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ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF CHROMIUM TOLERANT BACTERIA FROM INDUSTRIAL EFFLUENT AND THEIR EFFICACY STUDY

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ABSTRACT

All over the world soil and water is being polluted because of industrial waste. Chromium is a virulent heavy metal in environment becomes a serious problem. This is a major contaminant in industrial effluent and pollutes the soil and water as well. This study based on the isolation and identification of Cr resistance microorganisms isolated from industrial effluents collected from the heavy metal contaminated areas in and around the Visakhapatnam. Cr resistant microorganisms were isolated and the identification of the isolates were done by 16s rRNA sequencing followed by BLAST results confirming the novel *Bacillus benzoevorans* strain. For isolation 1% chromium nutrient agar medium was used. Total 12 bacteria were isolated from the soil samples. Only isolate-7 showed highest growth (OD-0.33 at 600 nm) at 5% Cr. The bacterial strain *Bacillus benzoevorans* was isolated and identified through molecular techniques. This novel Bacillus species would be helpful for the bioremediation of polluted soils. It is concluded from this study that the bacterial strains from soil samples and effluent use their innate ability to degrade pollutants like chromium is economically viable when compared to conventional methods.

(Key words: Chromium, isolation, bacteria, heavy metals, bioremediation)

INTRODUCTION

Many densely populated cities facing environmental pollution due to toxic heavy metals released in industrial effluents. Heavy metals are a massive term, which applies to the group of metals and metalloids with an atomic density higher than 4 g / cm^3 or 5 times or more higher than water (Paul and Sinha, 2017). In nature also, heavy metals are found at various background levels due to various concentrations in the bedrock. The presence of heavy metals in industrial effluence is known to have major hazard to natural water animal and human health.

Many toxic metals, among chromium is widely used in leather tanning, metal finishing and chromate preparation of all the heavy metals chromium (Cr) is the seventh most abundant metal in the soil (Katz and Salem, 1994). Naturally chromium exists in two different states either Cr (III) or as Cr (VI). Hexavalent chromium is known to be more toxic than the trivalent form. High levels of chromate in the environment also has and inhibitory effect on most organisms. Chromium affects the plant physiological function like inhibition of seed germination, nutrient balance, inductive oxidative stress (Barcelo and Poschenrieder, 1997; and Patra and Panda, 1998).

Heavy metal ions removal is not easy in the environment that are already contaminated, contrary to many other organic pollutants, heavy metals cannot be biologically or chemically degraded and therefore difficult to control (Ranjith Kumar and Mahalingam, 2016). The commonly used physico chemical methods for heavy metal removal include chemical precipitation, solvent extraction and membrane technology. Since, these all the methods are very expensive, therefore it is important to develop a low cost and ecofriendly method to remove the toxic heavy metals from the soil. Biosorption, a biological method for removal of heavy metal ions may provide and attractive alternative to physic-chemical methods (Kapoor and Viraraghavan, 1995).

The response of microorganisms towards toxic heavy metals is of importance in view of their interest in the reclamation of polluted sites. However, microorganisms have evolved resistance mechanism that led to the selection of resistant variants that can tolerate metal toxicity (Srinath and Verma, 2002). Till now many bacterial strains such as Bacillus, *Shewanella, microbacterium* etc., have been reported to reduce the toxic $Cr^{6+}$to the less toxic Cr^{3+}. Bioremediation using soil bacteria is regards as the most suitable technique since bacterial population can show

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resistance to as much as 500 mg l^{-1} of Cr (VI). Microorganisms provide higher surface area to the volume due to the small size and assimilate metals from surrounding settings (Zouboulis *et al*., 2004). However, the availability of an effective Cr^{6+} removing bacterial strain is an essential prerequisite for the developing bio remediation process.

Bacteria survive in polluted environment because they are metabolically capable of utilizing its resources from it and can occupy a suitable niche. Identification and characterization of suitable bacteria is useful for further applications in the field of bioremediation. A wide range of bacteria have been reported for reducing or transforming Cr (VI) to Cr (III) under aerobic and anaerobic conditions. e.g. *Bacillus* sps (Masood and Malik, 2011). Therefore, present study focussed on screening and characterization of Cr tolerant bacterial strains from the industrial waste for bioremediation. The objective of the study was to isolate the chromium tolerant bacterial strains from the industrial effluent, and to measure minimum inhibitory concentration of bacterial strains that can be used for bioremediation process.

MATERIALS AND METHODS

Sample collection

Isolation and enumeration of microorganisms

One gram of the mixed soil was added to 9 ml of deionized water and 1 ml of the diluted sample was spread by plating on nutrient agar medium from the appropriate dilution tubes and then incubated at the room temperature for 24 hours. The soil samples were collected from the ten contaminated areas from Parawada, Srikakulam, Visakhapatnam port area, Divis Laboratories getty etc. known to support the requirement and extent of pollutants made by varied industries, situated in Visakhapatnam. Total ten samples were collected from completely different spots to hide a high vary of soil metal concentration. The soil samples were collected aseptically in sterilized sealed pack bags and sieved through two millimetre pore size sieve. The collected samples were maintained at or below 4°C and were used for microbial enumeration immediately after collection.

Plates showing isolated colonies were estimated and the results were determined for each soil sample. Isolated colonies were plated on specific agars, which were used for identifying specific microorganisms in the contaminated soil. The colonies emerged after 1 week were streaked onto nutrient agar plates and stored the pure colonies at 4 ^{0}C. for further studies.

Characterization of isolates

Morphological characters of the isolates were studied. The macroscopic studies of microorganisms growing on agar medium were useful for rapid identification of their respective genus, which includes characters such as colony characteristics (configuration, surface, pigment, shape, elevation, colour margin and arrangement), absence or presence of aerial mycelium and extent of spore formation. Bergey's Manual of Determinative Bacteriology (1930) was followed for the structure resemblance and comparison for genus identification of purified isolates. Total 12 bacterial isolates were isolated.

Chromium tolerance estimation

All twelve bacterial isolates were tested for the chromium tolerance efficiency at various concentrations using spectrophotometer. Twelve bacterial isolates were inoculated individually in nutrient broth medium, after 24 hours of incubation 5% inoculum was inoculated in nutrient broth with gradient concentration of Chromium (0.5% to 5.0%). Inoculated vials were incubated in orbital shaker incubator at 37 ^{0}C for 72 hours. After 72 hours of incubation bacterial growth was calculated at 600 nm using spectrophotometer.

16s RNA sequencing analysis

Bacterial DNA isolation

2 ml overnight culture was taken and the cells were harvested by centrifugation for 10 minutes. 875 ìl of TE buffer was added to the cell pellet and the cells were resuspended in the buffer by gentle mixing. 100 ìl of 10% SDS and 5 ìl of Proteinase K were added to the cells. The above mixture was mixed well and incubated at 37 ºC for an hour in an incubator. 1 ml of phenol-chloroform mixture was added to the contents, mixed well by inverting and incubated at room temperature for 5 minutes. The contents were centrifuged at 10,000 rpm for 10 minutes at 4 ºC. The highly viscous jelly like supernatant was collected using cut tips and was transferred to a fresh tube. The process was repeated once again with phenol-chloroform mixture and the supernatant was collected in a fresh tube. 100 ìl of 5M sodium acetate was added to the contents and was mixed gently. 2 ml of isopropanol was added and mixed gently by inversion till white strands of DNA precipitates out. The contents were centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed and 1ml 70% ethanol was added. The above contents were centrifuged at 13,000 rpm for 10 minutes. After air drying for 5 minutes 100 ìl of TE buffer or distilled water was added. 10 ìl of DNA sample was taken and diluted to 1 or 2 ml with distilled water. The concentration of DNA was determined using a spectrophotometer at 260 / 280 nm. (Calomoris *et al.,* 1984).

PCR and sequencing

DNA sequence analysis for four bacterial isolates with potential activities was carried. DNA isolated from overnight grown cultures on nutrient broth, and amplification of DNA with two primers 27F and 907R were carried. The reaction conditions for PCR reaction was as fallows each reaction tube have 20µl of reaction mixture containing 1/10 volume 10× Taqbuffer, 2 mm MgCl2, 1 unit TaqDNA polymerase, 0.2 mMdNTP, 20 pmol primer (Table 2), and 10 ng soil DNA.DNA amplification was carried out in a Biorad Mini thermocycler with the following procedure: an initial denaturing step at 94°C for 1 min; 40 cycles for 1

min at 94 °C (denaturation), 1 min at 49°C (annealing), 2 min at 72°C (extension) and a final elongation step at 72 °C for 5 min. PCR products were separated by electrophoresis on 1.8% agarose gel containing 0.5 ìg/ml ethidium bromide, and photographed. The standard DNA samples (100 bp DNA ladder marker) were used as molecular size marker.PCR products were sequenced and the DNA sequences were analysed using BLAST tool, and MEGA software program for sequence analysis and alignment analysis (Altschul *et al.*,1990). Phylogenetic trees were constructed using UPGMA program and sequences were submitted in NCBI open access website.

Biochemical tests

Biochemical tests, which encompasses Indole utilization, Methyl red, Voges Proskauer, and Citrate utilization tests were carried out to identify the isolates up to their species level. Pure cultures of bacteria isolated were presumptively identified on basis of their morphological and biochemical characteristics. Species were identified as described by Buchanan and Gibbons (1974).

RESULTS AND DISCUSSION

Isolation of bacterial strains

An investigation of bacteriological quality assessment of effluent discharged from industrial waste in and around Visakhapatnam was carried out with a view to isolate and identify, the bacterial species present in the industrial effluent for bioremediation. The isolated Chromium tolerant strains were characterized by colony characteristics, morphology and physiology. From the results it was observed total 12 isolates in treated sample.

Table 1. Morphological and physical characters of isolated bacteria

Bacterial Isolate	Colony colour	Colony shape	Pigmentation	Grams Staining
1	Yellow	Round	No	+ rods
2	Cream	Irregular	No	+ rods
3	Yellow	Round	No	+ cocci
4	Pale yellow	Round	No	- cocci
7	White	Irregular	No	+ rods
8	Orange	Irregular	Yes	+ rods
9	White	Irregular	No	+ rods
10	Yellow	Round	No	+ rods
11	Pale yellow	Irregular	No	+ cocci
12	Pale yellow	Round	No	+ cocci

Table 2. Biochemical characteristics of isolated bacteria

S.no.	Biochemical tests	VPCR1	VPCR2	VPCR3	VPCR4	VPCR7	VPCR8	VPCR9	VPCR10	VPCR11	VPCR12
1	MR Test	-	-	-	-	-	-	-	+	+	+
2	VP Test	-	-	-	+	-	-	-	+	+	+
3	Citrate Test	-	-	-	-	-		+	-	-	-
4	Catalase Test	+	-	-	+	+	-	-	+	+	+
5	Starch	-	+	-	-	-	+	+	+	+	+
6	Nitrate Test	-	-	+	+	-	+	+	+	+	-
7	Salt Analysis	+	-		+	+	-	+	+	+	+
8	Motility (Hanging drop)	Non motile	Non motile	Motile	Non motile	Motile	Non motile	Non motile	Non motile	Non motile	Motilee

Figure 3. Effect of chromium on bacterial isolates

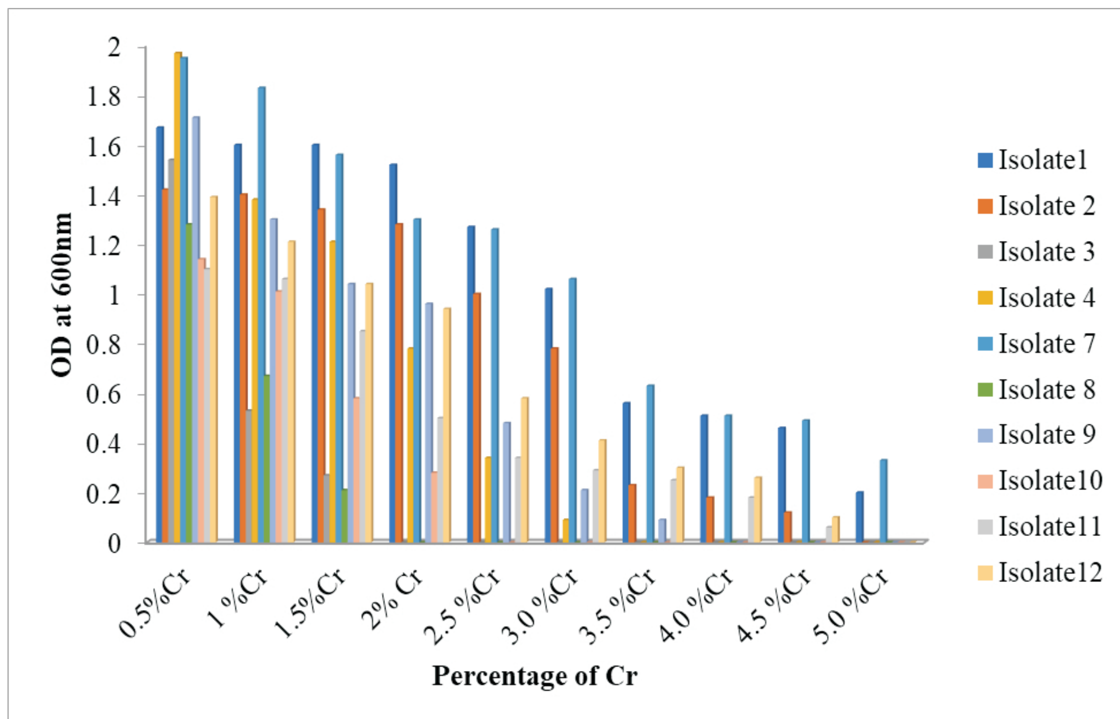

Table 1 shows the growth of bacterial colonies in serial dilution test tubes. Many colonies were found at lower concentration and finally 12 bacterial isolates could isolate for the further investigation. 5 and 6 isolates showed very poor growth, so that the results for these two isolates are not included. Isolate 1 had shown growth in catalase test and salt analysis. Isolate 2 showed good growth in starch test. Isolate 3 showed growth in nitrate test and salt analysis. Isolate 4 growth was good in VP test, catalase and nitrate tests and salt analysis. Isolate 7 growth was good in catalase test and salt analysis. Isolate 8 showed good growth in starch and nitrate tests. Isolate 9 growth was good in citrate, salt, starch and nitrate tests. Isolates 10 and 11 growth was good in all tests except for citrate test. Out of seven tests isolate 12 showed growth in five tests. In hanging drop technique isolates 3,7 and 11 observed as motile.

Chromium tolerance of isolated bacteria was assessed and selected bacteria reacted in different manner to different concentrations of chromium as illustrated in the above graph. Out of 12 bacterial strains one bacterial strain exhibited chromium tolerance to most of the chromium concentrations. From the above Table 2 it is clearly explaining that the out of 12 isolates on 12 different concentrations of chromium, isolate 1 and isolate 7 were grown. The two bacterial isolates were able to grow even at 5.0% Cr^{6+} both isolates showed a more or less similar pattern of growth in chromium containing media and decreased in biomass production. The isolates were characterized morphology and biochemistry and identified as *Bacillus benzoevorans*. The results indicated that Bacillus strain can significantly reduce chromium. The growth of bacterial isolates was studied in the presence and absence of Cr^{6+}.

Previous experiments taken from the references, explaining that 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).

The present study focused on the isolation of chromium tolerant bacteria. Reviews shown the effect of microorganisms such as bacteria (Srinadh *et al*., 2002), Fungi (Sudha Bai and Abraham, 2003) in biosorption studies. Other experimental evidence is Micrococcus species survived to a concentration 8,000 mg $l^{-1}$of Cr (VI) (Shankar Congeevaram *et al*., 2006). 16s rRNA gene sequence and nucleotide blast from NCBI revealed that the isolate- 7 was *Bacillus benzoevorans*. Our present sequence has shown 99.05% similarity with *Bacillus benzoevorans*.

It is concluded from this study that the bacterial strains from soil samples and effluent use their innate ability to degrade pollutants like chromium is economically viable when compared to conventional methods. The bacterial strain *Bacillus benzoevorans* was isolated and identified through molecular techniques. It has significant potential to reduce the hexavalent chromium which is useful for bioremediation process.

VPCR Isolate-7

TGCAAGTCGAGCGGACTTAAAAAGCTTGCTTTTTAAGTTAGCGGCGGACGGGT
GAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGGAG
CTAATACCGGATAATCCTTTCCTACTCATGTAGGAAAGCTGAAAGACGGTTTACGC
TGTCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCAC
CAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCT
GTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAAC
CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATG
TGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGC
AGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGG
AACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
GCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTC
CGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATG

16S rRNA sequence-based phylogenetic tree of *Bacillus benzoevorans*

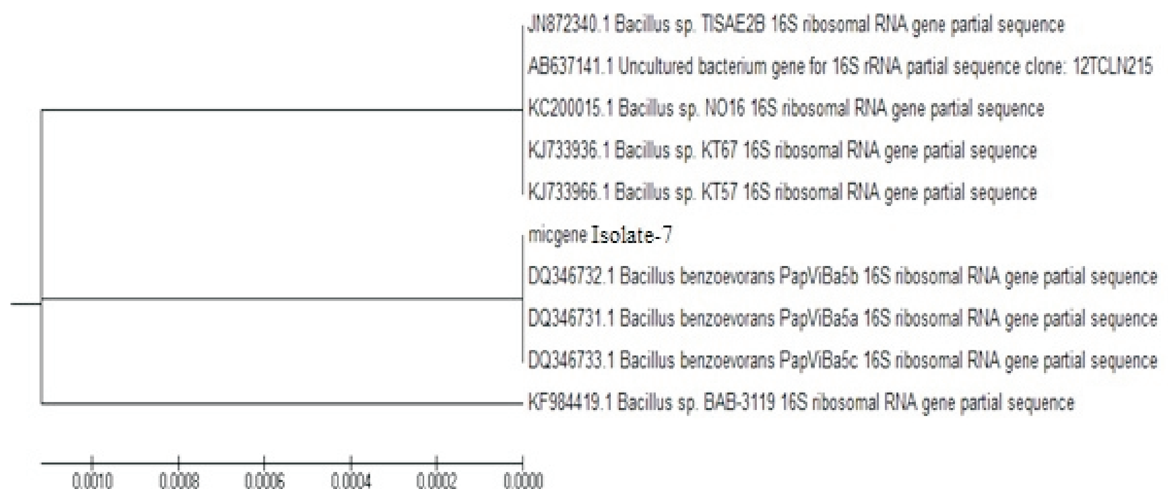

Figure 1. Nutrient agar with 1.5% chromium plates showing different chromium tolerant bacteria

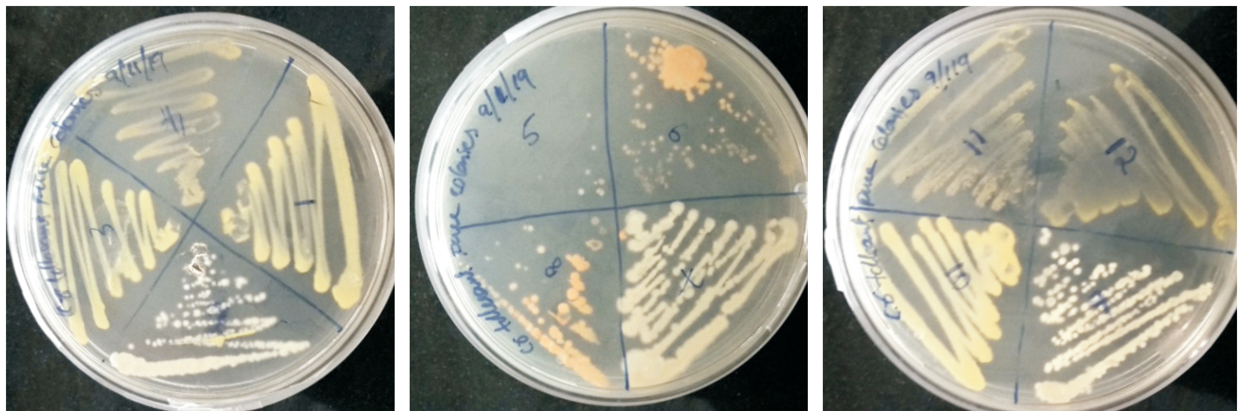

Figure 2. Pure colonies of chromium tolerant bacteria on nutrient agar medium

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