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Application of Genetic Engineering for Chromium Removal from Industrial Wastewater

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Abstract**—**The treatment of the industrial wastewater can be particularly difficult in the presence of toxic compounds. Excessive concentration of Chromium in soluble form is toxic to a wide variety of living organisms. Biological removal of heavy metals using natural and genetically engineered microorganisms has aroused great interest because of its lower impact on the environment. *Ralston metallidurans*, formerly known as *Alcaligenes eutrophus* is a L-Proteobacterium colonizing industrial wastewater with a high content of heavy metals. Tris-buffered mineral salt medium was used for growing *Alcaligenes eutrophus* AE104 (pEBZ141). The cells were cultivated for 18 h at 30 ^{o}C in Tris-buffered mineral salt medium containing 3 mM disodium sulphate and 46 mM sodium gluconate as the carbon source. The cells were harvested by centrifugation, washed, and suspended in 10 mM Tris HCl, pH 7.0, containing 46 mM sodium gluconate, and 5 mM Chromium. Interaction among induction of *chr* resistance determinant, and chromate reduction have been demonstrated. Results of this study show that the above bacteria can be very useful for bioremediation of chromium from industrial wastewater.

Keywords**—**Chromium, Genetic Engineering, Industrial Wastewater, Plasmid

I. INTRODUCTION

MOST heavy metals are well-known toxic and carcinogenic agents and when discharged into the wastewater represent a serious threat to the human population and the flora and fauna of the receiving water bodies. Living organisms require trace amounts of some heavy metals, including cobalt, copper, iron, manganese, molybdenum, vanadium, strontium and zinc. Excessive levels of essential metals, however, can be detrimental to the organism. Non-essential heavy metals of particular concern to surface water systems are cadmium, chromium, mercury, lead, arsenic and antimony. Heavy metals which are relatively abundant in the Earth's crust and frequently used in industrial processes or agriculture are toxic to humans. These can make significant alterations to the biochemical cycles of living things [1]. Most of the point sources of heavy metal pollutants are industrial wastewater from mining, metal processing, tanneries, pharmaceuticals, pesticides, organic chemicals, rubber and plastics, lumber and wood products, etc. [1]-[5].

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The heavy metals are transported by runoff water and contaminate water sources downstream from the industrial site. All living things including microorganisms, plants and animals depend on water for life. Heavy metals can bind to the surface of microorganisms and may even penetrate to the inside of the cell.

The treatment of the industrial wastewater can be particularly difficult in the presence of toxic compounds. Chromium is largely present in the industrial wastewater coming from tanning industry, electroplating industry, metal fabrication and finishing industry, textile dyeing industry, steel industry and wood preservation [6]-[8]. Both Hexavalent Chromium Cr(VI) and Trivalent Chromium Cr(III) exist in wastewater, but Cr(III) is 500 times less toxic and less soluble than Cr(VI) [9]-[12]. Excessive concentration of Chromium in soluble form is toxic to a wide variety of living organisms, from bacteria to humans. Chromium is a known mutagen, with Cr(VI) causing mitotic inhibition, reduction of cell growth and cell death. Chromium is considered by IARC as a powerful carcinogenic agent that modifies the DNA transcription process causing important chromosomic aberrations. In humans, it causes irritation and corrosion of skin and respiratory tract and is suspected to be responsible for lung carcinoma. Chromate is also hazardous to flora and fauna in natural aquatic ecosystem [13]-[18].

Due to severe toxicity of Cr(VI), the Agency for Toxic Substances and Diseases Registry (ATSDR) classifies Cr(VI) as the top eighteenth hazardous substance and the Minimal National Standards (MINAS) upper limit of Chromium in industrial wastewater is 0.1 mg/L. The USEPA has set the maximum contaminant level for Cr(VI) in domestic water supplies to be 0.05 mg/L [19]. Hexavalent Chromium toxicity to wastewater treatment system is significantly influenced by abiotic variables such as salinity, pH and temperature of water and is not removed from the wastewater by conventional treatment systems and strongly reduces microbial activity of the wastewater bodies [20]-[22].

Several physico-chemical methods have been widely used for Cr(VI) removal from industrial wastewater, such as ion-exchange, activated charcoal, chemical precipitation, chemical reduction, reverse osmosis, electrodialysis, ultrafiltration and adsorption etc. [23]-[26]. The conventional methods used for the treatment of heavy metals from industrial wastewater present some limitations. There are still some common problems associated with these methods such as incomplete metal removal, high reagent and energy requirement, cost-expensiveness and can themselves produce other waste products that require careful disposal, which in turn have limited their industrial applications [27]-[29].

II. Materials and Methods

Ralstonia metallidurans, formerly known as *Alcaligenes eutrophus* and thereafter as *Ralstonia eutropha*, is a L-Proteobacterium colonizing industrial sediments, soils or wastes with a high content of heavy metals. The type strain CH34 carries two large plasmids (pMOL28 and pMOL30) bearing a variety of genes for metal resistance. A chronological overview describes the progress made in the knowledge of the plasmid-borne metal resistance mechanisms, the genetics of *R. metallidurans* CH34 and its taxonomy, and the applications of this strain in the fields of environmental remediation and microbial ecology. The sequence draft of the genome of *R. metallidurans* has now become available.

R. metallidurans strain contains at least eight determinants encoding resistance to heavy metals, located either on the bacterial chromosome or on one of the two indigenous megaplasmids pMOL28 and pMOL30. Chromate resistance in *R. metallidurans* is based on chromate efflux catalyzed by ChrA efflux pumps. The bacterium harbours two chromate resistance determinants, the previously known chr_1 on plasmid pMOL28 (genes *chrI*, $chrB_1$, *chrA1*, *chrC*, *chrE*, $chrF_1$) and chr_2 on the chromosome (genes $chrB_2$, $chrA_2$, $chrF_2$). *R. metallidurans* strains AE128 (pMOL30) and AE126 (pMOL28) contain only one of the two megaplasmids, respectively and strain AE104 is plasmid free. The plasmid borne character of the resistance to Cr(VI), Cd(II), Co(II), Cu(II), Mn(II), Ni(II), Hg(II) and Zn(II) [30].

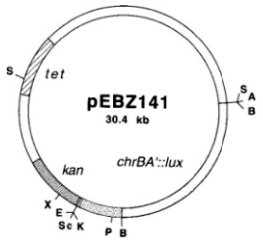

Fig. 1 Map of the chromate sensor plasmid pEBZ141. The genes encoding resistances to tetracycline (*tet*) and kanamycin (*kan*) and the *chrBA'*::*lux* gene fusion are indicated. Restriction endonucleases were *Sal*I (S), *Xba*I (A), *Bam*HI (B), *Pst*I (P), *Kpn*I (K), *Sac*I (Sc), *Eco*RI (E), and *Xho*I (X).

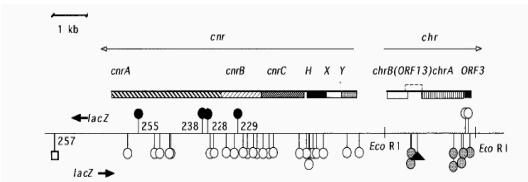

Fig. 2 Insertion points of Tn*5*-*lacZ* transposons in the *cnr*-*chr* region of plasmid pMOL28

A. Bacterial Strains and Growth Conditions

The bacterial strains and the plasmid used in this study have been mentioned. Tris-buffered mineral salt medium was used for growing *Alcaligenes eutrophus* and *Alcaligenes eutrophus* AE104 (pEBZ141). Solid Tris-buffered media contained 2% (w/v) agar [31].

B. Industrial Wastewater

Artificial industrial wastewater was composed of the following components (per litre):

10 mg of aniline, 5 mg of nitrobenzol, 10 mg of phenol, 2 mg of toluene, 50 mg of acetone, 50 mg of ethanol, 100 mg of isopropanol, 300 mg of methanol, 29 mg of urea, 6.5 g of chloride anions, 138 mg of nitrate anions, 1.7 mg of phosphate anions, and 3.5 g of sulphate anions. The artificial industrial wastewater resembles in its composition from that of an industrial plant [30].

C. Chromium Reduction and Uptake

The cells were cultivated for 18 h at 30 ^{o}C in Tris-buffered mineral salt medium containing 3 mM disodium sulphate and 46 mM sodium gluconate as the carbon source. The cells were harvested by centrifugation, washed, and suspended in 10 mM Tris HCl, pH 7.0, containing 46 mM sodium gluconate, and 5 mM Chromium. The hexavalent Chromium in the supernatant was measured with diphenylcarbazide as described in the literature.

III. Results and Discussion

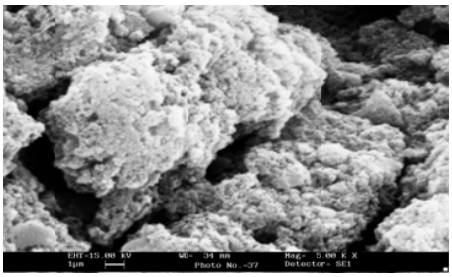

Fig. 3 SEM image of Chromium deposition of AE104 (pEBZ141) on Granulated Activated Carbon (GAC)

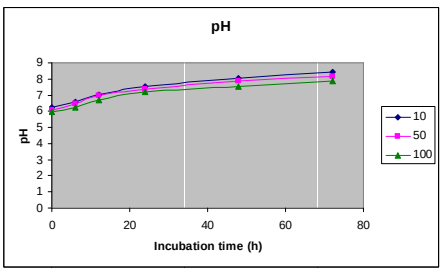

Fig. 4 pH for different Chromium concentration for *Alcaligenes eutrophus* AE104

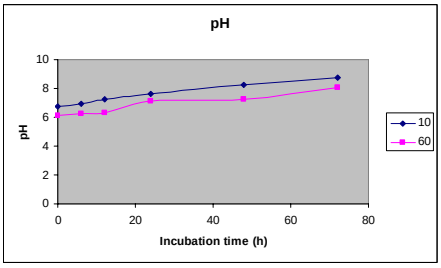

Fig. 5 pH for different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

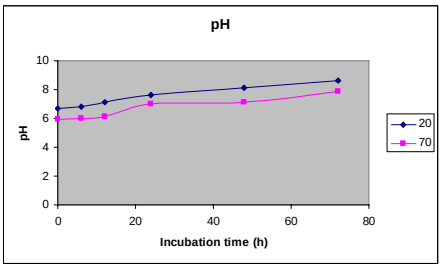

Fig. 6 pH for different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

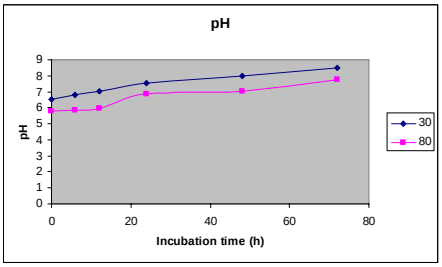

Fig. 7 pH for different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

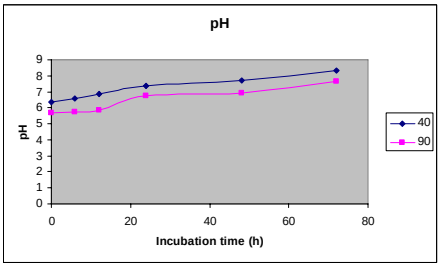

Fig. 8 pH different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

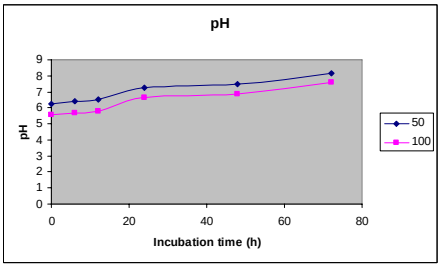

Fig. 9 pH for different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

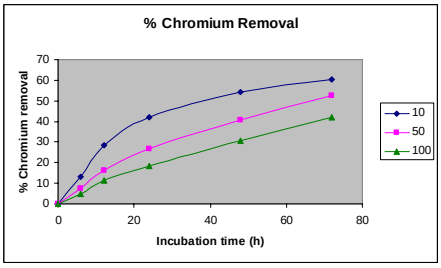

Fig. 10 % removal for different Chromium concentration for *Alcaligenes eutrophus* AE104

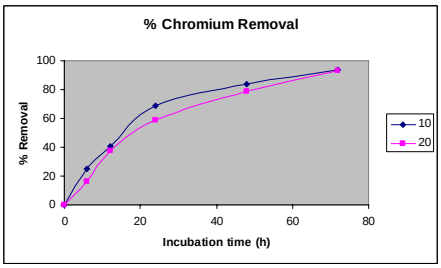

Fig. 11 % removal for different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

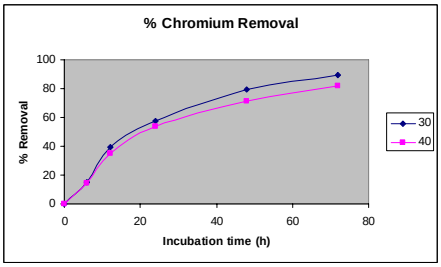

Fig. 12 % removal for different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

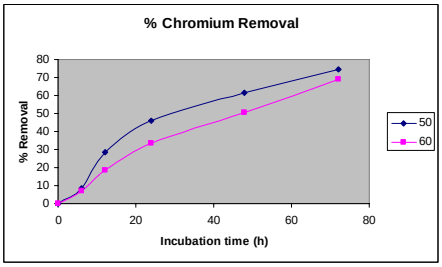

Fig. 13 % removal for different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

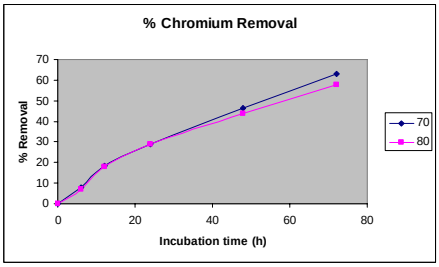

Fig. 14 % Chromium removal for different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

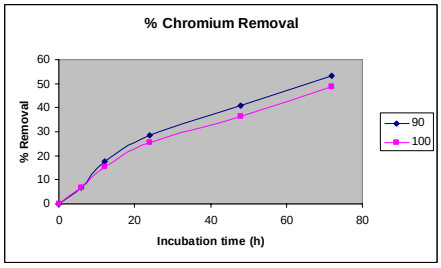

Fig. 15 % Chromium removal for different Chromium concentration for *Alcaligenes eutrophus* AE104 (pEBZ141)

Configuration:	1 (0.1A)	Counts:	83270
Cell Type:	Magnetic	S.N.F.:	1.00
Sample Type:	Regular	S.D.U.:	3760
Acq. Range:	0 - 300	Solids:	2.67e-005 %
Acq. Mode:	S.Size(2)	Conc.:	1.20e+006 #/ml
Acq. Time:	75	Sp.Area:	7.21e+004cm²/ml

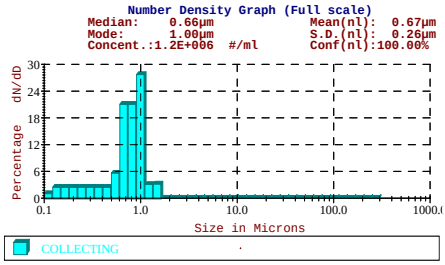

Fig. 16 Particle size analysis of the treated industrial wastewater sample of Chromium concentration of 50 mg/L after 72 h incubation time by *Alcaligenes eutrophus* AE104

Configuration:	1 (0.1A)	Counts:	63327
Cell Type:	Magnetic	S.N.F.:	1.00
Sample Type:	Regular	S.D.U.:	96
Acq. Range:	0 - 300	Solids:	4.26e-006 %
Acq. Mode:	S.Size(2)	Conc.:	1.75e+004 #/ml
Acq. Time:	1474	Sp.Area:	1.64e+004cm²/ml

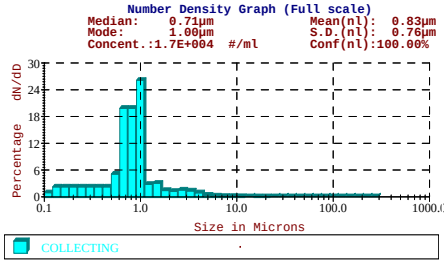

Fig. 17 Particle size analysis of the treated industrial wastewater sample of Chromium concentration of 50 mg/L after 72 h incubation time by *Alcaligenes eutrophus* AE104 (pEBZ141)

A. Cell growth

Bacterial growth was measured by measuring optical density at 540 nm using UV-Visible spectrophotometer (Perkin Elmer model Lambda 35). Optical densities have been measured at every two hours interval time till one day. The maximum growth was observed during first 8-20 h acclimatization time. Recombinant cells showed higher growth rate in Nutrient Broth medium than in Nutrient Agar. The plasmid free strain *Alcaligenes eutrophus* AE104 had taken 24 h of cultivation time to reach stationary phase where as recombinant bacterium *Alcaligenes eutrophus* AE104 (pEBZ141) came to stationary phase in 18 h.

B. Calibration curves

The calibration curves have been plotted by measuring absorbance by spectrophotometer for different Chromium concentrations from 0 to 100 mg/L using diphenylcarbazide method. The Potassium Dichromate stock solution has been prepared by dissolving 141.4 mg of Potassium Dichromate in 1 L of distilled water. The 10 ml of Potassium Dichromate stock solution has been diluted 10 times to prepare 100 mL of Potassium Dichromate standard solution. 1 mL of the above standard solution is equivalent to 5 µg Chromium.

The colour development is produced by transferring 95 ml of the extract into 100 ml volumetric flask and adding 2 mL of diphenylcarbazide. Sulphuric acid is added to get the pH value of 2.0 and distilled water is added to make up the volume upto 100 mL. The extract was allowed to stand for 10 min. for the development of full colour. The absorbance is measured at 540 nm using UV-Visible spectrophotometer. The calibration curves have been plotted by making different dilutions of Chromium. The absorbance of standard solutions of Chromium was nearly as reported in the literature. The liner equations and the R^2 values have been taken to calculate the concentration of the unknown Chromium in the treated wastewater.

C. Scanning electron microscopic image of bacteria

To get the Scanning Electron Microscopic images of the bacteria, washing is done to remove any foreign contaminant. Fixing is done with 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.2-7.4 for 24-48 hours. Washing is again done with 0.1M phosphate buffer for 15 minutes. The bacteria were rinsed with distilled water for 15 minutes. Various ethanol concentration were used for dehydration of the bacteria, first in 50% ethanol 20 minutes, then in 70% ethanol 20 minutes, in 80% ethanol 20 minutes, 90% ethanol 20 minutes, in 95% ethanol 20 minutes and finally in 100% ethanol for 2 hours. Samples were then ready for Critical Point Drying. It was dried in air for 15 min. These plates were analyzed by scanning electron microscope (SEM, U.K).

The SEM image of the 2-5 mm granulated activated carbon particles has been taken. The point-to-point length has been shown of the CAG particle in the image. The deposition of the Chromium on CAG alone has been taken at Chromium concentration of 50 mg/L. The Chromium deposition is clearly visible in the SEM image. The comparison of the deposition of

the Chromium has been taken after treatment of Chromium concentration of 50 mg/L after 72 hours of incubation time. The deposition on recombinant strain *Alcaligenes eutrophus* AE104 (pEBZ141) was more pronounced than that of plasmid free strain *Alcaligenes eutrophus* AE104 as shown in SEM images of Chromium deposition. This shows the enhanced absorption capacity of recombinant bacterium *Alcaligenes eutrophus* AE104 (pEBZ141) than that of plasmid free strain *Alcaligenes eutrophus* AE104.

D. Chromium concentration

The optical density of the wastewater after treatment with plasmid free strain grown on GAC for different Chromium concentrations of 10, 50 and 100 mg/l have been taken than those of recombinant strain for different Chromium concentration of 10-100 mg/L. The optical densities of the treated wastewater have been plotted at different incubation times of 0, 6, 12, 24, 48 and 72 hours. The optical density data has been found as expected. The concentration of Chromium in the treated wastewater was more when the initial concentration of the Chromium was more. The best results are obtained when the initial Chromium concentration was 10-20 mg/L.

The pH value of the treated wastewater has been found to be slightly more than the neutral range for the recombinant strain but more or less in the neutral range for the plasmid free strain as shown in fig. 4-9. The pH value increased with the increase in the incubation time from 0-72 hours but decreased with the increase in the Chromium concentration from 10-100 mg/L for the same incubation period of 0-72 hours as expected.

Based on the calibration curves for different Chromium concentration and the data from optical density measurement, Chromium concentrations in the treated wastewater have been calculated. The % removal of Chromium from the above graphs has been shown from fig. 10-15. It has been found that the % removal capacity of the plasmid free strain varied from 60.2 to 41.8 for Chromium concentrations of 10 and 100 mg/l respectively, while those of recombinant strain the % removal capacity varied from 93.8 to 48.7 for Chromium concentrations of 10 and 100 mg/L respectively. This showed that the Chromium sensor plasmid pEBZ141 has transformed the biofilm mechanism to enhance the Chromium uptake capacity.

The particle size analysis of the artificial industrial wastewater has been taken. The big particle sizes of the 1-10 µm showed the presence of big molecules of Chromium in the artificial wastewater. There is considerable reduction in the particle size of the treated wastewater of initial Chromium concentration of 50 mg/L after 72 h of incubation time for both the plasmid free and recombinant strain.

IV. Conclusion

Alcaligenes eutrophus AE104 (pEBZ141) may be readily used for the treatment of Chromium from industrial wastewater. The Chromate resisting process is highly specific. The results of percent Chromium removal as a function of incubation time for various Chromium concentrations have been plotted. Results show that the biosorption of Chromium increases with various Chromium doses with incubation time from 0 to 72 hours. The removal of Chromium ranges from 48% to 93% for the recombinant strain than those of 41% to 60% for the plasmid-free strain after the incubation period of 72 hours for various Chromium concentrations. It can be concluded that the rate of Chromium binding with the biomass increases gradually and remains almost constant after an optimum period. The obtained results are in good agreement with the previous results. As a result, it can be concluded that this strain can be used successfully in the removal and recovery of Chromium from the wastewater containing higher levels of Chromium ions. Further studies are needed to increase the biosorption capacities of biomass and to develop appropriate technologies applicable in the treatment of industrial wastewater.

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