Studies on the Bioremediation of Chromium (VI) Through Bioleaching by Thiobacillus ferrooxidans

VIJAYANAND. K1*, P. GANESH PRABU2, A. JAISON RATHINA RAJ1 AND ANANT ACHARY1

1,1* Department of Biotechnology, Kamaraj College of Engineering and Technology Virudhunagar - 626 001. Tamilnadu, India.
2 Department of Zoology, Annamalai University, Annamalainagar – 608 002, Tamilnadu, India.
1*Corresponding author: K. Vijayanand, E-mail: vijayvpk605@gmail.com

Received 27 June 2012; accepted 10 July 2012

Abstract
Chromium (VI) is the toxic substances that are released from the electroplating and the tannery industries, it is caused serious environmental problems and is toxic to organisms. These toxic substances are effectively remediated by microorganisms such as Thiobacillus ferrooxidans. Their toxic effect is minimized by these types of microorganisms. These properties can be effectively studied in Stirred Tank Reactor (STR). In STR of about 2 litres capacity 1 litre medium was used along with sludge. The sludge taken was 2%, 4% and 6% of Chromium (VI) respectively. The pH, temperature, air flow rate and few other parameters were kept constant varying only sludge concentration. Each sludge concentration was run for 10 days. The concentration of Chromium (VI) after the 10 day run showed that there was a feasible amount of Chromium (VI) leached. Chromium (VI) was leached higher in 2% sludge than in 4% and 6% sludge’s respectively.

Key Words: Bioremediation, Chromium (VI), Bioleaching, Thiobacillus ferrooxidans.

Introduction
Bioremediation is the use of microorganism metabolism to remove pollutants. Technologies can be generally classified as in situ or ex situ. In situ bioremediation involves treating the contaminated material at the site, while ex situ involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are phytoremediation, bioventing, bioleaching, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration and biostimulation. Bioremediation can occur on its own (natural attenuation or intrinsic bioremediation) or can be spurred on via the addition of fertilizers to increase the bioavailability within the medium (biostimulation). Recent advancements have also proven successful via the addition of matched microbe strains to the medium to enhance the resident microbe population's ability to break down contaminants. Microorganisms used to perform the function of bioremediation are known as bioremediators.

Due to industrial expansion, large quantities of industrial wastes are accumulating in many countries and cannot be disposed without prior special treatments. In particular, waste products from the mining and metal refining industries, sewage sludges and residues from power station and waste incineration plants can contain heavy metals at high concentrations. Usually, these heavy metals can be leached from the soil to the surface water system (Chang et al. 1984; Dowdy et al. 1991) at concentrations higher than they are allowed (CEC, 1986). For this reason, they cannot be disposed into wastewaters plant and must be submitted to special treatment in order to reduce metals contents. Therefore, the management of waste sludge produced from the industrial activity becomes the most important issue of environmental protection. Among heavy metals presents in sludge, chromium is one of the most common. This metal exists in two stable oxidation states, trivalent and hexavalent chromium. The trivalent chromium state is less toxic and mobile, while hexavalent chromium is easily soluble and 100 fold more toxic than trivalent chromium. So, the reduction of Chromium (VI) to Chromium (III) is an attractive and useful process for remediation of Chromium (VI) pollution, and the technologies focusing on transformation of Chromium (VI) to Chromium (III) have accordingly received much more concerns. Many studies have reported that hexavalent chromium could be reduced into trivalent chromium by chemical methods (Panswad et al. 2001; Erdem 2006; Guo et al. 2006; Souza et al. 2006). Although these physical and chemical treatment techniques have been extensively applied in practice, they show some limitations such as low...
efficiency and high cost (Rulkens et al. 1995). Generally, biotechnology is a powerful and versatile alternative to chemical and physical methods for resolving many problems of environmental pollution because of low demand of energy and materials, and low generation of waste and emissions. Recently, biological chromium (VI) reduction has been developed with some advantages due to the lower costs and the significant smaller quantities of the produced sludge (Sisti et al. 1996; Ganguli and Tripathi 1999; Konovalova et al. 2003; Liu et al. 2006). Industrial residues can be finally disposed and even utilized as fertilizer on agricultural lands (Davis 1987; Scheltinga 1987). However, if their metal content of metals as Zn, Al, Mn, Ni or Cu is high (Sreekrishan et al. 1996; Tichy et al. 1998; Chen et al. 2004), previous leaching to decrease the content below the guidelines set for each country should be done. Bioleaching process is largely documented in literature (Porro et al. 1990; Krebs et al. 1997; Gadd 2000; Solisio et al., 2002) it could be to the extraction of metals from various ore concentrates (Ebner et al., 1978; Veglio et al., 1999) but it has been recently developed to remove heavy metals from sludges, sediments and soils containing metals at high concentrations (Sreekrishan et al. 1996; Tichy et al. 1998; Chen et al. 2004).

Chromium is used in different industrial processes and released into the environments. Leather processing industries (LPI) use chromium material (chrome liquor or chrome powder) for tanning of leather. Residual chromium thus is discharged in solid or liquid effluents. Soluble hexavalent chromium (Chromium (VI)) is extremely toxic and shows mutagenic and carcinogenic effects on biological systems due to its strong oxidizing nature (McLean and Beveridge, 2001). Chromium (III) is less toxic and bioavailable than Chromium (VI), as it readily forms insoluble oxides and hydroxides above pH 5 (Rai et al., 1987). A wide variety of bacteria have been reported for reducing / transforming Chromium (VI) to Chromium (III), under aerobic and anaerobic condition, e.g. Intrasporangium Sp. Q5-1, Bacillus sp. ES29, Escherichia coli, Enterobacter cloaceae, Pseudomonas fluorescens LB300 (Bopp, et al., 1983; Wang, et al., 1989; Shen and Yi-Tin, 1993; Camargo, et al., 2003a; Yang et al., 2009). Application of Chromium resistant bacteria for detoxification of Chromium (VI) has been considered as an economical, effective and safe procedure over conventional physical and chemical methods (Ganguli and Tripathi, 2002). Chromium VI is a transition element that is extensively used in tanning, metal finishing, petroleum refining, iron and steel industries, inorganic chemical production, and textile processing and pulp production (Srinath et al., 2002; Meriah and Tebo, 2002). Tanneries are a major source of chromium pollution and release Chromium (VI) ranging from 40-25,000 mg/l of wastewater. The maximum tolerance of total Chromium for public water supply has been fixed at 0.05 mg/l as per Indian standards. The environmental protection agency has formulated the maximum permissible levels of Chromium (VI) into water bodies at 50 g/dm³ and in drinking water as 3 μg/dm³ and that of Chromium (III) as 100 μg/dm³ (Lee and Jones, 1998; Palmer and Puis, 1994). Hexavalent Chromium compounds pose health risks to humans, plants, animals and fishes (Lee and Jones, 1998; Srinath et al., 2002). Due to its carcinogenicity and mutagenicity, the United States Environment Protection Agency (USEPA) has designated Chromium as a “Priority pollutant” or Class A” pollutant (Lee and Jones, 1998; Srinath et al., 2002). At high levels, heavy metals like chromium damage cell membranes, alter enzyme specificity; disrupt cellular functions and damage structure of DNA (Bruins et al., 2000).

**Materials and Methods**

**Collection of Chromium (VI) Sludge Samples and Thiobacillus ferrooxidans Bacteria**

Chromium sludge collected from electroplating chemical industries, Trichy. Samples were stored at 4°C. The water content of the sludge was 80.7%. The laboratory strain of *Thiobacillus ferrooxidans* was isolated from Chitra Durga acid mine drainage.

**Chromium (VI) Assay**

Hexavalent chromium was determined calorimetrically with a spectrophotometer (Jasco V-530) using the S-diphenylcarbazide (DPC) (Nacalai Tesque, Inc., Japan) method (Camargo, et al., 2003). The DPC reagent was prepared by adding 24 ml of 85% H₃PO₄ to 56 ml distilled water. This solution was mixed with 0.076 g DPC previously dissolved in 20 ml of 95% ethanol. The reagent was stored in dark at 4°C. Chromium (VI) in the sample was assayed by adding 125 ml of the DPC reagent to 1 ml of chromium samples, mixed gently and kept at room temperature for 20 min. The absorbance of the color produced was measured at 540 nm using a spectrophotometer. Chromium (VI) concentration in the sample was calculated from a standard curve using K₂Cr₂O₇ as standard.

**Experimental Condition**

The different concentration sludges, 2%, 4% and 6% chromium (VI) are taken and run in the STR. The Composition of sludge (2%) 20 g of sludge + 900 ml of 9K media + 100ml of *Thiobacillus ferrooxidans* (7 day culture), Composition of sludge (4%) 40 g of sludge + 900 ml of 9K media + 100ml of *Thiobacillus ferrooxidans* (7 day culture), Composition of sludge (6%) 60 g of sludge + 900 ml of 9K media + 100ml of *Thiobacillus ferrooxidans* (7 day culture) (Fig.1). In this experiment, three different concentrations of sludges were used (2%, 4% and 6%). The other parameter are air flow rate, temperature (25-30°C),
pH (1.5-1.8) were maintained throughout the process. The sample was collected every day and pH, ORP is analyzed and readings are noted.

**Isolation of *Thiobacillus ferroxidans* Bacteria**

Collected samples were allowed to grow in different medium, 9K, ATCC medium 64, NB Medium, LB medium. Since *Thiobacillus* species will grow in Silverman 9K medium (Silverman and Lundgren, 1959) growth can be seen in 9K medium alone. Also aeration is required for its growth. *Thiobacillus* will not grow in static condition. So, every medium was prepared in double-one was places in static condition and other was placed in agitator at 200 rpm. Bacterial stain was allowed to grow for 7 days at 28°C, inoculum size is about 10% V/v. Totally six experiments were done by isolation of *Thiobacillus ferroxidans* (Fig.2), all the experimental samples were collected from Chitra Durga acid mine drainage and experimental sample culture is about 10^7 serial dilution.

**Analytical Methods**

Sludge pH was determined with a Metler Toledo 320 pH meter. Samples were first centrifuged at 10,000 x g for 10 min, and the supernatants were collected for chromium (VI) assay. Chromium concentration in supernatants was determined calorimetrically with 1,5-diphenylcarbazide method (Apha and Awwa, 1995) by Jasco V-530 Spectrophotometer at 540 nm.

**Table 1. Phenotypic and Biochemical Characterization of *Thiobacillus ferroxidans* bacteria**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Phenotypic and Biochemical Characteristics</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cell morphology</td>
<td>colony</td>
</tr>
<tr>
<td>2</td>
<td>Endospores</td>
<td>-ve</td>
</tr>
<tr>
<td>3</td>
<td>Motility</td>
<td>+ve</td>
</tr>
<tr>
<td>4</td>
<td>Reactions to gram’s staining</td>
<td>-ve</td>
</tr>
<tr>
<td>5</td>
<td>Fe²⁺ oxidation</td>
<td>Yellowish red colonies</td>
</tr>
<tr>
<td>6</td>
<td>S² oxidation</td>
<td>+ve</td>
</tr>
<tr>
<td>7</td>
<td>Utilization of yeast extract/glucose</td>
<td>-ve</td>
</tr>
<tr>
<td>8</td>
<td>Growth at 50°C</td>
<td>-ve</td>
</tr>
<tr>
<td>9</td>
<td>Ferrous agarose</td>
<td>+ve</td>
</tr>
<tr>
<td>10</td>
<td>Sodium thiosulphate agar</td>
<td>Light green colour</td>
</tr>
<tr>
<td>11</td>
<td>Oxidase test</td>
<td>+ve</td>
</tr>
<tr>
<td>12</td>
<td>Catalase test</td>
<td>+ve</td>
</tr>
</tbody>
</table>

**Table 2. The variations of pH, ORP, concentrations of ferrous, ferric and chromium (VI) in the different concentrations of sludge**

<table>
<thead>
<tr>
<th>Days</th>
<th>pH</th>
<th>Oxidation Reduction Potential (ORP)</th>
<th>Concentration of ferrous (Fe²⁺) (mg/ml)</th>
<th>Concentration of ferric (Fe³⁺) (mg/ml)</th>
<th>Concentration of chromium (VI) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2%</td>
<td>4%</td>
<td>6%</td>
<td>2%</td>
<td>4%</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0.09</td>
</tr>
<tr>
<td>1</td>
<td>2.3</td>
<td>2.4</td>
<td>2.2</td>
<td>4.50</td>
<td>560</td>
</tr>
<tr>
<td>2</td>
<td>2.1</td>
<td>2.1</td>
<td>1.8</td>
<td>444</td>
<td>451</td>
</tr>
<tr>
<td>3</td>
<td>1.9</td>
<td>1.9</td>
<td>1.6</td>
<td>450</td>
<td>560</td>
</tr>
<tr>
<td>4</td>
<td>1.9</td>
<td>1.9</td>
<td>1.5</td>
<td>450</td>
<td>601</td>
</tr>
<tr>
<td>5</td>
<td>1.9</td>
<td>1.8</td>
<td>1.4</td>
<td>451</td>
<td>623</td>
</tr>
<tr>
<td>6</td>
<td>1.8</td>
<td>1.8</td>
<td>1.2</td>
<td>451</td>
<td>701</td>
</tr>
<tr>
<td>7</td>
<td>1.7</td>
<td>1.7</td>
<td>1.3</td>
<td>458</td>
<td>725</td>
</tr>
<tr>
<td>8</td>
<td>1.6</td>
<td>1.6</td>
<td>1.3</td>
<td>458</td>
<td>741</td>
</tr>
<tr>
<td>9</td>
<td>1.7</td>
<td>1.7</td>
<td>1.2</td>
<td>457</td>
<td>735</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>1.4</td>
<td>1.1</td>
<td>456</td>
<td>735</td>
</tr>
</tbody>
</table>
Results
The characterization of isolated bacteria were done by biochemical assay, it very important for the confirmation of selected bacterial strain from the different samples. Table 1 shows that the phenotypic and biochemical characterization of *Thiobacillus ferrooxidans* bacteria. The biochemical characterization confirmed the isolated bacteria is colony form, gram negative, absence of endospores formation, Motility is positive, yellowish red colonies were formed due to ferrous oxidation process, S² oxidation were positive, utilization of yeast extract or glucose is absent, growth is not occurred in 50°C, growth were occurred in ferrous agarose medium, light green colour colony occurred in sodium thiosulphate agar medium, involved in oxidation and catalase process. This biochemical assay was confirmed that the isolated sample is *Thiobacillus ferrooxidans* bacteria.

In a subsequent series of experiments, the effect of the initial pH, ORP, concentration of ferrous, ferric and chromium (VI) of the leaching sludges on the efficiency of the process was studied. Table 2 shows that the variation of pH, ORP, concentration of ferrous, ferric and chromium (VI) in the three different concentrations (2%, 4% and 6%) of sludges. The leaching process started at the three different concentrations of sludges (2%, 4% and 6%) in the range of pH is 2.0, 2.0 and 2.0 respectively; range of ORP is 405, 420 and 410 respectively; concentration of ferrous (Fe²⁺) is 8.96, 8.20 and 9.90 mg/ml respectively; concentration of ferric (Fe³⁺) is 0.99, 0.7 and 0.9 mg/ml respectively, concentration of chromium (VI) is 0.0001, 0 and 0 µg/ml respectively. After 10 days the variations of pH, ORP and concentrations of ferrous, ferric and chromium (VI) were recorded for the three different concentrations (2%, 4% and 6%) of sludges. The variation of pH range is 1.5, 1.4 and 1.1 respectively; the variation of ORP range is 456, 735 and 717 respectively; the concentration of ferrous is 7.83, 7.86 and 6.01 mg/ml respectively; the concentration of ferric is 2.05, 1.5 and 2.34 mg/ml respectively; the concentration of chromium is 0.28, 0.08 and 0.09 µg/ml respectively. In these three different concentrations (2%, 4% and 6%) of sludges, the pH range was increased suddenly and then gradually decreases as the day progresses. The ORP rate was suddenly increased and gradually reached the stable state. The ferrous concentration gradually decreased as the day progresses. The ORP concentration increased as the day progresses due to the conversion of ferrous to ferric. The chromium (VI) is leached by the *Thiobacillus ferrooxidans*. The leached chromium (VI) is present in the medium rather than the sludge hence the increase in chromium (VI) concentration clearly indicates the effective leaching of chromium (VI). In these three different concentrations of sludges, the maximum leaching of chromium (VI) were obtained from 2% sludge (0.28 µg/ml) when compared to 4% (0.08 µg/ml) and 6% (0.09 µg/ml) sludges. The pH range was rapidly decreased and the Oxidation Reduction Potential (ORP) rate was rapidly increased as the day progresses.

Discussion
Bioremediation has developed from the laboratory to a fully commercialized technology over the last 30 years in many industrialized countries. Camargo *et al.* (2003a) isolated some chromium resistant bacteria that can tolerate or reduce Chromium (VI) at concentrations of 1,500–2,500 mg/l. Chromium (VI) was completely reduced after 6 and 24 h respectively by isolates IFR-2 and IFR-3. It has been reported that *Bacillus* Sp. ES29 reduced 90% of Chromium (VI) added (2 mg/l) to the medium in less than 6 h (Camargo *et al.*, 2003b). Thacker and Madamwar, (2005) have shown that the bacterial isolate DM1 reduces 50 ppm of chromium to 0 ppm in 54 h. They added a second aliquot of chromium which was reduced to 0 ppm in 99 h, and the third aliquot was reduced to 21.8 ppm in 126 h. Camargo *et al.* (2003b) observed chromate reductase activity in the cell-free extract and soluble fraction but very low activity in the membrane fraction of *Bacillus* sp. ES29. Wang et al. (1990) reported that chromate reductase activity is preferentially associated in the membrane fraction of *E. cloacae* HO1. Membrane bound chromium reduction activity has also been reported in *P. fluorescens* LB300 (Bopp et al., 1983). Chromate reduction activities of whole cells of bacterial isolates were determined at 37°C. Whole cells of IFR-2 and IFR-3 reduced 24 and 30% of the initial Chromium (VI) concentration (1 mg/l) in 45 min respectively. Initial cell density has been shown to have a dramatic effect on Chromium (VI) reduction (McLean, et al., 2000). A number of factors may be contributing to the observed trends, including Chromium (VI) toxicity acclimation time needed to readjust cells to low nutrient loads before initiation of reduction.

Thacker and Madamwar, (2005) reported a maximum reduction of Chromium (VI) at 35°C by the bacterial isolate DM1. An NAD(P)H dependent hexavalent chromium reductase was purified from *Pseudomonas ambigua* which showed activity over a wide range of temperature from 40 to 70°C (Suzuki et al., 1992). Optimal Chromium (VI) reduction was shown to be directly related to the optimum pH (8.0) for the growth of *Bacillus* isolates (Camargo *et al.*, 2003a). McLean *et al.* (2000) reported a wide range of pH between 6.0 to 9.0 for Chromium (VI) reduction by *P. syxanthana*. Chromate tolerance mechanisms in bacteria have been reported to include reduction, methylation, precipitation at the cell surface, blocking cellular uptake by altering the uptake pathway and removal from the cytoplasm by efflux pumps (Shuttleworth and Richard, 1993; Lovely and Philips, 1994; Rami rez-Dr‘az *et al.*, 2008). The profiles of sludge pH and chromium solubilization were similar to the earlier reports on removal of metals from tannery sludge and other wastes by sulfur-oxidizing bacteria using S² (Blais et al., 1993; Shen et al., 2003; Zhou et al., 2004a; Zhou et al., 2004b; Zhou et al., 2005). Data of above experiments strongly demonstrated that chromium solubilization was negatively related to sludge pH. According to the results, pH 2.0 seemed to be a threshold for considerable chromium solubilization during the bioleaching experiment. This was similar to earlier reports on microbial leaching of chromium from tannery sludge (Shen et al., 2003; Zhou et al., 2004b; Zhou et al., 2005). Chromium solubilization using the indigenous *A. thiooxidans* at low sludge
concentration might not require the preacidification process. Moreover, with a shorter time and lower amount of sulfur, chromium release efficiency of this experiment was almost the same as demonstrated (Shen, et al., 2003; Zhou, et al., 2004b; Zhou, et al., 2005). However, efforts should be made to improve the efficiency of chromium bioleaching at high sludge concentration. Tseng and Benefieldt, (2002) also reported a higher Chromium (VI) reduction by indigenous microbes under aerobic condition especially when the initial Chromium (VI) concentration was higher. Anaerobic microbes are usually more sensitive to toxic compounds like heavy metals. There was no Chromium (VI) reduction in the control reactor without any microorganisms, which demonstrates the role of microorganism in the reduction of Chromium (VI) (Mosey, 1976).

Conclusions
Experiments on bioremediation of chromium (VI) in Stirred Tank Reactor (STR) using Thiobacillus ferrooxidans bacteria were performed over a period of 10 days using different concentration (2%, 4% and 6%) of sludges at regular interval of days and results were analyzed. Based on the analysis the following are concluded, The isolated microorganism is Thiobacillus ferrooxidans, Feasible amount of chromium (VI) is leached in all the three concentration of sludges, The maximum leaching was obtained in the 2% sludge, because in 4% and 6% the toxicity of chromium (VI) inhibits the leaching hence little amount of leaching in 4% and 6%. Rapidly decreased the pH and rapid increased the reduction of oxidation potential (ROP) were found in all the different sludges.

Acknowledgement
This work was supported by the Department of Biotechnology, Kamaraj College of Engineering and Technology Virudhunagar, Tamilnadu, India. I am very grateful to express my heartfelt thanks to my guide for his benevolent and noble ideas, imminent guidance with sustained interest, learned council and constant encouragement.

References


F.E. Mosey, Assessment of the maximum concentration of heavy metals in crude sewage, which will not inhibit anaerobic digestion of sludge, Water Pollut. Contr., 75 (1976) 10–17.

O. Ohnura, H. Saiki, Desulfurization of Coal by Microbial Column Flotation, Biotechnology and Bioengineering, 44 (1994) 125.


Source of support: Nil; Conflict of interest: None declared