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Characterization of chromium remediating bacterium *Bacillus subtilis* isolated from electroplating effluent.

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ABSTRACT

Chromium, especially chromium (VI), is the main pollutant of the tannery and electroplating industries. Cr (VI) is said to be a hazardous pollutant as it is highly toxic, mutagenic as well a carcinogen. Cr (VI) is mobile as it is soluble in nature. In our present study, indigenous bacteria that are tolerant to high concentrations of Cr (VI) were isolated from chromium contaminated electroplating effluents which were collected and analyzed in different seasons of a year. Among many isolates of season 1, a highly chromium tolerant bacterial strain named PESA, was isolated from effluents of electroplating industries. This study, reports the tolerance ability of the bacteria as well as the identification of the strain PESA by different morphological, biochemical and 16s rRNA gene analysis as *Bacillus subtilis* and the sequence of which is deposited with NCBI Genbank. The optimal pH value for chromium biosorption was found to be 3.0 and optimum temperature being 30-32^{o}C. It is found to reduce Cr to >50% upto 93 % in 10 days in a 500 mg/L concentration of media at pH 3.0. Hence the organism has great potential for cleaning of Cr (VI) polluted effluents.

Keywords: **Hexavalent chromium, chromate tolerant bacteria, electroplating effluent, *Bacillus subtilis*, 16 s rRNA sequencing.**

I. INTRODUCTION

Chromium pollution is due to large number of industrial operations like mining, petroleum refining, leather tanning, wood preserving, textile manufacturing, chrome plating and electroplating industries (Wang, 1995). Chromate is a strong oxidizing agent that can be reduced intracellularly into Cr 5+ and this reacts with nucleic acids and other components of the cell to produce mutagenic and carcinogenic effects in humans and animals. (Mc lean and Beveridge, 2001; Clark, 1994).This can be further reduced to trivalent chromium Cr (III) which is stable, less soluble and less toxic. However Cr (VI) is a very toxic form and more hazardous as it is known to cause many health effects like skin infections or rashes, stomach upset and ulcers, respiratory problems, kidney and liver damage, alteration of genetic damage, lung cancer and may also cause death. World Health Organization has recommended maximum allowable concentration of 0.05mg/L in drinking water for Cr (VI). According to the Indian Standard Institution, the desirable limit for Cr (VI) in drinking water is 0.05mg/L and With reference to Central pollution control board, the allowable chromium concentration in effluents is 2.0-5.0 mg/L.

There are many methods by which Cr (VI) can be removed, some of the methods being chemical precipitation, membrane processing, ion exchange, liquid extraction and electro dialysis (Verma et al, 2006). These methods are quite expensive and have many other disadvantages like generation of toxic sludge and other waste products, high energy requirements and incomplete metal removal. These days thus bioremediation is the best choice of treatment and much work is in progress in identifying the bacterial strains for removal of Cr contaminated effluents and use of adsorption technique where many different forms of bioadsorbants are prepared for removal of the heavy metal and this method is said to be efficient and cost effective (Li et al, 2007). Bacterial and fungal remediation has been worked out in various aspects. There is a strong correlation between the chromium content and the number of metal resistant and tolerant bacteria in the soil (Viti and Giovannetti, 2001).

The genus *Bacillus* was identified for the first time in soil contaminated with Cr (VI) in 1995 (Campos et al, 1995; Wang and Xiao, 1995). There is presence of chromate-reductase in *Bacillus* (Campos Garcia et al, 1997). Some bacterial strains such as *Pseudomonas sp* (Bopp and Erlich, 1988; Cervantes and Ohtake, 1988), *Enterobacter cloacae* (Wang et al, 1989), *Arthrobacter sp* (Megharaj et al, 2003), and *Bacillus sp* (Megharaj et al, 2003; Camergoetal, 2005) are resistant to Cr (VI). Some chromate resistant bacteria use Cr (VI) as an electron donor, reducing it to Cr (III) (Wang and Shen, 1995). The ability of chromate resistant bacteria to reduce Cr (VI) varies from species to species (Cervantes et al, 2001). Chromium resistant bacteria have been isolated from tannery effluent (Basu et al, 1997; Shakoori et al, 2000), discharge water (Campos et al, 1995), activated sludge (Fransisco et al, 2002), electroplating effluent (Ganguli and Tripati, 2002). All these have shown high potential for the removal of toxic chromium.

The main objective of the present study is to isolate and characterize the microbial populations from 5 different electroplating industrial effluents collected in different seasons of a year, located in Peenya Industrial area, Bangalore, India. The tolerance ability of the organisms were recorded by growing the bacterial strain in different Cr(VI) concentration provided medium and was characterized and identified by 16 s rRNA sequencing. The optimal conditions like pH and temperature for growth pattern were standardized and percentage of remediation at different parameters was also recorded.

II. MATERIALS AND METHODS:

2.1: Sampling:

Effluent samples were collected in sterilized polysterene bottles of one liter capacity from 5 electroplating industries of Peenya Industrial area, Bangalore, India for four seasons of the year.100ml of samples were preserved immediately by acidifying with concentrated nitric acid, stored at 4 degree. It is then subjected to chromium analysis after nitric acid digestion and analyzed using AAS (chemito AA201) to find out the chromium concentration (APHA 2005). Other samples were preserved at 4^{o}C for microbial analysis.

2.2 Isolation of chromium tolerant bacteria by Enrichment:

For isolation and enumeration of bacteria, 1ml samples were inoculated in 100ml Luria Bertani (LB) broth medium (1% tryptophan, 0.5 % yeast extract, 1% NaCl, pH 7) supplemented with 100 mg/L concentration of Cr (VI) as Potassium dichromate and grown in a shaker incubator at 30^{o}C with 160 rpm for 5 days. The culture was then plated on LB agar plates supplemented with similar Cr concentration using spread plate method and incubated at 30^{o}C for 24 to 48 hours. Initially the bacterial isolates were identified on the basis of colony morphology, gram staining and biochemical tests. Total number of chromate tolerant bacteria was expressed as CFU/ml. A single strain which is gram positive rod with endospore was capable of growing at this condition and was selected for further experiments. The isolate was preserved in LB slants at 4^{o}C as well in glycerol stocks at -20 ^{o}C.

2.3 Heavy metal tolerance and Biosorption experiments:

The isolate was tested for chromium tolerance by growing in LB broth containing

different concentrations 100,200,400,600,800 mg/L of Cr (VI) Supplemented as potassium dichromate. For the study of tolerance, the cell growth was monitored as absorbance at 600nm using a UV-Vis Spectrophotometer at several interval of time like 24, 48, 72 and 96 hours. For biosorption studies, at the same intervals of time, 5 milliliters of culture solution was taken and then centrifuged at 8000 rpm for 8 mins. The supernatant was analyzed for Cr (VI) concentration. The concentration of chromium was determined using Atomic Absorption Spectrophotometer (Chemito AA201) after nitric acid digestion pretreatment.

2.4 Identification of chromium resistant bacteria:

The bacterial isolate was identified by 16s r RNA sequencing as follows: Bacterial colony grown on Luria-Bertani agar was inoculated into Luria Bertani broth for 24 hours at 37℃. Cells were pelleted down by centrifugation at 8000rpm for 10 minutes .Genomic DNA was isolated from the culture using GeneiUltrapure TM Bacterial Genomic DNA Purification KT 162**.** Using consensus primers, approximately 1.5 kb sized 16S rDNA fragment was amplified by using *Taq* DNA polymerase. **U**sing the forward, reverse and an internal primer, the PCR product was sequenced**.** Sequence data was aligned and analyzed to find the closest homologous microbes using BLAST tool.

2.5 Effect of pH and Temperature:

The influence of pH on the microbial growth and chromium reduction was investigated at different pH values 3.0, 5.0, 7.0, 9.0 and 11.0 by adjusting the media with 0.1N NaOH or 0.1N Hcl with 500 mg/L Cr. 1 ml log phased culture inoculum was inoculated and incubated at 30^{o}C in 150 rpm. The influence of temperature on microbial growth and chromate reduction were investigated at different temperatures like 28, 32, 35 and 40 ^{o}C in shaking incubators.

III. RESULTS AND DISCUSSION

3.1 Sampling:

Samples were collected and stored at 4^{o}C for analysis.

3.2 Isolation of chromium tolerant bacteria by enrichment:

The bacterium isolated from enrichment of effluent was found to be Gram positive rod with endospore. This bacterial strain was designated as PESA. The morphological characteristics and biochemical tests of strain PESA are shown in table 1.

Table 1: Morphological and Biochemical characteristics of strain PESA

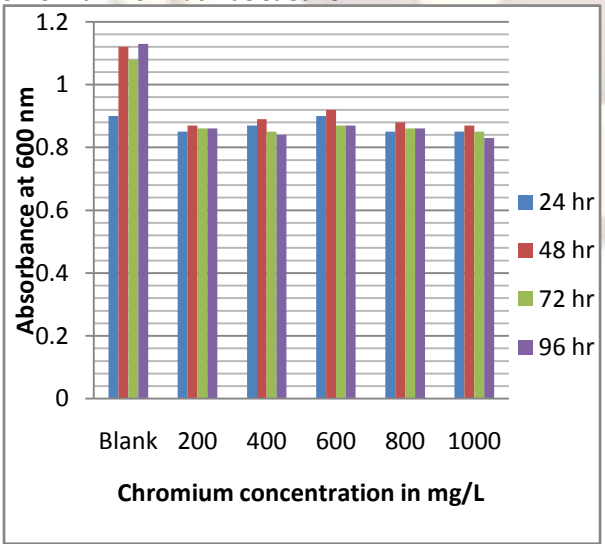
Morphological characteristics	***Bacillus subtilis***
Form	Irregular
Elevation	Flat
Margin	Undulate
Color	White

Biochemical characteristics	***Bacillus subtilis***
Gram's Staining	Gram positive rods (bacillus)
Endospore Staining	Positive with oval endospore
Negative Staining	Positive
Motility Test	Positive
Oxidase Test	Negative
Catalase Test	Positive
Indole Test	Negative
Methyl Red Test	Negative
Voges-Proskauer test	Positive
Citrate Utilization Test	Positive
Starch Hydrolysis Test	Positive
Glucose Test	Negative
Gelatinase Test	Positive

3.3 Heavy metal tolerance and Biosorption studies:

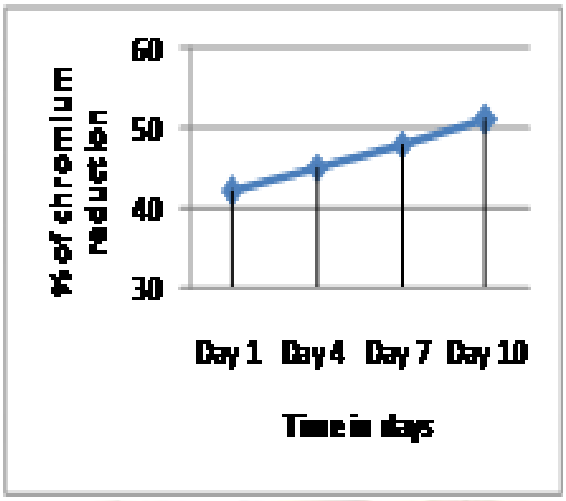
The absorbance value at 600nm of PES A strain subjected to tolerance studies at varied chromium concentration at different time intervals is plotted in graph 1 with a reference to a blank.

Graph 1. Graph of growth in O.D. vs conc. of chromium for *Bacillus subtilis*



To analyze accumulation or biosorption 1ml culture from each of the flask was centrifuged at 6000 rpm for 10 minutes at 4℃ and the supernatant was analyzed for Cr (VI) concentration using Flame Atomic Absorption Spectrophotometry after pre-treatment of digestion with nitric acid. The percentage of remediation increased from day 1 to day 10 and PES A was found to remediate > 50% of chromium in the medium and the results are plotted (graph 2).

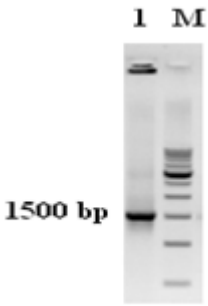
Graph 2. Percentage of Chromium reduction by PES A



3.4 Identification and Characterization of chromium resistant bacteria:

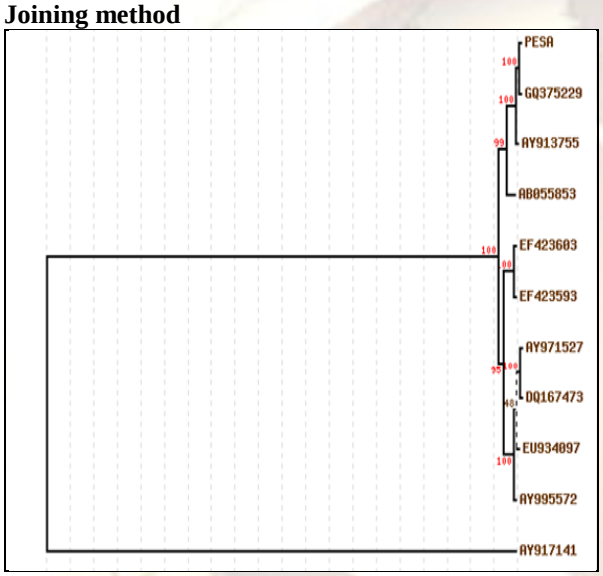
The molecular characterization was done for the isolate PES A which showed maximum tolerance to chromium and remediation ability . The bacterial genomic DNA was extracted from the isolate, purity was checked and quantified using UV- spectrophotometer and approximately 20-50ng of DNA was used as template in PCR reaction using the universal primer. By the use of these universal primers 1.5kb gene fragment was isolated and visualized on 2% agarose gel as shown in fig 1. The purified PCR product was subjected to 16 s r RNA sequencing by automated DNA analyzer. Sequence was recorded and analyzed. BLAST program was used to identify and download the nearest neighbor sequences from the NCBI database. The sequences was aligned using clustal W 1.6 program and based on nucleotide homology and phylogenetic analysis the microbe Sample PES A was detected to be *Bacillus subtilis (*fig 2). The sequence of the same is deposited in NCBI Genbank (accession number JX081251).

Fig. 1: Agarose gel showing 1.5 kb 16S rDNA fragment



Lane 1: Sample PESA
M: Marker- StepUpTM 500bp DNA ladder

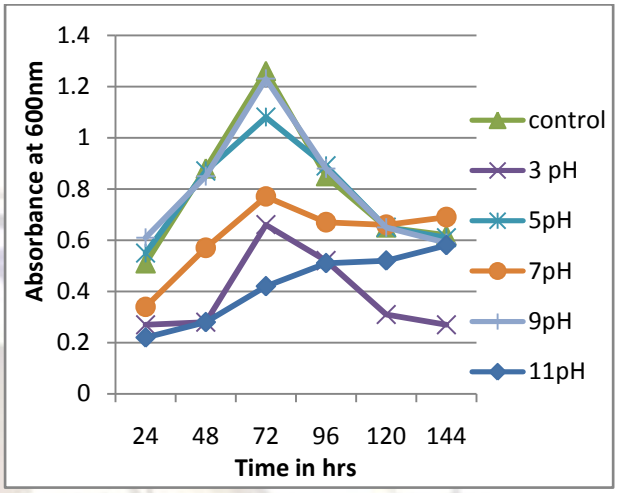
Fig. 2. Phylogenetic Tree made using Neighbour Joining method



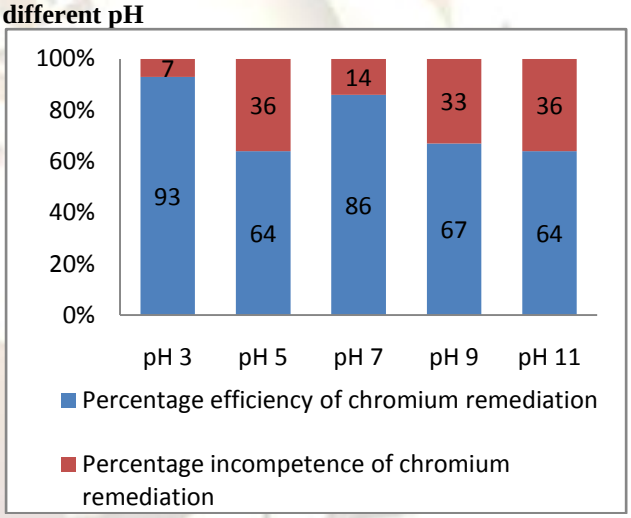
3.5 Effect of pH and Temperature:

A comparative analysis of the growth of *B. subtilis* at various pH revealed that the growth is maximum in media without chromium at pH 7. From the graph 3 it is observed that the growth is moderately high in media set at pH 7 and pH 9. The growth at pH 3, pH 5 and pH 11 is moderately low is shown in graph 3. The percentage efficiency of accumulation of chromium by bacterial strain inoculated in media set at pH 3 was observed to be the highest. The percentage efficiency of chromium remediation at pH 3.0 by *B. subtilis* was 93% and is as shown in graph 4.This suggests that chromium remediation is facilitated and augmented by a highly acidic pH. The presence of chromium reductase in the bacterial strain is responsible for this remediation. It has been found that the activity of chromium reductase is high in acidic conditions (pH 2 to 3) and therefore chromium remediation is better under acidic conditions. A moderate growth and highest biosorption percentage of Cr being 50-55 % was observed at temperature range of 28-32 ^{o}C and the results are shown in graph 5, 6.

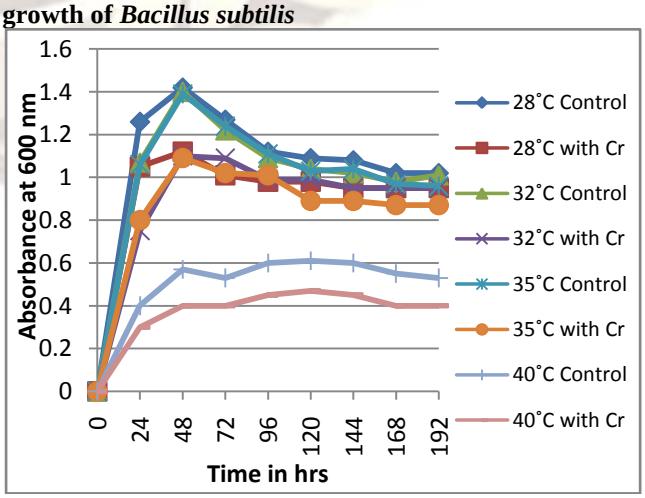
Graph 3. Effect of different pH on chromium reduction at 30^{o}C



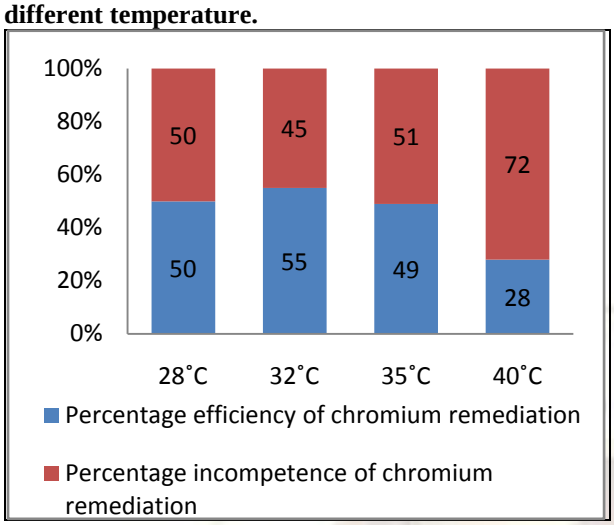
Graph 4. Percentage of chromium remediation at different pH



Graph 5: Effect of different temperatures on growth of *Bacillus subtilis*



Graph 6: Percentage of chromium remediation at different temperature.



IV. CONCLUSION

The study establishes the role and efficiencies of *Bacillus subtilis*, in the absorption, accumulation and remediation of chromium. Heavy metals can be toxic to microorganisms and to humans alike due to their strong affinity to form complexes with the cell membrane constituents, causing loss of integrity and impairment of their functions. However, microbial resistance to heavy metals is attributable to a variety of detoxifying mechanisms developed by using resistant microorganisms. The heavy metal resistant organisms could be potential agents for bioremediation of heavy metal pollution.

The study revealed the capacity of the bacterial strains to tolerate and grow at different concentrations on chromium (VI). Chromium (VI) concentration can be an important environmental factor regulating tolerance to the metal. The bacterial strains exhibited optimal growth at high chromium (VI) concentration. The strain, which can tolerate up to 500 mg/L of chromium (VI), having remediation ability upto 93% at optimized conditions can be effective in remediation strategies for ecosystem polluted with hexavalent chromium.

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