria)is the second mechanism. The most commonly found mechanism of plasmid-controlled bacterial metal ion resistance, involving highly specific cation or anion efflux systems encoded by resistance genes (analogous to multidrug resistance of animal tumor cells), is the third mechanism. Method used for detoxification of the toxic cation or anion by enzymatically convert it into a less toxic from a more toxic form, is the fourth mechanism. The fourth mechanism is excellent for detoxification of inorganic and organomercurials. It may also be utilized for oxidation of As (III) and the reduction of Cr (VI) to less toxic forms, but these known microbial processes here have not been associated with plasmids [21]. The major assemblies of resistance system function by energy-dependent efflux of toxic ions. Metal-binding proteins (for example, metallothionein SmtA, chaperone CopZ and periplasmic silver binding protein SilE) or enzymatic transformations (oxidation, reduction, methylation, and demethylation) are involved in smaller amount. Several efflux resistance systems are ATPases and others are chemiosmotic ion/proton exchangers. For example, Cd^{2+}-efflux pumps of bacteria are either inner membrane P-type ATPases or three polypeptide RND chemiosmotic complexes comprising of an inner membrane pump, a periplasmic-bridging protein and an outer membrane channel. In addition, the best studied three-polypeptide chemiosmotic system, Czc (Cd^{2+}, Zn^{2+}, and Co^{2+}), others are known that efflux Ag^{+}, Cu^{+}, Ni^{2+}, and Zn^{2+}. To transfer more toxic to less toxic forms this process is used, resistance to inorganic mercury, Hg^{2+}(and to organomercurials, such as Ch_3Hg^{+} and Phenyl mercury) involve a series of metal binding and membrane 15 transport proteins as well as the enzymes mercuric reductive and organomercurial lyase. Three patterns takes placein Arsenic resistance and metabolizing systems, the extensively *ars* operon present in several plasmids and mainly bacterial genomes, recently recognized genes for the periplasmic arsenate reductase are*arr* genes that functions in anaerobic respiration as a terminal electron acceptor, and the genes for the periplasmic arsenite oxidase are*a so* that functions as an initial electron donor in aerobic resistance to arsenite [22]. The mechanism of resistance involves cellular uptake is chromate. Currently, it is unknown whether there is a block directly on uptake or accelerated chromate efflux [21] [Fig-1] and [Fig-2].

Different phenotypic and genotypic methodologies are being used to catalog and characterize bacteria to recognize the variation within a group of Chromium highly resistant bacterial strains and also, to understand the microbial diversity within and across the group. Whereas phenotypic methods play a significant role in identification but the molecular methods are more consistent and authenticated for identification and to study genetic diversity of bacterial isolates. The SSCP analysis is a simple and valuable method for detection of minor sequence changes in PCR amplified DNA [23].

Major molecular techniques include Polymerase chain reaction (PCR), Randomly amplified polymorphic DNA (RAPD), Restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP), Single sequence repeats (SSR) and 16S-rRNA gene sequencing. RAPD is the more consistent,

rapid and practical method [24] utilized for phylogenetic relationships along with closely related species [25]. In addition to highly conserved primer binding sites, 16S rRNA gene sequences holding hyper-variable regions that can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification [26].

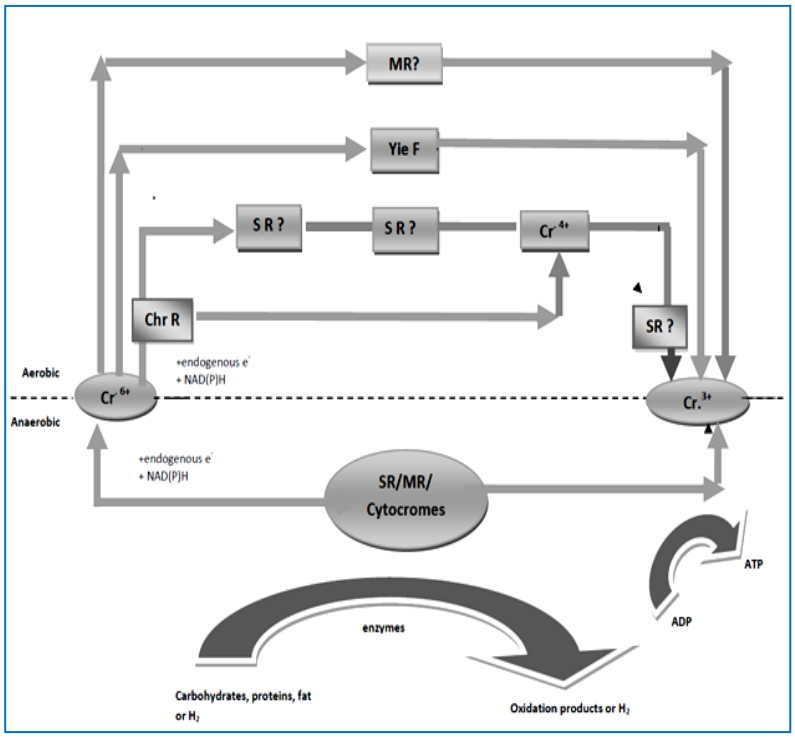


Fig-1 Plausible mechanism of enzymatic Cr $^{6+}$ reduction under aerobic (upper)& anaerobic (lower) condition (Wang and Shen, 1995).

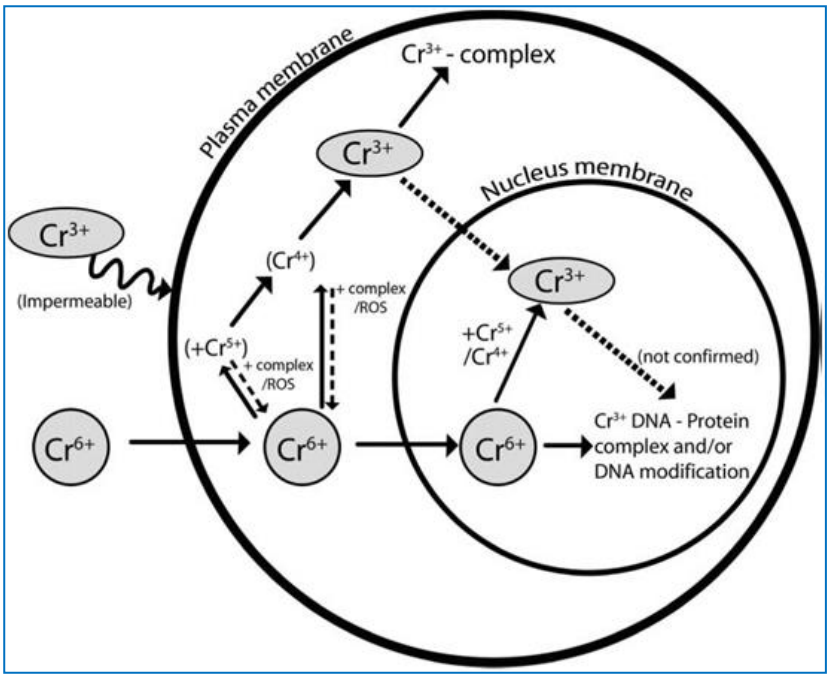


Fig-2 Mechanisms of Cr^{6+} reduction in the bacterial cell (Cheung, 2007)

Some bacteria are difficult to identify with phenotypic identification methods commonly used outside reference laboratories. 16S ribosomal DNA (rDNA) based identification method of bacteria potentially offers alternative method when phenotypic characterization fails. However, as yet, the usefulness of 16S rDNA sequence analysis in the identification of generally unidentifiable isolates has not been evaluated with a large collection of isolates [27]. However, these techniques are reported to be impractical due to the high operation cost and subsequent invention of solid waste, which is difficult to treat. Research in recent years indicated that many microorganisms accumulate large concentrations of metals [28]. Microbial tolerance to hexavalent chromium has practical importance because it can serve as a basis for selecting organism that can be used to detoxify chromium in the environment [29]. Quantities of chromium tolerant microorganisms have been reported including different concentration of chromium (VI). Bacteria were isolated from sewage sludge in the oxidation ditch and chromium (VI) tolerance of the isolates determined by plating on media amended with different concentrations of the chromium. The tanning industry generally utilizes chrome liquor in the tanning process, thus chrome salts discharged (solid or liquid form) through effluents and effects on biological systems due to its strong

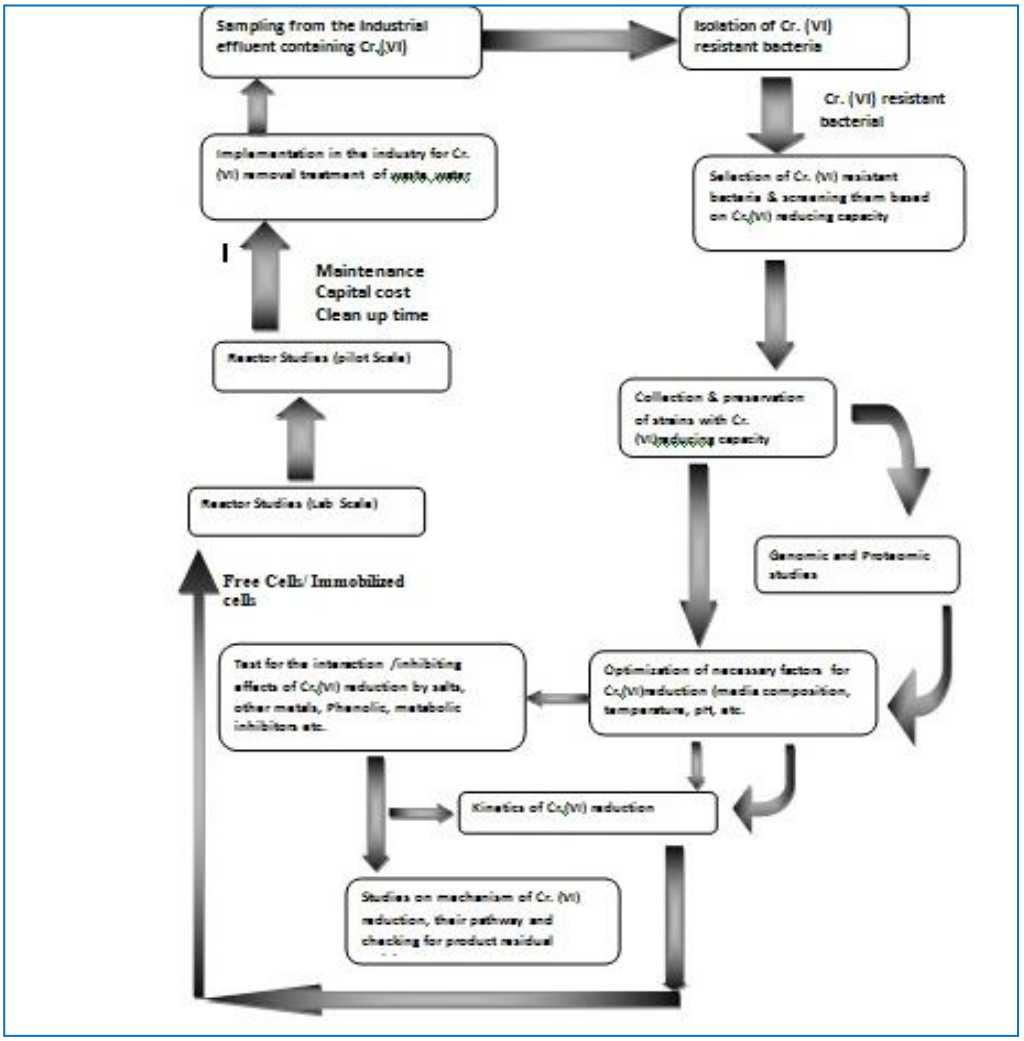
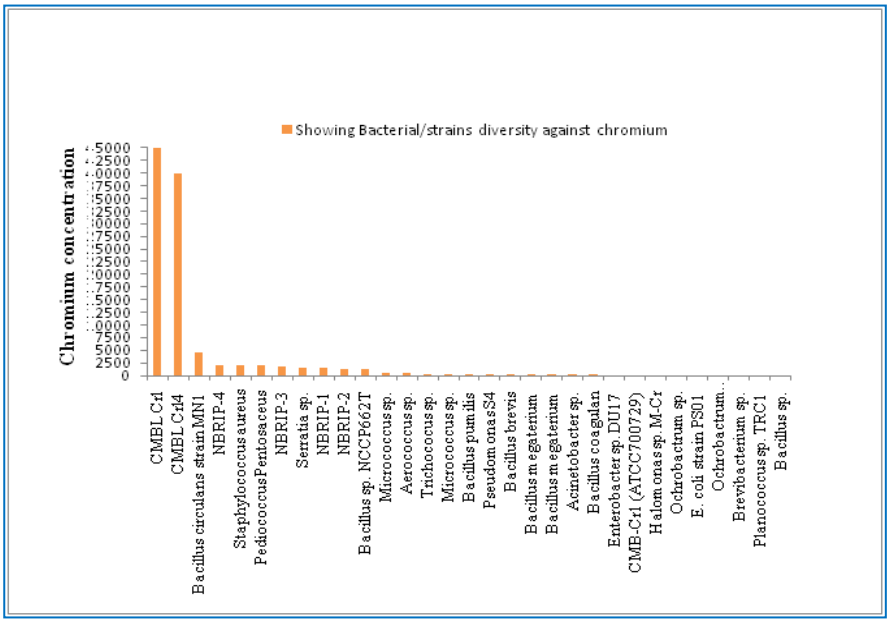


Fig-3 Schematic representation of hexavalent chromium removal from waste water by bacteria

oxidizing nature [30, 31]. Therefore, provides a natural environment for enrichment of chromium-resistant bacteria. Chromium-resistant bacterial strains have been isolated by several investigators from such chromium-contaminated sediments [13, 32, 33].

The isolates were identified as members of *Staphylococcus* spp., *Bacillus* spp., *Pseudomonas* spp., *Micrococcus* sp. and *E.coli* [34]. *Pseudomonas* spp. [35], *Desulfovibrio* spp. [36], *Enterobacter* spp [37], *Escherichia coli* [38], *Bacillus* spp [39, 40], *Bacillus brevis*[41]and several other bacterial isolates [42]. Some are given in [Table-1] and [Graph-1].



Graph-1 Diversity of Bacteria/strains isolated from tannery effluents at different chromium concentrations

Table-1 *List of chromium-resistant bacteria isolated from a source with their maximum resistance capability*

S. No	Cr(VI) Resistant/Tolerant bacteria	Gram (+)/(-)	Mol. characterization	Cr(VI) Concentration (µg/ml)	Culture media	Source of isolation	References
1	CMBL Crl3	+	No	45000	Luria-Bertani (LB)	Effluents of leather industry	[52]
2	CMBL Crl4	–	No	40000	Luria-Bertani (LB)	Effluents of leather industry	[52]
3	*Bacillus circulans strain* MN1	+	No	4500	Nutrient agar	Spent chrome effluent	[49]
4	NBRIP-4	–	No	2100	Nutrient broth	Tannery effluent	[53]
5	*Staphylococcus aureus*	+	16S rRNA	2000	Luria-Bertani (LB)	Tannery effluent	[54]
6	*Pediococcus Pentosaceus*	+	16S rRNA	2000	Luria-Bertani (LB)	Tannery effluent	[54]
7	NBRIP-3	–	No	1800	Nutrient broth	Tannery effluent	[53]
8	*Serratia sp.*	–	16S rRNA	1500	Nutrient agar	Chromium-contaminated site	[55]
9	NBRIP-1	–	No	1400	Nutrient broth	Tannery effluent	[53]
10	NBRIP-2	–	No	1200	Nutrient broth	Tannery effluent	[53]
11	*Bacillus* sp. NCCP662T	+	16S rRNA	1200	Nutrient agar	Tannery effluent	[56]
12	*Micrococcus sp.*	+	No	400	Anaerobic agar	Tannery effluent	[57]
13	*Aerococcus sp.*	+	No	400	Anaerobic agar	Tannery effluent	[57]
14	*Trichococus sp.*	+	No	250	Peptone Yeast Extract (PYE) agar	Tannery-effluent sediments	[58]
15	*Micrococcus sp.*	+	No	250	Peptone Yeast Extract (PYE) agar	Tannery-effluent sediments	[58]
16	*Bacillus pumilis*	+	No	200	Nutrient agar	Treated tannery effluent	[50]
17	*Pseudomonas S4*	–	16S rDNA	200	Luria-Bertani (LB)	Tannery effluent	[59]
18	*Bacillus brevis*	+	No	180	Nutrient agar	Treated tannery effluent	[50]
19	*Bacillus megaterium*	+	No	170	Nutrient agar	Treated tannery effluent	[50]
20	*Bacillus megaterium*	+	No	170	Nutrient agar	Treated tannery effluent of a common effluent treatment plant	[60]
21	*Acinetobacter sp.*	–	16S rDNA	160	Luria-Bertani (LB)	Tannery effluent	[59]
22	*Bacillus coagulans*	+	No	140	Nutrient agar	Treated tannery effluent	[50]
23	*Enterobacter sp. DU17*	+	16S rRNA	100	Nutrient agar	tannery waste dump site	[61]
24	CMB-Cr1 (ATCC 700729)	+	No	80	Luria-Bertani (LB) Agar	Tannery effluent	[62]
25	Halomonas sp. M-Cr	+	16S rDNA	50	Luria-Bertani (LB) medium	Tannery effluent	[63]
26	*Ochrobactrum sp.*	–	16S rRNA	40	Nutrient agar	Tannery effluent	[64]
27	*E. coli strain PS01*	_	16S rRNA	40	Nutrient agar	Tannery effluent	[65]
28	*Ochrobactrumanthropi STCr-1*	-	16S rRNA	40	Nutrient agar	Tannery effluent	[66]
29	*Brevibacterium sp.*	+	16S rRNA	40	Nutrient agar	Tannery effluent	[67]
30	*Planococcus*sp. TRC1	+	16S rDNA	25	Nutrient agar	Tannery effluent	[68]
31	*Bacillus sp.*	+	No	10	Nutrient agar	Tannery effluent contaminated soil	[69]

Proposed methodology

The identification of more bacterial strains that could uptake metals with high efficiency and specificity has attracted increasing attention to both medical and biotechnological points of view. Tannery-effluent sediments were collected for isolation of highly chromium resistant bacterial strains from different tannery industry.

Identification of different bacteria in different source samples

⇩

Evaluation of chromium-tolerance

⇩

Identification of Hexavalent Chromium Resistant Bacterium

⇩

Morphological characteristics (shape and size, gram reaction, motility)

⇩

Culture method: Different culture media (Nutrient agar colonies, salt agar, stab culture by streak plate method, pour plate method and spread plate method and incubate for 24-48 hours at 30-37°C.

⇩

Quantification of growth of chromium-resistant strains: Isolates monitored by measuring the optical density by spectrophotometer.

The observable growth isolated different colonies which are classified in to no growth, scanty, moderate or heavy growth found on the following criteria.

No growth: No colonies in any of the 4 quadrants
Scanty growth: Growth in quadrant 1 only
Light growth: Growth in quadrant 1 and 2
Moderate growth: Growth in quadrant 1, 2 and 3
Heavy growth: Growth in quadrant 1, 2, 3 and 4

⇩

Estimation of highly multiplication rate of chromium-resistant bacterial strains and its potential to remove these metals from water resources frequently using human population:

⇩

Microscopic examination: After incubation period the cultured check for Gram's staining, motility and endospore staining *etc.*

⇩

Biochemical method: Behind microscopic examination the culture tested for the different biochemical tests like(Growth on MacConkey agar, Indole test, Methyle Red test, Voges Proskaure test, Citrate Utilization, Casein hydrolysis, Starch hydrolysis, Urea hydrolysis, ONPG hydrolysis, Nitrate reduction, Nitrite reduction, H_2S production, Cytochrome Oxidase test, Catalase test, Gelatine liquefaction, Arginine dihydrolase, Lysine decarboxylase, Ornithine decaroxylase, Milk clotting, NH_3 production, Coagulase test, IMViC test and Sugar fermentation/Oxidation tests *etc.*)

⇩

Antibiotic sensitivity of different isolated bacterial strains

⇩

Molecular method: Characterization of bacterial strains by 16S rDNA based molecular characterization

⇩

DNA isolation

⇩

Amplification of 16S rDNA fragment

⇩

Sequencing and sequence analysis of 16S rDNA amplicons: BLAST (http://www.ncbi.nlm.nih.gov/BLAST) is used to reads sequence of Nucleotide of each different bacterial strain will be further subjected to get conclusion about the bacterial sp. (based on nucleotide similarity/ E-value). Molecular phylogeny analysis (of 16S rDNA sequence) of each isolated bacterial strain with others (already submitted in public domain) will be study to estimate historical branching order of the species using appropriate software.

⇩

Diversity analysis using software

Future prospects

In India, environmental pollution increasing metal toxicity such as cadmium and chromium is high in many rural and urban areas due to low to moderate sanitation. Earlier identified Chromium resistant bacterial strains are not much effective as increasing toxicity of Chromium in water resources and air. Therefore it's a need to identify, characterize new bacterial strains which have higher resistant then earlier identified.

There is a need to identify higher multiplication rate of such kind bacterial strains then earlier.

Conclusion

Current study is the capability of biochemical and molecular methods to identify and characterize natural culturable bacterial communities isolated from polluted surroundings, and the potential operation of chromium-resistant bacterial strains in bioremediation process aids removal of heavy metal from contaminated environment. Most of the information related to chromium resistant bacterial strains are scattered in different publications. We conclude all updated information at one platform. Further, it will very useful for researchers who are working in field.

Conflict of interest: The authors declare that they have no conflict of interest.

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