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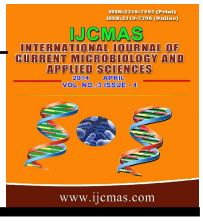

Original Research Article

Isolation and molecular characterization of bacterial Strains from tannery effluent and reduction of chromium

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A B S T R A C T

Industrial wastes are capable of generating soil and groundwater pollution. The tanning industry is considered an activity with elevated potential for environmental pollution all over the world. The chromium (Cr), a toxic heavy metal, is a major contaminant in tannery waste, and its accumulation in soil and water is an environmental issue of increasing public concern in India particularly in Tamil Nadu. The present study deals with isolation, identification and characterization of chromium resistant bacteria were isolated from tannery effluent collected from Dindigul, south India. Two chromium resistant bacteria were isolated. These isolates displayed different degrees of chromium reduction under aerobic condition. One of the two bacterial isolates were screened and characterized by using the 16s rRNA based PCR amplification and it was named as *Escherichia coli* strain PS01. Microbes related technology may 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.

Keywords

Industrial wastes; chromium; *Escherichia coli*.

Introduction

In the past few decades, uncontrolled urbanization has caused a serious pollution problem due to the disposal of sewage and industrial effluents to water bodies. Unlike many other pollutants, heavy metals are difficult to remove from the environment (Ren *et al*.,2009).

Heavy metals are recognised to be powerful inhibitors of biodegradation activities (Deeb and Altalhi., 2009). These metals cannot be degraded, and are These heavy metals such as copper, cadmium, lead, zinc, nickel, mercury, and chromium when accumulated in soils, water bodies they can also be present in concentrations toxic to plants, animals, humans, and aquatic life (Dowdy and Volk., 1983). Each heavy metal has unique bio functions or bio toxicities.

The tanning industry is one of the major sources of pollution in Tamil Nadu, India, as it releases large quantities of effluents

and sludge rich in chromium (Cr) and salts into the environment (Ramasamy *et al*.,2000). Notably, there are several contaminated sites located in the Vellore, Erode and Dindigul districts in Tamil Nadu, where more than 60% of Indian tanneries are located (Ramasamy and Naidu, 2000).

To survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate and uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, and reduction of the heavy metal ions to a less toxic state (Niles, 1999; Spain, 2003). Therefore this study was performed to determine the antibiotic and heavy metal resistance patterns of bacteria which were isolated from tannery waste water.

Materials and Methods

Collection and transport of effluent

The tannery effluent sample was collected from leather industry located at Dindigul district during January-March 2012. The samples were collected in sterile plastic container and transported to laboratory for bacteriological analysis.

Physical and chemical analysis of tannery effluent

Tannery effluent was collected from tannery industry located in Dindigul and was analysed for number of characteristics. such as colour, odour, turbidity, total dissolved solids, pH, electrical conductivity, BOD, COD, Carbonate, bicarbonate, chloride, sulphate, phosphate, silicate, nitrate, nitrite, fluoride, aluminium, calcium, magnesium, sodium, potassium, zinc, copper, iron, manganese, chromium and lead.

Isolation of bacteria from tannery effluent

The bacterial isolates were screened on Nutrient Agar (NA) plates. The plates were incubated at 37º C for 24 hours and colonies differing in morphological characteristics were selected and used for further studies.

Characterization of the isolates

Selected tannery effluent isolates were grown on MacConkey agar (Himedia, India). The shape and colors of the colonies were examined under the microscope after Gram staining followed by capsule study, spore structure, motility were also observed under the microscope. Then the isolates were biochemically analysed for the activities of Oxidase, Catalase, MR-VP test, Urease test, Indole test, Citrate utilization, Acid production from carbohydrates. The tests were used to identify the isolates according to Bergey's Manual of Determinative bacteriology (Holt *et al.,* 1994-1999).

Isolation of chromium resistant bacteria

Chromium-resistant bacteria were isolated from tannery. For the isolation and enumeration of bacteria, samples were serially diluted and plated on Nutrient agar adjusted at normal pH value (7.0). The molten medium was amended with Cr (VI) as $K_2Cr_2O_7$ to final concentration 40 mg/l using sterile filtered Cr (VI) stock solutions. Plates were incubated at 30º C in the dark and read after two days. Subsequently four isolates were selected according to their morphological shapes for further studies.

Determination of MIC

The minimum inhibitory concentration (MIC) of two Cr (VI) resistant isolates were determined by broth dilution method (Calomoris *et al.,* 1984) in LB medium with Cr (VI) concentrations ranging from 20 to 500 mg/l. The minimum concentration of metal in the medium inhibiting complete growth was taken as the (MIC). Based on the evaluation MIC was determined at 37 ºC /24 hours. The minimum Concentration of the chromium ($K_2Cr_2O_7$) at which no turbidity was observed by Spectrophotometer at 600nm was considered as the MIC of bacterial isolates against heavy metals.

Reduction of chromium by the isolates

Chromate-resistant bacterial isolates (1 and 2) were inoculated into LB broth (pH 7.0) containing different concentration of Cr (VI) (from 20 to 200mg/l) and incubated for 72 hrs at 30 ºC with orbital shaking (200 rpm). The inoculum was 2% of the total volume of medium. Bacterial cell density (diluted 10-fold with water) of the liquid cultures was determined by measuring optical density at 600 nm by use of UV/Vis. Reduction of chromium was determined from extracted solution by using UV spectrophotometers at 540 nm with 1,5-diphenylcarbazide as a pink coloured complex agent (APHA, 1992).

Molecular Characterization

For molecular characterization the genomic DNA were isolated from the isolates. Then it was amplified with universal bacterial primers. The unknown bacterium was identified using Gen Bank database. The sequences obtained were initially estimated by the BLAST facility of NCBI (J.D. Thompson *et al.,* 1994). The identification of ribosomal RNA as a premier molecule for evaluating evolutionary relationships and the application of molecular techniques to microbial systematics has revolutionized the concept of phylogenetic relationships among bacteria (C.R.Woese, 1987 and G.J.Olsen *et al.,* 1994). Sequence data was aligned and analyzed for finding the closest homology for the microbes.

Results and Discussion

Physical and Chemical analysis of tannery effluent

The results of physical and chemical parameters of the effluent were tabulated.

Isolation of bacteria from tannery effluent

A total of six chromium resistant bacteria were isolated from tannery effluent in the present study. Out of six bacterial strains three were Isolate I (*E.coli*) and the other three were Isolate II (*B.subtilis*). Two selected isolates according to the morphological shape were plated in media with 40 mg/l of potassium chromate.

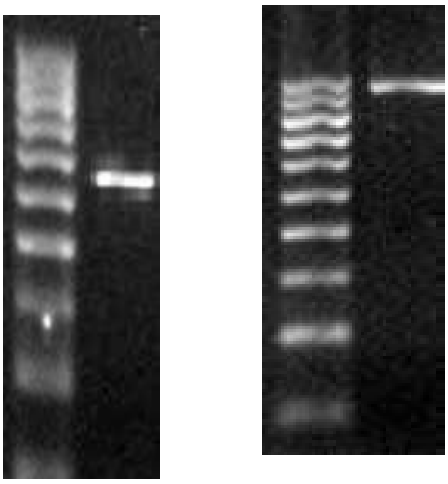
Molecular Characterization

The highest yield of DNA to be obtained was preserved with TE buffer, yields of 25 µl total DNA from the 1.5ml broth. The extracted DNA was electrophoresed on 1% Agarose gel with ethidium bromide. The bands were observed under UV-transilluminator were showed in figure .1. (a). The amplified sample and 100bp DNA ladder was electrophorosed on 2% Agarose gel and compared. The bands were observed under UV-transilluminator were showed in figure.1.(b).

Table.1 Characterization of Physical and Chemical parameters of effluent sample

S.no	Name of the parameter	Sample details
Physical Parameter		
1.	Colour	Dark brown
2.	Odour	Dis.Agree
3.	Turbidity	65 NTU
4.	Total dissolved solids (mg/l)	1683
5.	pH	8.45
6.	Electrical conductivity (dsm^{-1})	2.63
7.	BOD (mg/l)	2250
8.	COD (mg/l)	3200
Anions		
9.	Carbonate (mg/l)	4.8
10.	Bicarbonate (mg/l)	489
11.	Chloride (mg/l)	539
12.	Sulphate (mg/l)	126
13.	Phosphate (mg/l)	0.19
14.	Silicate (mg/l)	4.26
15.	Nitrate (mg/l)	0.42
16.	Nitrite (mg/l)	0.08
17.	Fluoride (mg/l)	5.69
18.	Aluminium (mg/l)	Nil
Cations		
19.	Calcium (mg/l)	589
20.	Magnesium (mg/l)	126
21.	Sodium (mg/l)	236
22.	Potassium (mg/l)	46
Heavy metals		
23.	Zinc (mg/l)	1.97
24.	Copper (mg/l)	1.23
25.	Iron (mg/l)	3.56
26.	Manganese (mg/l)	1.89
27.	Chromium (mg/l)	280
28.	Lead (mg/l)	1.64

Fig.1 16S rRNA based identification



2= ~1500 bp

(a) **(b)**

ORIGIN

1 TGTTGGGAAA CTGCCTGATG GAGGGGGATA ACTACTGGAA ACGGTAGCTA ATACCGCATA
61 ACGTCGCAAG ACCAAAGAGG GGGACCTTCG GGCCTCTTGC CATCGGATGT GCCCAGATGG
121 GATTAGCTTG TTGGTGGGGT AACGGCTCAC CAAGGCGACG ATCCCTAGCT GGTCTGAGAG
181 GATGACCAGC CACACTGGAA CTGAGACACG GTCCAGACTC CTACGGGAGG CAGCAGTGGG
241 GAATATTGCA CAATGGGCGC AAGCCTGATG CAGCCATGCC GCGTGTATGA AGAAGGCCTT
301 CGGGTTGTAA AGTACTTTCA GCGGGGAGGA AGGGAGTAAA GTTAATACCT TTGCTCATTG
361 ACGTTACCCG CAGAAGAAGC ACCGGCTAAC TCCGTGCCAG CAGCCGCGGT AATACGGAGG
421 GTGCAAGCGT TAATCGGAAT TACTGGGCGT AAAGCGCACG CAGGCGGTTT GTTAAGTCAG
481 ATGTGAAATC CCCGGGCTCA ACCTGGGAAC TGCATCTGAT ACTGGCAAGC TTGAGTCTCG
541 TAGAGGGGGG TAGAATTCCA GGTGTAGCGG TGAAATGCGT AGAGATCTGG AGGAATACCG
601 GTGGCGAAGG CGGCCCCCTG GACGAAGACT GACGCTCAGG TGCGAAAGCG TGGGGAGCAA
661 ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG TCGACTTGGA GGTTGTGCCC
721 TTGAGGCGTG GCTTCCGGAG CTAACGCGTT AAGTCGACCG CCTGGGGAGT ACGGCCGCAA
781 GTTAAAACTC AAATGAATTG ACGGGGGCCC GCACAAGCGG TGGAGCATGT GGTTTAATTC
841 GATGCAACGC GAAGAACCTT ACCTGGTCTT GACATCCACA GAACTTTCCA GAGATGGAAA
901 GGTGCCTTCG GGAACCGTGA GACAGGTGCT GCATGGCTGT CGTCAGCTCG TGTTGTGAAA
961 TGTTGGGTTA AGTCCCGCAA CGAGCGCAAC CCTTATCCTT TGTTGCCAGC GGTCCGGCCG
1021 GGAACTCAAA GGAGACTGCC AGTGATAAAC TGGAGGAAGG TGGGGATGAC GTCAAGTCAT
1081 CATGGCCCTT ACGACCAGGG CTACACACGT GCTACAATGG CGCATACAAA GAGAAGCGAC
1141 CTCGCGAGAG CAAGCGGACC TCATAAAGTG CGTCGTAGTC CGGATTGGAG TCTGCAACTC
1201 GACTCCATGA AGTCGGAATC GCTAGTAATC GTGGATCAGA AGCCACGGT GAATACGTTC
1261 CGGGCCTTG

Phylogenetic tree

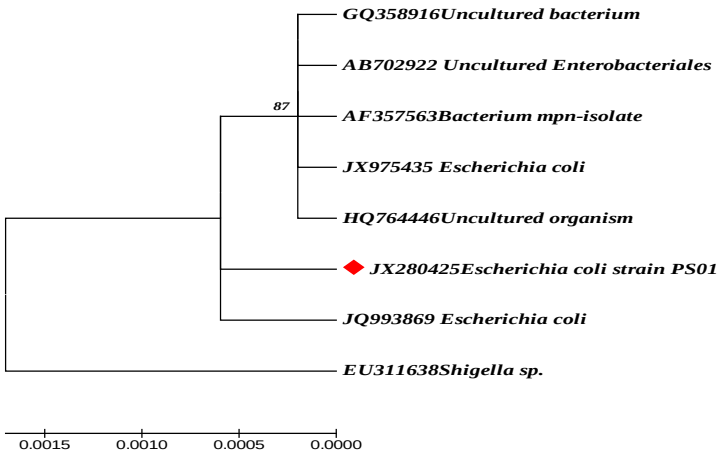
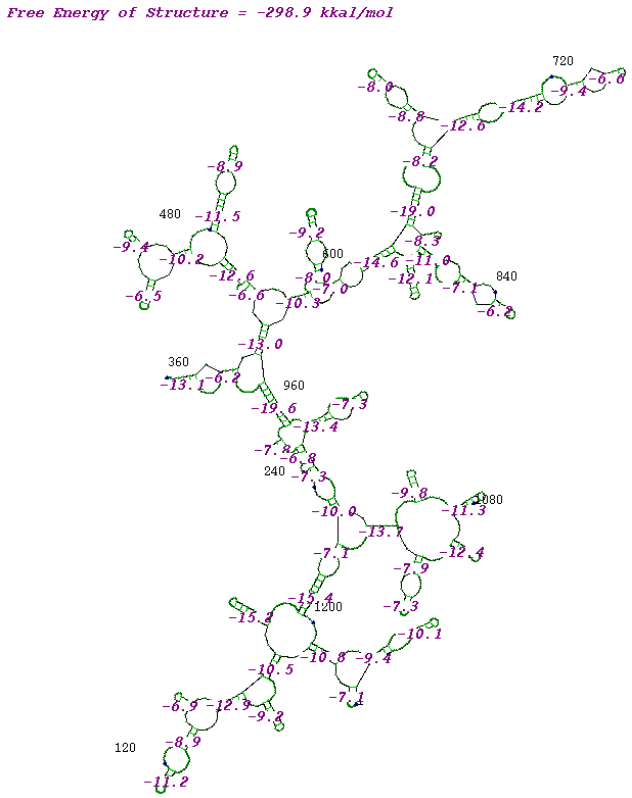


Fig.1 Phylogenetic tree of *E.coli sp* strain. The tree was constructed using the neighbor-joining method



Linear Sequence: Escherichia coli

Display : - All single cutter restriction enzymes
- Main non-overlapping, min. 100 aa ORFs

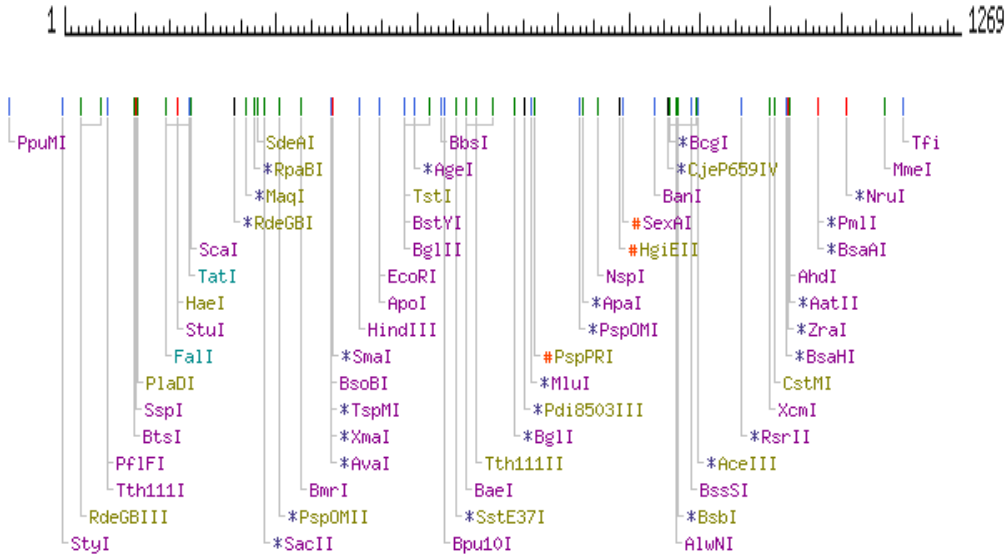


Table.2 Effect of various concentration of chromium on the growth of bacterial strains

Concentration mg /l	Growth in Isolate I	Growth in Isolate II
20	+	+
40	+	+
60	+	+
80	+	+
100	+	+
200	+	+

Table.3 Chromium reduction by the isolates

Concentration mg /l	OD values of The Isolates		% of chromium Reduction by the isolates	
	I	II	I	II
20	1.866	1.892	100	100
40	1.621	1.683	100	100
60	1.540	1.595	90.2	92.3
80	1.510	1.576	70.4	73.4
100	0.104	0.175	65.1	67.8
200	0.088	0.110	62.3	63.6

Reduction of chromium by the isolates

Significant difference in both growth rate and chromium reduction potential observed were shown in table 4. 100 % of reduction of chromium was observed in both strains 1 and 2 at concentration ranging from 20-40 mg/l. However by increasing the concentration of chromium ion the reduction ratio decrease significantly 63.6 % to Isolate II and 62.3 % of Isolate I concentration 200 mg/l. From the result it was observed a progressive decrease in growth with increasing chromium ion concentration was observed. Among the Isolates, Isolate II was found to be more potential for chromium reduction than the Isolate I.

Molecular characterization of the isolates

Identification of the bacterial strain by 16S r RNA Gene sequencing

In the present study it was evident that two bacterial strains such as Isolate I and Isolate II showing tolerance to chromium. The resistance of isolates to chromium ions was neither lost nor altered when isolates were stored in Nutrient agar at refrigerator temperature. A total of six chromium resistant bacteria were isolated from the tannery effluent in the present study. Two selected isolates according to their morphological shape were plated in medium amended with 40 mg/l of chromium ion.

It is imperative to keep its concentration low and bacterial process offer a strategy to extract and reuse of spent chromium. Hence two chromium resistant bacterial strains were isolated. These strains could grow in a very high concentration of potassium chromate, both in Nutrient agar and broth.

They could resist against potassium chromate at a high concentration in Nutrient agar medium as compared to broth. This may be due to the binding of metal ions by various components of culture media. Growth was better in chromate free Nutrient broth as compared to chromate supplemented Nutrient broth. Itoh *et al.,* (1994) reported that the viability of *E.coli* cells shows decreased in the presence of chromate. Genomic DNA was isolated from the pure culture. The PCR product was bi-directionally sequenced using the forward, reverse and primer. Sequence data was aligned and analyzed for finding the closest homolog for the microbe. The strain was named as *Escherichia coli* strain PS01.

In the present investigation two bacterial strains were isolated form tannery effluent collected from Dindigul. Based on the morphological, cultural characteristics and biochemical characteristics they were identified as *E. Coli* and *B. subtilis*. Both strains are capable of growing medium supplemented with chromium i.e., 40 mg/l. It shows that these organisms are resistant to chromium ions. MIC value of chromium was also determined. Both strains are significantly able to reduce chromium. Among the Isolates, Isolate II was found to be more potential for chromium reduction than the Isolate I. Chromium reduction Potential of this strains exhibit so that the strains would be useful for the treatment of industrial waste water.

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