BIOREDUCTION BASED BIOREMEDIATION OF HEXAVALENT CHROMIUM Cr (VI) THROUGH POTENTIAL INDIGENOUS MICROBES

Submitted *by*

ALOK PRASAD DAS

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Dr. SUSMITA MISHRA

AND

Prof. SOMESH JENA

DEPARTMENT OF CHEMICAL ENGINEERING

NATIONAL INSTITUTE OF TECHNOLOGY,

ROURKELA-769008, ORISSA, INDIA.

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CERTIFICATE

This is to certify that the thesis entitled **"Bioreduction based bioremediation of hexavalent chromium through potential indigenous microbes"** is submitted by **Mr. ALOK PRASAD DAS**, (Roll NO- **60700002**) to this Institute in partial fulfillment of the requirement for the award of the degree of Master of Technology by Research in **Department of Chemical Engineering,** is a bonafied record of the work carried out under my supervision and guidance. It is further certified that no part of this thesis is submitted for the award of any degree.

(Dr. Susmita Mishra)

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Date:

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ABSTRACT

Sukinda Valley, of Orissa, contains 97% of India's chromite ore deposits and one of the prime open cast chromite ore mines in the world. (ENVIS Newsletter, 2007). Hexavalent chromium pollution in this area has caused a major health hazard affecting 2,600,000 people in this area (Blacksmith Institute report, 2007). Our investigation involved biological treatment of Cr (VI) without producing any byproduct. Bacterial cultures tolerating high concentrations of chromium were isolated from the soil sample collected from the chromite contaminated sites of Sukinda chromite mines and their bioaccumulation properties are investigated. Strains capable of growing at 300 mg/l of Cr (VI) are considered as chromium resistant. The mentioned stain was capable of resisting Cr (VI) up to 500 mg/l. Its resistance to different metals such as Ni, Zn, Cu, Cd and Fe were also investigated. The effects of different operating parameters such as initial pH, temperature and initial Cr (VI) concentrations on bioaccumulation of Cr (VI) by enriched cultures were studied in a batch system. The experimental investigation showed the maximum specific chromium uptake at pH 7 and temperature 30^0C. With increasing initial Cr (VI) concentration from 5 mg/l to 50 mg/l showed increase in Cr (VI) uptake. At about 50 mg l^{-1} initial Cr (VI) concentrations, uptake of the selected potential strain exceeded 98% within 12 hours of incubation. The bacterial isolate is identified by 16S rRNA sequencing as *Brevebacterium casei*. Data indicated that isolate culture can be utilized to improve efficiency of biological treatment processes for effluents containing higher levels of Cr (VI).

INTRODUCTION

Effluents from textile, leather, tannery, electroplating, galvanizing, dyes and pigment, metallurgical and paint industries and other metal processing and refining operations at small and large-scale sector contains considerable amounts of toxic metal ions. These metal ions from metal mining pose problems to the water environment by discharging mine water from underground and open pit mines (Moncur et al 2005). Leachate water and runoff water from overburden/ waste rock dumps also contaminate nearby water streams. The potential impacts from leaching operations on the environment are most likely to be experienced as changes to surface and groundwater quality. The principle pathways by which leached contaminants can enter into groundwater are leakage or spills from storage ponds, leach pad liners, subsequent leaching to groundwater, storm water run-on/off, uncontrolled leaching from heaps and dumps following closer (Moncur et al 2005). These toxic metals ions not only cause potential human health hazards but also affect other life forms. Cr (VI) is toxic, carcinogenic and mutagenic to animals as well as humans and is associated with decreased plant growth and changes in plant morphology. They cause physical discomfort and sometimes life-threatening illness including irreversible damage to vital body system (Malik, 2004).

Chromium is the most abundant of the Group VIA family of metallic elements. At a concentration of nearly 400 parts per million in the earth's crust as various minerals, it is the 13th most common element**.** It is one of the world's most strategic, critical & highly soluble metal pollutant having wide range of uses in the metals and chemical industries (Kotas and Stasicka 2000, Das and Mishra 2008). Chromium exists in the environment in several diverse forms such as trivalent Cr (III) and hexavalent Cr (VI), of which hexavalent chromium Cr (VI) is a so-called carcinogen and a potential soil, surface water and ground water contaminant (Cervantes et al.,

2001). A slight elevation in the level of Cr^{6+} elicits environmental and health problems because of its high toxicity (Sharma et al., 1995), mutagenicity (Nishioka, 1975) and carcinogenicity (Venitt and Levy, 1974). Whereas it's reduced trivalent form, (Cr^{3+}) is less toxic, insoluble and a vital nutrient for humans. Due to its toxicity stringent regulation are imposed on the discharge of Cr (VI) to surface water to below 0.05 mg/l by the U.S. EPA (Baral et al., 2002) and the European Union, while total Cr, including Cr (III), Cr (VI) and its other forms to below 2 mg/l.

Since the industrial revolution, the anthropogenic inputs of chromium have increased rapidly (Nriagu and Pacyna, 1988; Baral et al., 2006). Chromium is extensively used in electroplating (as chrome plating), resistant alloys (e.g., stainless steel), leather tanneries and dye productions (United States Environmental Protection Agency, 1998; Ryan et al., 2002). Mine tailing and effluents from non-ferrous metals industry are the major sources of Cr (VI) in the environment (Moore and Ramamoorthy, 1984).

Many such ferro alloy industries and chromite mines are located in the Sukinda area of Jajpur district in Orissa state. About 97% of India's chromite ore deposits are present in this region. (ENVIS Newsletter, 2007). Mining activity in this region generate around 7.6 million tonnes of solid waste in the form of rejected minerals, overburden material/waste rock and sub grade ore.

Due to the seepage of water from the dumped waste the nearby water stream shows contamination due to Cr (VI) much above their permissible limits. OVHA (Orissa Voluntary Health Association) reports contaminations in the different water bodies of Sukinda area such as wells, ponds rivers etc. Damasala River is the main water source to the inhabitants nearby who have fallen prey to innumerable diseases. The OVHA reported health hazards due to Cr (VI) contamination leading to death. The main diseases includes, "gastrointestinal bleeding, tuberculosis asthma Infertility, birth defects, and stillbirths". The survey report further mentioned

that villages at a distance of less than one km from the sites were the worst affected, with 24.47 per cent of the inhabitants found suffering from pollution-induced diseases (ENVIS Newsletter, 2007). In spite of the repeated warning by OSPB and other regulatory bodies the industries have not employed adequate treatment facilities to reduce Cr (VI) contamination. Currently the effluents are treated with ferrous sulphate method that suffers from precipitation, so additional treatment methods to remove those are sorted. Hence our investigation would involve a means to reduce Cr (VI) from the effluent in a cost effective and eco-friendly manner.

Biological reduction of Cr (VI) using indigenous microorganism offer a new cost-effective and environmentally compatible technology (Camargo et al., 2005). Many researchers have explored the potential of microorganisms such as Aerococcus sp. isolated from tannery effluents in kanpur by Ramteke et al (2001), Arthrobacter sp. and Bacillus sp. from Tannery effluents in Australia by Megharaja et al, (2003), Bacillus sphaericus from Serpentine Soil by Paul et al, (2004), Acinetobacter haemolyticus from Heavy metal contaminated waste water by Zakaria et al, (2007) and Sphaertilus natans from activated sludges by caravelli et al, (2008). Most of these have been investigated using Cr (VI) aqueous solution and have not shown significant or complete removal of Cr (VI). All the reported microbial strains have lower MIC for Cr (VI).

There is only limited investigation for mining effluent treatment particulary Cr (VI) contaminants using microbial strains. Till date there is no literature cited on any molecularly identified and sequenced microbial species for chromium resistant and removal from sukinda region. Thus our present study uses potential indigenous microbial strains for treatment of industrial and mining effluent that may be suitable for biological treatment of Cr-contaminated waste of Sukinda mines. This study proposes a remediation route for detoxification of Cr (VI) using an indigenous microorganism.

2. REVIEW OF LITERATURE

2.1 Introduction

Chromium (Cr) is a transition metal present in group VI-B of the periodic table. Although it can exists in nine valence states, from−2 to+6 (Smith et al., 2002) only trivalent chromium Cr (III) and hexavalent chromium Cr (VI) are ecologically important because these are the most stable oxidation states in the natural environment. Hexavalent chromium polluted anthropogenic effluents are principally answerable for environmental contamination by toxicity and carcinogenicity. Chromium polluted soils and sediments are usually the result of sewage sludge disposal or dumping of chromate wastes from industrial and manufacturing activities (McGrath and Smith, 1990). Chromium contamination of the environment is of concern because of the mobility and toxicity of Cr (VI). Trivalent and hexavalent chromium differ widely in physicochemical properties and biological reactivity. While Cr (VI) species and dichromate's are extremely water-soluble and mobile in the environment, Cr (III) species are much less soluble and comparatively immobile (Viamajala et al., 2004). Moreover, Cr (VI) is recognized to be highly toxic, carcinogenic, mutagenic and teratogenic for mammals including humans (A. Flores et al., 1999), whereas Cr (III) is an essential trace element necessary for glucose, lipid and amino-acid metabolism as well as a popular dietary supplement (S. Viamajala et al., 2004). Studies have revealed that Cr (VI) is approximately 100 times more toxic (Beszedits, 1988) and 1000 times more mutagenic than Cr (III) (Lofroth et al., 1978).

2.2 Chromium

Chromium is a chemical element which has the symbol Cr and atomic number 24. It is a steely-gray, lustrous, hard metal that takes a high polish and has a high melting point. It is odorless, tasteless, and malleable metal. The name of the element is derived from the Greek word

"chrōma" meaning color, because many of its compounds are intensely colored. Chromium is important metal due to its high corrosion, resistance and hardness. It is used extensively in manufacturing of stainless steel. Although trivalent chromium (Cr (III) or Cr^{3+}) is required in trace amounts for sugar and lipid metabolism in humans, however its deficiency causes disease. Hexavalent chromium (Cr (VI) or Cr^{6+}) is a toxin and a carcinogen metal pollutant that tremendously affects the environment at abandoned chromium production sites. Hence its environmental cleanup is highly essential. Comprehensive data on the chemical element Chromium is provided in Table 1.

Table 1: Comprehensive data on the chemical element Chromium

Name, symbol, number	chromium, Cr, 24
Group, period, block	6, 4, d
Element category	Transition metal
Appearance silvery metallic	Silvery metallic
Standard atomic weight	51.9961(6)g·mol^{-1}
Electron configuration	[Ar] $3d^5$ $4s^1$
Crystal structure cubic body centered	Cubic body centered
Oxidation states	6, 5, 4, **3**, 2, 1
Atomic radius	140 pm
Thermal conductivity	(300 K) 93.9 W·m^{-1}·K^{-1}

2.3 Uses and Toxicity

Elemental chromium (Cr) does not occur in nature, but is present in ores, primarily chromite ($FeOCr_2O_3$). Hexavalent chromium is the main chromium species used in industrial processes, including manufacturing of metallic alloys (the most important use of Cr), chrome leather tanning, metal cleaning processing, wood preservation, ceramics, pyrotechnics, electronics and so on, and is therefore the most common pollutant in a wide variety of industrial wastes (Middleton et al., 2003). Considering its potential for hazardous toxicity and exposure, Cr (VI) has been designated as a priority pollutant in many countries.

Non-occupational exposure to the metal occurs via the ingestion of chromium-containing food and water, whereas occupational exposure occurs via inhalation (Pedersen, 1982). Workers in the chromate industry have been exposed to estimated chromium levels of 10-50 $\mu g/m^3$ for Cr (III) and 5-1000 $\mu g/m^3$ for Cr (VI). Humans and animals localize chromium in the lung, liver, kidney, spleen, adrenals, plasma, bone marrow, and red blood cells (RBC) (ATSDR, 1989). The main routes for the excretion of chromium are via the kidneys/urine and the bile/feces (Guthrie, 1982; Langard, 1982). Hexavalent chromium is transported into cells via the sulfate transport mechanisms, taking advantage of the similarity of sulfate and chromate with respect to their structure and charge. Once developed, chrome sensitivity can be persistent. In such cases, contact with chromate-dyed textiles or wearing of chromate-tanned leather shoes can cause or exacerbate contact dermatitis. Vitamin C and other reducing agents combine with chromate to give Cr (III) products inside the cell (Salnikow et al., 2008). Hexavalent chromium compounds are genotoxic carcinogens. Chronic inhalation of hexavalent chromium compounds increases risk of lung cancer (lungs are especially vulnerable, followed by fine capillaries in kidneys and intestine). The mechanism of genotoxicity relies on pentavalent or trivalent chromium.

According to some researchers, the damage is caused by hydroxyl radicals, produced during reoxidation of pentavalent chromium by hydrogen peroxide molecules present in the cell. Zinc chromate is the strongest carcinogen of the chromates used in industry. Soluble compounds, like chromic acid, are much weaker carcinogens (Salnikow et al., 2008). The sub chronic and chronic oral RFD for Cr (VI) are 0.02 and 0.005 mg/kg/day, respectively (U.S. EPA, 1991a, b; 1992). The accumulated chromium in soil can also cause acute and long term toxic effects on soil ecosystems (Viti. 2006). Based on adequate proof for humans and animals, Cr (VI) has been placed in the EPA weight-of-evidence classification a human carcinogen (U.S. EPA, 1991a). The major industrial source of Cr(VI) emissions includes chemical manufacturing industry, e.g., dyes for paints, rubber, and plastic products, metal finishing industry, e.g., chrome plating, manufacturers of pharmaceuticals, wood, stone, clay, and glass products, electrical and aircraft manufacturers, steam and air conditioning supply services, cement-producing plants as cement contains chromium. The Cr (VI) concentrations in wastewater produced by industries are estimated to be between 0.1 and 200 mg/l. The concentrations of Cr (VI) in the wastewater of some industries are listed in Table 1

Table 1: Industrial wastewater containing Cr (VI)

Industry	**Cr(VI) concentration (mg/L)**	**Reference**
Hardware factory	60.0	Xu et al. 2005
Chrome tanning plant	3.7	Gupta et al. 1999
Electroplating plant	20.7 – 75.4	Davis et al. 1995, Kiptoo et al. 2004, Tukaram Bai et al. 2005
Tannery plant	8.3 - 3,950.0	Esmaeili et al. 2005, Song et al. 2000, Onyancha et al. 2008

2.4 Cr (VI) Regulations

Presence of Cr (VI) more than the standard limit in the water bodies causes many adverse effects to human beings, animals, plants etc. Hence stringent regulations have been imposed by various organizations. According to the World Health Organization (WHO) drinking water guidelines, the maximum allowable limit for hexavalent chromium and total chromium (including Cr (III), Cr (VI) and other forms) are 0.05 and 2mg/L, respectively (Gupta and Rastogi 2009). According to Safe Drinking Water Act, Maximum Contaminant Level (MCL) is 0.1 mg/L (total chromium). Maximum permissible level of chromium in bottled water is 0.1 mg/L. Specific color additives may contain chromium at levels no greater than 50 ppm. Chromium may be used in hydrolyzed leather meal used in feed for animals provided it contains chromium at levels below 2.75% of the total by weight. Occupational Safety and Health Administration (OSHA) prescribes the Permissible Exposure Limit (PEL) for Cr (VI) as 0.1 mg/m3 (based on chromic acid & chromates listing). National Institute for Occupational Safety and Health (NIOSH) indicates Immediately Dangerous to Life and Health (IDLH) limit as 15 mg/m^3 (as Chromium (VI)) (For chromic acid & chromates listing). Recommended Exposure Limit (time-weighted-average workday) is restricted to 0.001 mg/m3 (for chromic acid & chromates and chromyl chloride listings).

2.5 Conventional methods for heavy metal removal from industrial effluents

Various conventional methods to reduce Cr (VI) from the waste water stream includes physical and chemical methods such as ion exchange, filtration, precipitation, electrochemical treatment, chemical reduction, adsorption, membrane technologies and evaporation recovery (Patterson, 1985., Nyer, 1992., Camargo et al., 2003 and Rama Krishna et al., 2005., Ahluwalia and Goyal 2007, Sikaily et al 2007).

2.6 Electrochemical Precipitation:

This method utilizes an electrical potential to maximize the removal of heavy metal from contaminated wastewater over the conventional chemical precipitation method (Kurniawan et al. 2006). It is the most common method for removing toxic heavy metals up to parts per million (ppm) levels from water. Kongsricharoern and Polprasert in 1995 investigated the Cr (VI) removal from an electroplating wastewater using the ECP process. Using this process Cr (VI) concentration could be removed from 3,860 mg/l to less than 0.2 mg/l.

Although the process is cost effective its efficiency is affected by low pH and the presence of other salts (ions). The process requires addition of other chemicals, which finally leads to the generation of a high water content sludge, the disposal of which is cost intensive. Precipitation with lime, disulphide or ion exchange lacks the specificity and is ineffective in removal of the metal ions at low concentration.

2.7 Ion exchange:

Among the physicochemical methods developed for chromium removal from wastewater, ion exchange is becoming a popular method that has received much attention in recent years. Ion exchange is a unit process by which ions of a given species are displaced from an insoluble exchange material by ions of a different species in solution. The chromium-containing solution enters one end of the column under pressure, passes through the resin bed, and the chromium is removed from the solution. When the resin capacity is exhausted, the column is backwashed to remove trapped solids and then regenerated. Commonly used matrices for ion exchange are synthetic organic ion exchange resins.

Synthetic Dowex 2-X4 ion exchange resin was employed to investigate the uptake of Cr (VI) from real plating wastewater (Sapari et al. 1996). A strongly basic anion resin in hydroxide form

was used in the columns as anionic exchangers. About 100% removal of Cr (VI) was achieved in the studies. Another synthetic ion exchange resin, Ambersep 132 was also explored to recover chromic acid from synthetic plating solution in a four-step ion exchange process (Lin and Kiang 2003). Ion exchange resin was used in the first step to capture chromic acid which was converted to sodium chromate by sodium hydroxide solution in the second step. Sodium chromate was converted back to chromic acid using strong base cationic (H-type) ion exchange resin in the third step, and in the fourth step, the exhausted ion exchange resin was regenerated by hydrochloric acid solutions. The four-step process was proved to be efficient and was capable of providing continuous chromic acid recovery operation.

A disadvantage of an ion exchange method for chromium removal is that ion exchange resins are very selective. A resin must be chosen that selectively removes the metal contaminant of concern. Further, ion exchange equipment can be expensive and there can be incomplete removal of the chromium from the salt solution. Besides, it cannot handle concentrated metal solution as the matrix gets easily fouled by organics and other solids in the wastewater. Moreover ion exchange is nonselective and is highly sensitive to pH of the solution.

2.8 Biosorption:

Biosorption of chromium from aqueous solutions is relatively a new process that has proven very promising in the removal of contaminants from aqueous effluents. Adsorbent materials derived from low-cost agricultural wastes can be used for the effective removal and recovery of chromium ions from wastewater streams (Basso et al. 2002; Park et al. 2006). Metal biosorption is a rather complex process affected by several factors. Mechanisms involved in the biosorption process include chemisorption, complexation, adsorption–complexation on surface and pores, ion exchange, microprecipitation, heavy metal hydroxide condensation onto the biosurface, and

surface adsorption (Gardea-Torresdey et al. 2004). This method suffers from low adsorption capacity and less intensity of biosorption.

2.9 Adsorption using Activated Carbon

The most studied adsorbent in adsorption of chromium is found to be the activated carbon derived from various raw materials such as sawdust, nut shells, coconut shells etc. (Mohan and Pittman 2006). (Hamadi et al. 2001) studied the removal of Cr (VI) from aqueous solution using GAC type Filtrasorb Water Air Soil Pollut 400. It was found that reduction in particle size of adsorbents increases its surface area for metal adsorption, and it results in higher removal efficiency on Cr6+. It was also indicated that the adsorption of Cr6+ was more favorable at higher temperature. (Mohanty et al. 2005) prepared several activated carbons from Terminalia arjuna nuts, an agricultural waste, by chemical activation with zinc that showed maximum removal of chromium at pH 1.0. (Natale et al. 2007) used activated carbon produced by Sutcliffe Carbon starting from a bituminous coal to adsorb Cr (VI). It was found that the adsorption capacity for the activated carbon strongly depends on solution pH and salinity. The main demerit of the process lies in its frequent desorption and regeneration and is dependent on the life of the adsorbent.

2.10 Membrane Filtration

Membrane filtration technique has received a significant attention for the wastewater treatment. It considers the application of hydraulic pressure to bring about the desired separation through the semipermeable membrane. Various types of membranes such as inorganic, polymeric, and liquid membranes can be employed for Cr (VI) removal. (Pugazhenthi et al. 2005) prepared supported non-interpenetrating modified ultrafiltration carbon membrane by gas phase nitration using NOx and amination using hydrazine hydrate. The membrane was used for the separation of

Cr (VI) from the aqueous solution. Separation experiments on the chromic acid solution have been carried out using unmodified (giving 96% rejection), nitrated (giving 84% rejection), and aminated (giving 88% rejection) carbon membrane. (Muthukrishnan and Guha 2008) studied removal of Cr (VI) with different nanofiltration composite polyamide membranes for varying concentration and pH of the membrane feed solution. Two membranes were used for this investigation: one, a high rejection membrane (NFI) and the other, a low rejection membrane (NFII). The percent rejection of chromium was found to increase with the increase of feed solution pH. It has been observed that the effect of feed concentration on the percent rejection was quite low, but the nature of effect varies with the pH of the solution with a transition happening at above pH 7.0.The major disadvantage this technique apart from being economically expensive have disadvantages like incomplete metal removal, high reagent and energy requirements, and generation of toxic sludge or other waste products that require disposal.

The drawback of these existing and Conventional treatment methods for Cr (VI) contaminated soil and groundwater include high energy expenditure in the process, use of expensive and toxic chemical reductants (Komori et al., 1990) as well as inefficient removal of low concentrations of Cr(VI) in wastewater (Kurniawan et al., 2006). These methods are also relatively expensive and sometimes generate secondary wastes that require subsequent disposal. Bioremediation is a more attractive option, in that, the technology is relatively cheap and environmentally compatible (Okeke et al., 2003).

In Situ bioremediation technology can be applied to circumvent the limitations of physical and/or chemical methods. Direct metabolic reduction of Cr (VI) by bacteria has been documented by several researchers (Fujie et al., 1996; Garbisu et al., 1998; Guha et al, 2001; Zhihui Yang et al 2009). Bioreduction of Cr (VI) appears to be ubiquitous since, Cr (VI)

reducing consortia were isolated from Cr (VI) contaminated sites as well as uncontaminated sites (Turick et al., 1996; Chen and Hao, 1998; Schmieman et al., 1998; Sani et al., 2002; Camargo et al., 2003). Following microbial reduction, it is commonly assumed that Cr (VI) species are transformed to insoluble and immobile chromium hydroxide. Hence, this technology has potential to be applied at field sites to immobilize Cr in the subsurface.

2.11 Bioremediation of Hexavalent Chromium

Bioremediation has developed from the laboratory to a fully commercialized technology over the last 30 years in many industrialized countries. A successful bioremediation scheme relies on the management of soil microbial populations capable of catabolising the contaminants. Heavy metals exhibit toxic effects on soil biota, and they can affect key microbial processes and decrease the number and activity of soil microorganisms (Obbard et al., 2001). Microbial population has often been proposed to be an easy and sensitive indicator of anthropogenic effects on soil ecology. Cr (VI) has been reported to cause shifts in the composition of soil microbial populations, and known to cause detrimental effects on microbial cell metabolism at high concentrations. Quite a few studies on soil contamination of heavy metal from industrial sites were reported (Schulin R et al., 2007). Since the discovery of the first microbe capable of reducing Cr^{6+} in the 1970s (Romanenko and Korenkov, 1977), the search for Cr^{6+}-reducing microorganisms (both aerobic and anaerobic) has been enthusiastically pursued, with numerous strains being isolated. Based on recent isolation and purification of Cr6+ reductases from aerobic bacteria and the fact that the process involved in Cr^{6+} reduction occurring under anaerobic conditions is starting to be understood, biological processes for treating chromium contaminated sites are becoming very promising. Some of the emerging technologies for the mitigation and remediation of Cr (VI) include microbial strategies for *in situ* and on-site bioremediation

strategies and use in permeable reactive barriers. Discovery of microorganisms capable of reducing Cr (VI) to Cr (III) have significant potential in development of *in situ* or on-site bioremediation strategies. In 1977, the first reported bacterial strains, *Pseudomonas,* were isolated from chromate ($CrO4^{2-}$ contaminated sewage sludge by Russian scientists N.A. Romanenko and V. Korenkov. Since 1977, several other $CrO4^{2-}$ reducing strains have been reported, including other strains such as B. cereus, B. subtilis, Ps. aeruginosa, Ps. ambigua, Ps. fluorescens, E. coli, Achromobacter eurydice, Micrococcus roseus, Enterobacter cloacae, Desulfovibrio desulfuricans and D. vulgaris (Lovley, 1994). A number of bacteria in other genera, viz. Bacillus spp., E. coli ATCC 33456, Shewanella alga BrY-MT and a few unidentified strains have also been shown to reduce Cr6+ (Guha et al., 2001; Camargo et al., 2003).

Terry J. Beveridge (2000) worked on isolation and characterization of a chromium-reducing bacterium from a chromated copper arsenate contaminated site. Reports conclude a gram-negative bacterium (CRB5) isolated from a chromium-contaminated site that was capable of reducing hexavalent chromium to an insoluble precipitate, thereby removing this toxic chromium species from solution. Analysis of the 16S rRNA from the isolate revealed that it was a pseudomonas with high similarity to Pseudomaonas synxantha. CRB5 was tolerant to high concentrations of chromate (500 mg l21) and can reduce Cr (VI) under aerobic and anaerobic conditions. It also exhibited a broad range of reduction efficiencies under minimal nutrient conditions at temperatures between 48^0C and 37^0C and at pH levels from 4 to 9. E. Donati in (2001) worked on Factors affecting chromium (VI) reduction by *Thiobacillus ferrooxidans.* In *T. ferrooxidans* cultures with sulphur as energy source, the capacity for Cr (VI) reduction was related to the generation of sulphur compounds (sulphite, thiosulphate and polythionates) with high reducing power. In contrast with other Cr (VI)-reducing microorganisms, *T. ferrooxidans*

showed higher Cr (VI) reduction at low pH. The reduction of Cr (VI) also increased with the age of the culture. A *T. ferrooxidans* cells were capable of growing under anaerobic conditions with chromium (VI) as the terminal-electron acceptor. J. Felix Gutierrez-Corona in (2003) reported Cr (VI) reduction in a chromate-resistant strain of *Candida maltose* isolated from the leather industry. Resistance of the strain to high Cr (VI) concentrations and its ability to chemically reduce chromium was studied. When compared to the three laboratory yeasts *Candida albicans*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*, the *C. maltosa* strain was found to tolerate chromate concentrations as high as 100 mg/ ml. In addition to this phenotypic trait, the *C. maltosa* strain showed ability to reduce Cr (VI). Chromate reduction occurred both in intact cells (grown in culture medium or in soil containing chromate) as well as in cell-free extracts. Jonnalagadda Raghava Rao (2007) reported biological removal of carcinogenic chromium (VI) using mixed *Pseudomonas* strains. In this study an aerobic reduction of Cr (VI) to Cr (III) by employing mixed *Pseudomonas* cultures isolated from a marshy land has been reported. The role of chromium concentration, temperature, pH and additives on the microbial reduction of Cr (VI) has been investigated. NADH was found to enhance the rate of reduction of Cr (VI). Complete reduction of Cr (VI) has been possible even at Cr (VI) concentrations of 300 ppm. Ions like SO_4^{2-} and poly-phenols inhibited the metabolic activity relating to Cr (VI) reduction. Under optimal conditions 100 mg/l of Cr (VI) was completely reduced within 180 min. Benedict C. Okeke (2008) worked on Bioremoval of hexavalent chromium from water by a salt tolerant bacterium, *Exiguobacterium* sp. GS1. His results suggest that the isolate significantly removed Cr (VI) at both high and low concentrations (1–200 µg mL/1) within 12 h. The Michaelis–Menten K_m and V_{max} for Cr (VI) bioremoval were calculated to be 141.92 µg ml/1 and 13.22 µg ml/1 h/1, respectively. Growth of *Exiguobacterium* sp. GS1 was indiverent at 1– 75 µg mL/1 Cr (VI) in

12h. At initial concentration of 8,000 µg L/1, *Exiguobacterium* sp. GS1 displayed rapid bioremoval of Cr (VI) with over 50% bioremoval in 3 h and 91% bioremoval in 8 h. Kinetic analysis of Cr (VI) bioremoval rate revealed zero-order in 8 h. *Exiguobacterium* sp. GS1 grew and significantly reduced Cr (VI) in cultures containing 1–9% salt indicating high salt tolerance. Similarly the isolate substantially reduced Cr (VI) over a wide range of temperature (18–45 °C) and initial pH (6.0–9.0). The T_{opt} and initial pH_{opt} were 35–40 °C and 7–8, respectively. Zhihui Yang et al (2009) reported Cr (VI) remediation by indigenous bacteria in soils contaminated by chromium-containing slag at a Steel-Alloy factory in Hunan Province, China. His results showed that when sufficient nutrients were amended into the contaminated soils, total Cr (VI) concentration declined from the initial value of 462.8 mg kg^{-1} to 10 mg kg^{-1} within 10 days at removal rate 97.8%. Water soluble Cr (VI) decreased from the initial concentration of 383.8 mg kg^{-1} to 1.7 mg kg^{-1}. Exchangeable Cr (VI) and carbonates-bound Cr (VI) were removed by 92.6% and 82.4% respectively.

2.12 Bacterial mechanisms of chromate resistance

It has been demonstrated in a variety of bacterial species that chromate actively crosses biological membranes by means of the sulfate uptake pathway, which reflects the chemical analogy between these two oxyanions (Cervantes and Campos- Garcıa 2007). Cr (III) crosses cell membranes with a low efficiency because it forms insoluble compounds (Cary 1982). Inside the cell, Cr (VI) is readily reduced to Cr (III) by the action of various enzymatic or nonenzymatic activities; the Cr (III) generated may then exert diverse toxic effects in the cytoplasm (Cervantes et al. 2001). A variety of chromate-resistant bacterial isolates has been reported, and the mechanisms of resistance to this ion may be encoded either by plasmids or by chromosomal genes (Cervantes and Campos-Garcıa 2007). Usually, the genes located in plasmids encode

membrane transporters, which directly mediate efflux of chromate ions from the cell's cytoplasm. On the other hand, resistance systems encoded within bacterial chromosomes are generally related to strategies such as specific or unspecific Cr (VI) reduction, free-radical detoxifying activities, repairing of DNA damage, and processes associated with sulfur or iron homeostasis. Table 2 summarizes the bacterial strategies and have related to chromate tolerance.

Table-2: Bacterial mechanisms of chromate resistance

Enzyme/system	Species	Function	Reference
Transport			
ChrA transporter	Pseudomonas aeruginosa	Efflux of cytoplasmic chromate	Alvarez et al. 1999
Cys operon products	Shewanella oneidensis	Sulfate transport	Brown et al. 2006
TonB receptor, hemin transporter	S.oneidensis	Iron transport	Brown et al. 2006
Reduction			
Chromate reductases	Diverse species	Reduction of Cr(VI) to Cr(III)	Cervantes et al. 2001
SOD, catalase	Escherichia coli	Combat of oxidative stress	Ackerley et al. 2004
Outer membrane proteins	Caulobacter crescentus	General stress response	Hu et al. 2005
DNA repair			
RecG and RuvB DNA helicases	Pseudomonas aeruginosa	Repair of DNA damage	Miranda et al. 2005
SO0368, UvrD, and HrpA helicases	Shewanella oneidensis	Repair of DNA damage	Chourey et al. 2006
Other mechanisms			
Cys operon products	S. oneidensis	Sulfur metabolism	Brown et al. 2006
Adenylyl sulfate kinase	S.oneidensis	Sulfur metabolism	Brown et al. 2006
Ferritin	S. oneidensis	Iron binding	Brown et al. 2006

2.13 Scope of the present investigation

In the present investigation we have isolated an indigenous bacterial strain and characterized it by 16 S RNA sequencing as *Brevebacterium casei* (GenBank Accession Number: EU781952) for remediation of Cr (VI) from Sukinda mining effluent. The main aim is to establish a remediation route for detoxification of Cr (VI) using this indigenous microorganism with all optimized process parameters and conditions.

3. MATERIALS AND METHODS

3.1 General:

This chapter describes materials used and outlines the experimental design for biodegradation of hexavalent chromium through batch process. It also gives an overview of the isolation and characterization of bacterial strains from soil. Characteristics of soil and water samples obtained from Sukinda area are well documented. Optimization of culture and process parameters for the biomass growth and Cr (VI) degradation kinetics is also studied. Treatment of mineral processing effluent at optimum conditions is studied to observe the efficiency of the indigenous strain.

3.2 Chemicals:

Pure and analytical grade chemicals were used in all experiments including media preparation for growth. Peptone, yeast extract, beef extract and nutrient agar and nutrient broth were supplied by M/s Hi Media chemicals, India. Potassium dichromate and 1,5-Diphenylcarbazide was procured from Merck, Chemical, India. Stock solution for Cr (VI) solution was prepared following standard procedure (EPA Methods 7196A). M9 minimal salt media was purchased from Sigma Aldrich

3.3 Glassware and Apparatus:

All glass wares (Conical flasks, Measuring cylinders, Beakers, Petriplates and Test tubes etc.) are purchased from M/s Bhattacharya & Co. Ltd (Kolkata, India) under the name Borosil. The instruments and apparatus used throughout the experiment are listed below in Table 4.

Table 4: List of Instruments used during the whole experiment their make and function

Instruments	Make	Function
Vertical Autoclave	Test Master	Sterilization
Analytical Balance	Sartorius	Weight Measurement
Laminar airflow	Zhichen (ZhJH-1109C)	Aseptic Environment
pH	EuTech Instruments	Measurement of pH
BOD Incubator	Vikram Scientific Instruments	Incubation of cultures
Ultra Low Temperature freezer	New Brunswick (U410)	Preservation of cultures
Ultra pure water system	Sartorius	Preparation of the stock solution, throughout the experiment etc.
Spectrophotometer(UV/Vis)	Jasco(V-530)	Estimation of Biomass and Cr (VI) degradation
Incubator shaker	Environmental Orbital Shaker	Batch degradation kinetics of Cr (VI)
Ultra Centrifuge	Hettich Universal (320R)	Collection of pellet and Cr (VI) estimation
SEM-EDX	JEOL JSM-6330F, Japan	Elemental analysis of sample
Biofermenter	New Brunswick(BIO FLO 410)	Batch degradation study
TOC analyzer	Schimadzu	Estimation of Total organic carbon

3.4 Sample collection and Characterization

The chromite mines in the Sukinda valley is one of the largest opencast mines in the world, and they contain over 97% of India's natural chromite. The mines, which are huge, open pits, operate without any external supervision. In order to clear the way for more mining they have dumped 30 million tons of contaminated rock all throughout the region, and the mining industries flush water over the dumped mineral during the mineral processing operation. These effluent flows down the Brahmani river, which is the only source of water for residents in the area. Although several treatment units are under operation, yet they are unable to treat the effluent effectively due to outdated process. The soil and water samples collected (0–15 cm depth) from different locations of the chromium deposited and contaminated site located at Sukinda mines, Jajpur, Orissa are examined to estimate the amount of pollutants in the water samples. Isolation of

cultures from the soil sample collected was carried out as per standard procedures (Cappuccino and Sherman, 1996). The physical & chemical properties such as Soil organic matter, Cr (VI), total chromium, pH of the soil samples obtained from these waste sites were characterized and shown in Table-1. For water sample characterization Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Dissolved Solids (TDS) and Total Suspended Solids (TSS) parameters are determined. Presence of other heavy metals was estimated by Atomic Absorption Spectroscopy (ASS) methods. Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) is evaluated to understand the morphology, elemental composition and particle density of the chromium contaminated soil sample. The SEM-EDX analyses were carried out with the help of a computer controlled field emission SEM (JEOL JSM-6330F, JEOL Ltd., Akishima, Tokyo, Japan) equipped with a EDX detection system. The SEM was used in its most common mode the emissive mode.

3.5 Isolation and Culture Conditions:

For isolation of chromium resistant bacteria, 1 ml of the wastewater sample was spread on PYE medium (Peptone, Yeast extract) agar plates containing 100 µg of Cr^{6+}/ml supplemented as $K_2Cr_2O_7$ to the medium. PYE agar plates were prepared by dissolving .1 g NaCl, 1 g Peptone and 0.5 g yeast extract in 100 ml distilled water, pH adjusted at 7.2–7.5 and then 1.5 g agar was added in the 250 ml flasks. The medium was autoclaved at 121^0C and 15 Lb pressure for 15 min. The growth of the bacterial colonies was observed after 24 h of incubation at 37^0C. Isolated colonies were picked up with sterilized wire loop and streaked on PYE agar medium plate containing 100 mg/ ml. Cr^{6+}/. It was again incubated at 37^0C for 24 h. This process was repeated with successively higher concentrations (12.5, 25, 50, 75, 100µl/ml) of Cr^{6+} until the minimum inhibitory concentration (MIC) of bacterial isolate was obtained. Significant growth and rapid Cr

(VI) degradation kinetics of the specific bacterial species in the presence of 25, 50 and 100 µl/l Cr (VI) during twenty four hours of incubation at 30°C, were considered as Cr (VI) resistant. A single strain capable of growing at this condition was selected for further experiments

3.6 16S rRNA **Identification and Phylogenetic analysis of the enrichment culture:**

16S rRNA gene sequencing is a powerful tool that has been used to trace phylogenetic relationships between bacteria, and to identify bacteria from various sources, such as environmental or clinical specimens. This technology is used today in clinical laboratories for routine identifications, especially for slow-growing, unusual or fastidious bacteria, but also for bacteria that are poorly differentiated by conventional methods; however, it provides no information about antibiotic resistance. Identification based on the 16S rDNA sequence is of interest because ribosomal small-subunit (SSU) exists universally among bacteria and includes regions with species-specific variability, which makes it possible to identify bacteria to the genus or species level by comparison with databases in the public domain. The molecular approach has been used for bacterial phylogeny and is of major importance for species definition and identification (Clarridge et al., 2004). The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level. The microbe was detected based on nucleotides homology and phylogenetic analysis (rRNA sequencing). The rRNA is the most conserved (least variable) gene in all cells. Portions of the rDNA sequence from distantly-related organisms are remarkably similar.

3.7 Minimum inhibitory concentration (MIC):

MIC in microbiology is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC of chromate for each isolate was

determined by colony counting method. Plates containing nutrient agar medium supplemented with different concentrations (100 -8000 mg/l) of $K_2Cr_2O_7$, were inoculated with 250 µl of a fresh overnight culture grown in nutrient broth medium and rinsed once in the same volume of a solution of MgSO4 (10 mM). All plates were incubated with shaking at 30°C for 48hours. The growth of bacteria was monitored by colony counting method.

3.8 Antibiotics Disk Sensitivity Test:

To determine the Antibiotic sensitivity of the bacterial strain, Antibiotic – Impregnated Discs are placed on freshly prepared agar plates at 30^0C for 24 hrs. The diameter of the inhibition zones was measured to the nearest cm and the isolate is classified as resistant, intermediate and susceptible following the standard antibiotic disc sensitivity testing method. The chromium resistance strain was tested for its sensitivity to 5 different antibiotics. Gentamycin (10 µg)**,** Kanamycin (15 µg), Streptomycin (10 µg), Paramycin (10 µg), Chloromphenicol (30µg).

3.9 Media Optimization:

The effect of several organic & inorganic nitrogen sources (Ammonium chloride, Ammonium sulphate, Urea Ammonium nitrate, Yeast extract, Beef extract & Peptone) and various carbon sources (Dextrose Fructose Glycerol, Maltose, Sucrose, and Starch soluble) in the form of saccharides and disaccharides are studied for hexavalent chromium Cr (VI) bioreduction and optimum biomass production. The optimized parameters are further considered for large scale degradation of Cr (VI) using fermenter.

3.10 Process optimization:

The optimization study was carried out for duration of 12 hours usuing m9 minimal salt media. Temperature and pH were the important parameter considered in the optimization experiments which were carried out in triplicates. For temperature optimization the five standard temperatures

considered were 26^0C, 28^0C, 30^0C, 32^0C and 34^0C while for pH optimization, the five standards used were 4, 5, 6, 7 and 8. The respective pH was adjusted with 0.5N NaOH and 0.5N HCl.

3.11 Heavy metal-resistance:

The Chromium resistant bacteria offer the unique opportunity to compare bacteria isolated from anthropogenically chromium-polluted ecosystems with those isolated from naturally Cr-percolated ecosystems. Resistance of selected bacterial isolate to other heavy metals was tested in nutrient agar supplemented with metals. Metals that were tested include Zn^{+2}, Co^{2+}, Ni^{2+}, Pb^{+2}, $Cu^{2+,}$ Mn^{2+} and Hg^{2+}.

3.12 Cr (VI) reduction:

The bacterial strain, are precultured overnight in PYE broth. Culture flasks (500-ml with a final liquid volume of 100 ml) containing minimal salts medium supplemented with Cr (VI) ranging from 10–50 mg Cr (VI)/l medium and 0.5% glucose were inoculated with the equal amounts of culture species. Media without Cr (VI) was inoculated with bacteria and uninoculated media containing Cr (VI) served as controls. All the cultures including controls (in duplicate) were incubated for 24 h at room temperature (30°C) with shaking speed (100 rpm). Growth of the bacteria was monitored at specific time intervals, by measuring optical density of the cultures at 600 nm. To compute the Cr (VI) reduction 1ml culture from each of the above flasks was centrifuged (6000 rpm for 10 min at 10°C) and the supernatant analyzed for Cr (VI).

3.13 Diphenyl carbazide assay:

Diphenyl carbazide assay for measurement of Cr^{6+} was developed from Standard Methods for the Examination of Water and Wastewater (Greenberg et al., 1992) as well as the methods listed in Turick et al., 1996). Absorbance readings for reduction cultures and Cr^{6+} standards of 0, 25, 50, 100 mg/l were recorded.

3.14 Measurement of cell Growth and Cr (VI) reduction:

The bacterial strain, are precultured overnight in PYE broth and the cell pellets were collected by centrifugation (6000*g* for 10 min at 10°C) followed by washing the cell pellets in phosphate buffer (0.1 M NaH_2PO_4-Na_2HPO_4; pH, 7.1). After two washes of the cells in phosphate buffer, the cells were re-suspended in the similar buffer. Culture flasks (150 ml with a final liquid volume of 30 ml) containing minimal salts medium supplemented with Cr (VI) ranging from 5–50 mg Cr (VI)/l medium and 0.5% glucose were inoculated with the equal amounts of culture species. Media without Cr (VI) was inoculated with bacteria and uninoculated media containing Cr (VI) served as controls. All the cultures including controls (in duplicate) were incubated for 72 h at 30^{o}C temperature with constant shaking at 100 rpm. Growth of the bacteria was monitored at specific time intervals, by measuring optical density of the cultures at 600 nm. To compute the Cr (VI) reduction by growing cells, a 1-ml culture from each of the above flasks was centrifuged (6000 rpm for 10 min at 10°C) and the supernatant analyzed for Cr (VI).

3.15 Batch degradation of Hexavalent chromium using Fermenter:

Large scale degradation of Cr (VI) at its optimum conditions by batch fermenter was performed using the indigenously isolated microbial strain of *B. casei*. In order to check the Cr (VI) reduction potential of the selected strain in mining & mineral processing industry, samples from Sukinda chromite mining effluent containing Cr (VI) 36 mg/l, Fe 22mg/l, Al 16 mg/l, Ni 12 mg/l, Co 11 mg/l and Si 8 mg/l in addition with microbial optimized nutritional medium are directly checked for hexavalent chromium reduction in fermenter (New Brunswick, BIO FLOW 410).

4. RESULTS AND DISCUSSIONS

4.1 Characteristics of Collected Samples:

Chromium contaminated soil and water samples were collected in screw capped sterilized bottles from different mining areas of at Jajpur district of Orissa State in India. Physicochemical parameters of contaminated soil and wastewater estimated are shown in Table 5 and Table 6. The pH of the samples was determined with an ion-specific electrode and the pH of the soil and waste water samples were in the range of 7.4 - 7.8 and 7 - 7.2, respectively. This indicated that the chromite contaminated sites are slightly alkaline in nature. The range of Cr (VI) in the contaminated soil sample is in the range of 2-6 mg/l and in case of effluent it varied from 36- 44 mg/l. Total Dissolved solids (TDS) and Total suspended solids (TSS) in the effluent varied form 480- 560 mg/l and 57 – 63 mg/l respectively. Our results showed similarity with the results reported elsewhere (Tiwary et al., 2005).

High degree of contaminants in the water is generally of inferior palatability and induces unfavorable physiological reactions in transient consumers. Analysis of the solids is important in the control of biological wastewater treatment process and for assessing compliance with regulatory agency limitations. The current waste water analysis indicates concentration of TDS and TSS as well within their permissible limits. COD/BOD ratio for industrial waste water ranges up to 10 or more (Markantonatos, 1990). A COD/BOD ratio in the range from 3 to 7 suggests that the waste water can be readily biodegradable. In our reported results COD/BOD ratio varies within 3.2 to 4.5 indicating it has a potential for biodegradation as indicated in Table 6. The maximum permissible limit for Cr (VI) in the treated effluent is 0.5 mg/L and the indicated effluent shows many folds increase in Cr (VI) concentration and hence needs to be addressed urgently.

Table 5: Physiochemical Properties of soil sample collected from Sukinda area

Parameters (SOIL)	Soil organic matter	Cr (VI)	Total chromium	pH
Estimated values	5.2 ± 0.5%	2–5.9 mg/g of soil	17-21 mg/g of soil	7.4-7.8

Table 6: Physiochemical Characterization of collected waste water from Sukinda region

Parameters (Water)	**Values**	**Metals**	**Concentration (mg/L)**
pH	7.0-7.2	Copper (Cu)	3.25-8
BOD_5	560 - 406	Iron (Fe)	36-42
COD	1794-1831	Aluminum (Al)	24-29
TDS (mg/L)	480- 560	Silicon (Si)	8-11
TSS (mg/L)	57 - 63	Cobalt (Co)	14-16
Total Cr (mg/L)	50 - 60	Nickel (Ni)	22-28
Cr (VI) (mg/L)	36 -44		

4.2 SEM- EDX analysis of the soil sample:

Scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX) is evaluated to understand the morphology, elemental composition and particle density of the chromium contaminated soil sample. The SEM-EDX analyses were carried out with the help of a computer controlled field emission SEM equipped with EDX detection system. Detailed examination by SEM/EDX of the soil sample and surfaces enabled us to detect its morphological characteristics; all the minerals to be associated with specific morphologies and some micrographs illustrate the shape of the soil particles. SEM results suggest that the soil is a heterogeneous material containing a large particle size distribution as well as small grumes adhered to particles of major

size, indicating that the material is a heterogeneous powder more importantly, allowed to understand the chemical composition of the Cr (VI) contaminated soil sample. The soil samples SEM analysis of soil samples determine its morphological characteristics. The micrograph illustrates the shape of the soil particles (Fig 1). Chemical composition was determined by using an EDAX spectrometer, the detection limit was 0.01 %. Analyses of these soil particles, with EDX, indicate that chromium is present in the soil sample along with other heavy metals such as Fe, Al and Si and is shown in Fig 2. Gutiérrez et al., in 2009 reported similar morphological characters and elemental chemical analyses of the soil performed with SEM- EDX collected from the facilities of the Química Central (QC) chromate factory, which is located at León-San Francisco del Rincón.

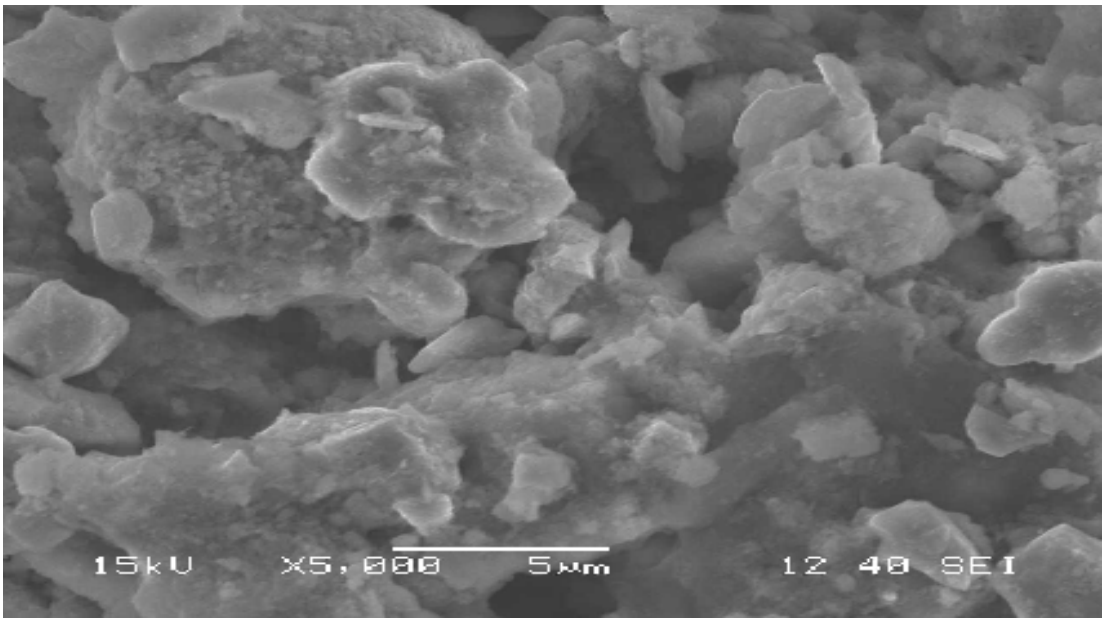


Fig 1: SEM-EDX of the soil sample (5-Nov-2008 05:00 PM 15 kV 35 Degree)

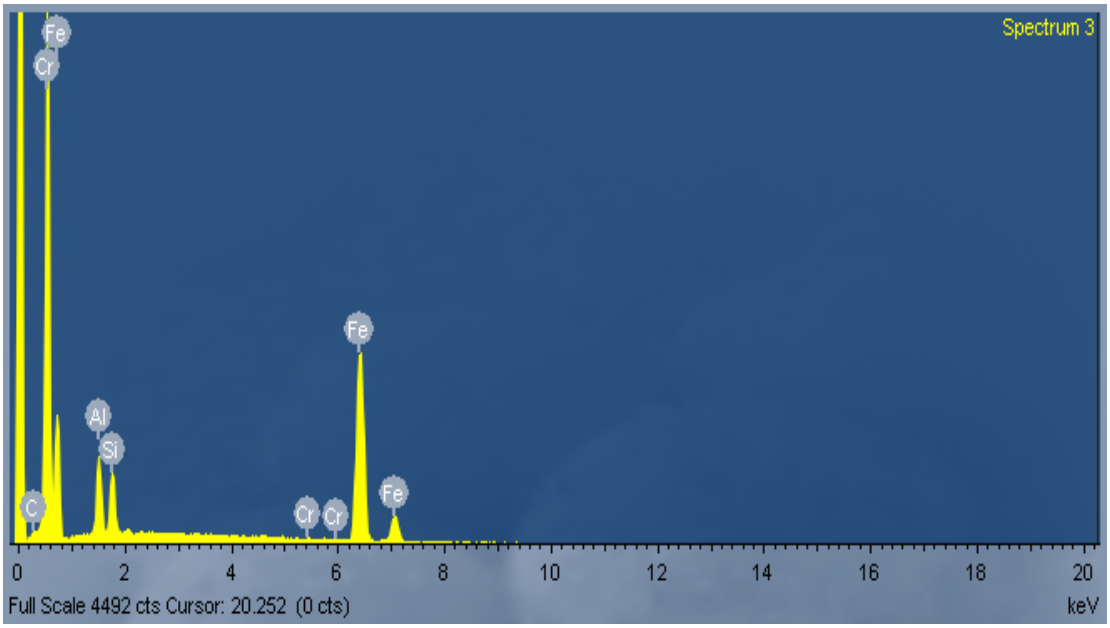


Fig 2: EDX of the soil sample (5-Nov-2008 05:00 PM 15 kV 35 Degree)

4.3 Isolation of Chromium Resistant bacterial Strains and Cr (VI) Tolerance study:

Soils contain a very large number of micro-organisms which can include a number of Cr (VI) utilizing bacteria. Three chromium resistance bacterial strains were isolated from the soil sample by serial dilution technique [Fig 3]. The isolates are tested for their chromate tolerance at different concentrations (25- 250µl/ml) in solid agar medium. All the three bacteria showed resistance to 100 mg/l of Cr (VI) in nutrient agar media amongst which *Brevibacterium sp* was able to grow to a concentration of 250 mg/l of Cr (VI) as illustrated in Fig 4. Megharaj, et al., in 2002 isolated two species such as *Arthrobacter* sp. and a *Bacillus* sp., from tannery waste contaminated soil that showed similar resistance to Cr (VI) and had the ability to reduce Cr (VI) to Cr (III). Both bacterial strains tolerated for Cr (VI) at 100 mg/ml on a minimal salt agar medium supplemented with 0.5% glucose, but only *Arthrobacter* could grow in liquid medium at this concentration. *Arthrobacter* sp. could reduce Cr (VI) up to 50 µg/ml, while *Bacillus* sp. was

not able to reduce Cr (VI) beyond 20 µg/ml. *Arthrobacter* sp. was distinctly superior to the *Bacillus* sp. in terms of their Cr (VI)-reducing ability and resistance to Cr (VI).

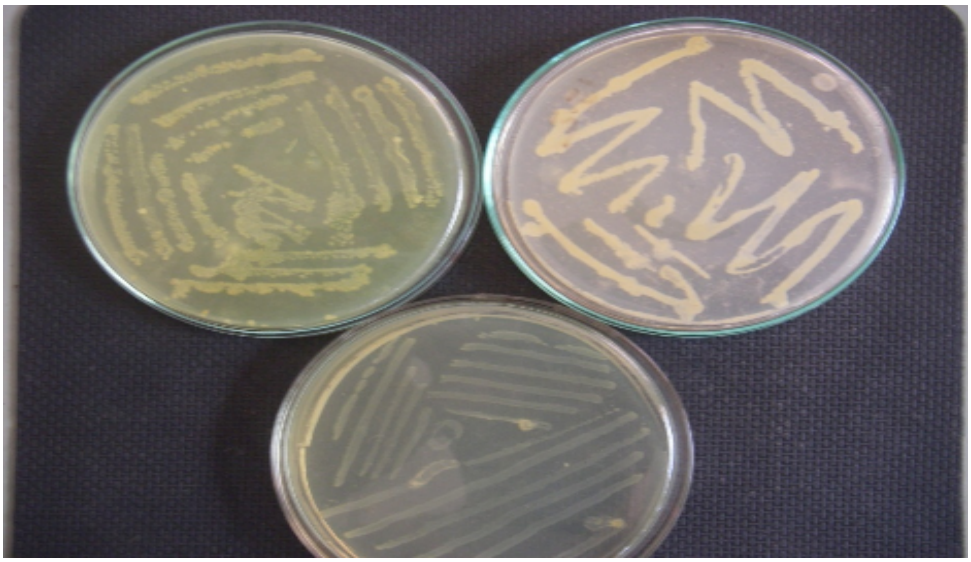


Fig-3: Isolates are tested for their chromate tolerance at different concentrations Cr (VI) in solid agar medium

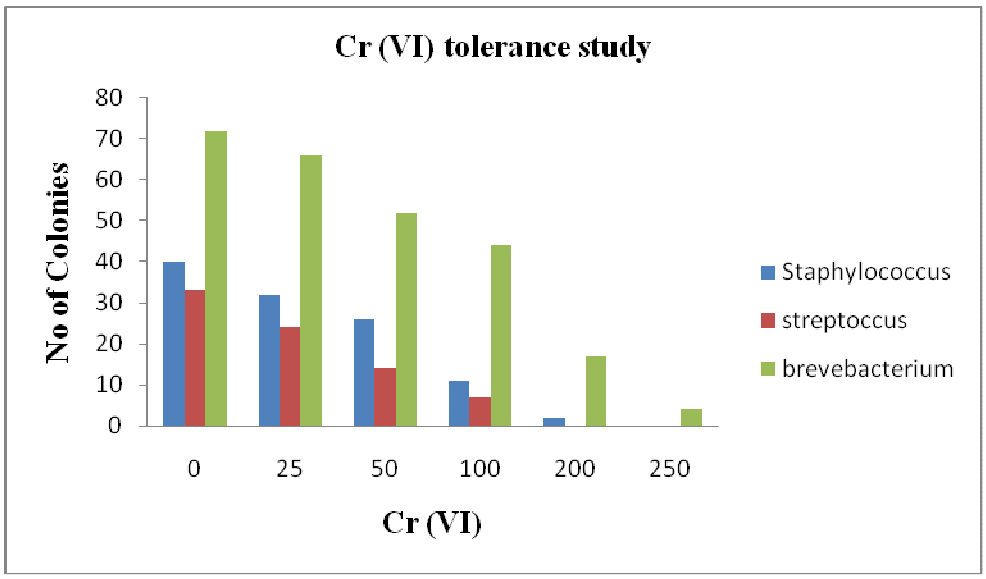


Fig-4: Cr (VI) degradation characteristics of the isolated strains at varying Cr (VI) concentrations ranging from 0- 250 mg/l.

Cr (VI) degradation kinetics were conducted using nutrient broth medium at initial Cr (VI) concentrations 25 mg/l, 50 mg/l and 100 mg/l as shown in Fig 5, 6 and 7 respectively. It was observed that *Brevibacterium sp* was able to degrade Cr (VI) rapidly as compared to the rest of the two isolates *Staphylococcus* and *Streptococcus*. *Streptococcus* showed reduced rate of degradation for all the concentrations under study. It was evident from Fig 5 at particular initial concentration 25 mg/l, that all the species reached equilibrium within 12 hours. However, within 9 hours incubation period *Brevibacterium, Staphylococcus and Streptococcus* achieved 99.3%, 96% and 91% degradation of Cr (VI) respectively. The removal response of two bacterial strains *Staphylococcus, Streptococcus* towards different concentrations of Cr (VI) differed greatly as compared to *Brevibacterium sp.* The maximum reduction capacity of *Brevibacterium sp* changed insignificantly with increasing Cr (VI) concentrations from 25 mg/l to 100 mg/l. However, the other two isolates showed extended equilibrium time for Cr (VI) degradation kinetics for Cr (VI) concentrations ranging from 25mg/l to 100 mg /l. Experimental results show that *Brevibacterium sp* was distinctly superior to *Staphylococcus* and *Streptococcus* in terms of its Cr (VI)-reducing ability and resistance to Cr (VI). The higher capability of B.Casei may be due to the higher tolerance ability of the gram negative bacteria to Cr (VI) than the gram positive species. Eagon 1984 and Vasanthy et al., 2003 explained the higher resistance ability of the gram negative bacteria to heavy metals than the gram positive species. Murugesan and Vasanthy reported that *Pseudomonas* sp. removed 92.7% of Cr at 10 ppm and 86% under 50 ppm concentrations. The maximum metal tolerance by the gram negative bacterial forms might be due to their abundant sedentary organism and also due to the metal precipitation in their peptidoglycan layers. The lipopolysaccharides nature of the outer membrane is responsible for efficient metal binding capacity. It can also be concluded that the reduction of Cr (VI) to Cr (III) was significantly

dictated by the presence of soluble enzymes. Similar findings were also reported using *Pseudomonas aeruginosa* isolated from soil sample (Liu et al., 2008).The rate of degradation of Cr (VI) by *Brevibacterium species* was more pronounced at lower incubation time with respect to other two species. It can be understood that *Brevibacterium sp* has maximum enzymatic degradation potential responsible for chromate metabolism that reduces Cr (VI) to Cr (III) more efficiently with respect to the rest of the two isolates. Moreover, the sensitivity of bacteria towards heavy metal is a characteristic of the strain, which leads to difference in the kinetics of Cr (VI) reduction. B. Chardin et al., in 2002 reported the effects of Cr (VI) on bioenergetics metabolism in two sulfate-reducing bacteria (SRB), *Desulfovibrio vulgaris* and *Desulfomicrobium norvegicum*, using isothermal microcalorimetry. His results revealed that Cr (VI) induces an inhibition of growth with concomitant production of energy, which can be compared to the reaction of the bacteria to a stress such as oxidative stress.

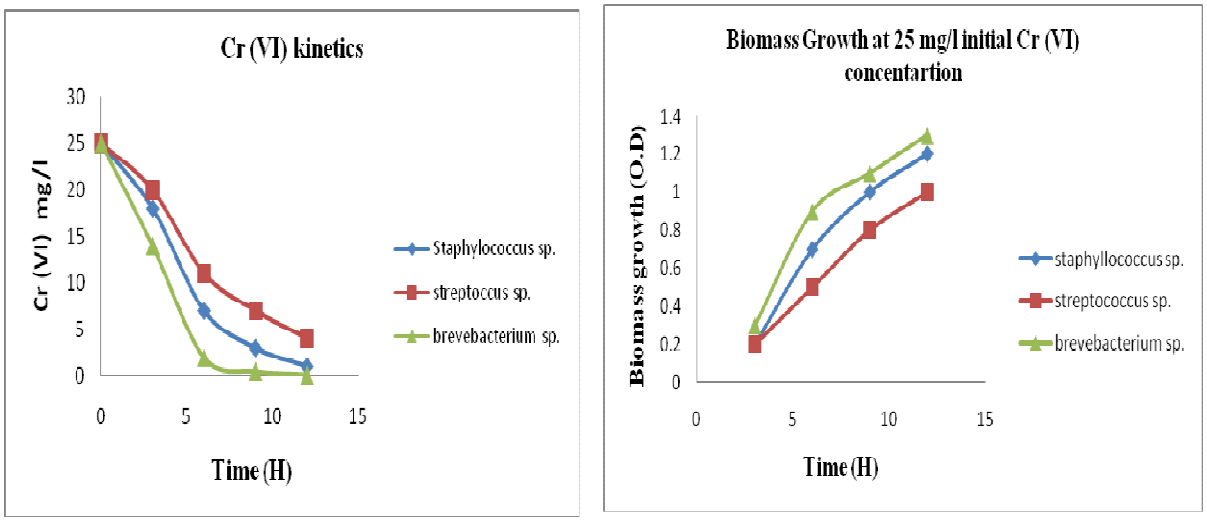


Fig-5 (a) & (b): Kinetics of Cr (VI) degradation and Biomass production by the isolated strains at Initial Cr (VI) concentration of 25mg/l at 30^0C, pH-7 and 100 rpm

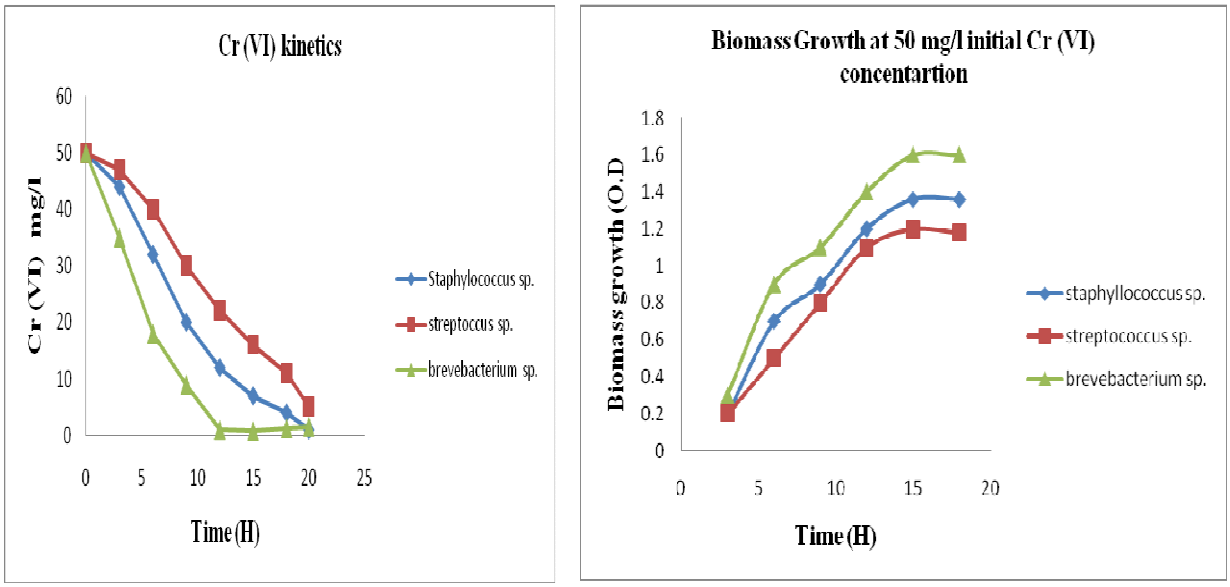


Fig-6(a) & (b): Kinetics of Cr (VI) degradation and Biomass production by the isolated strains at Initial Cr (VI) concentration of 50mg/l at 30^0C, pH-7 and 100 rpm

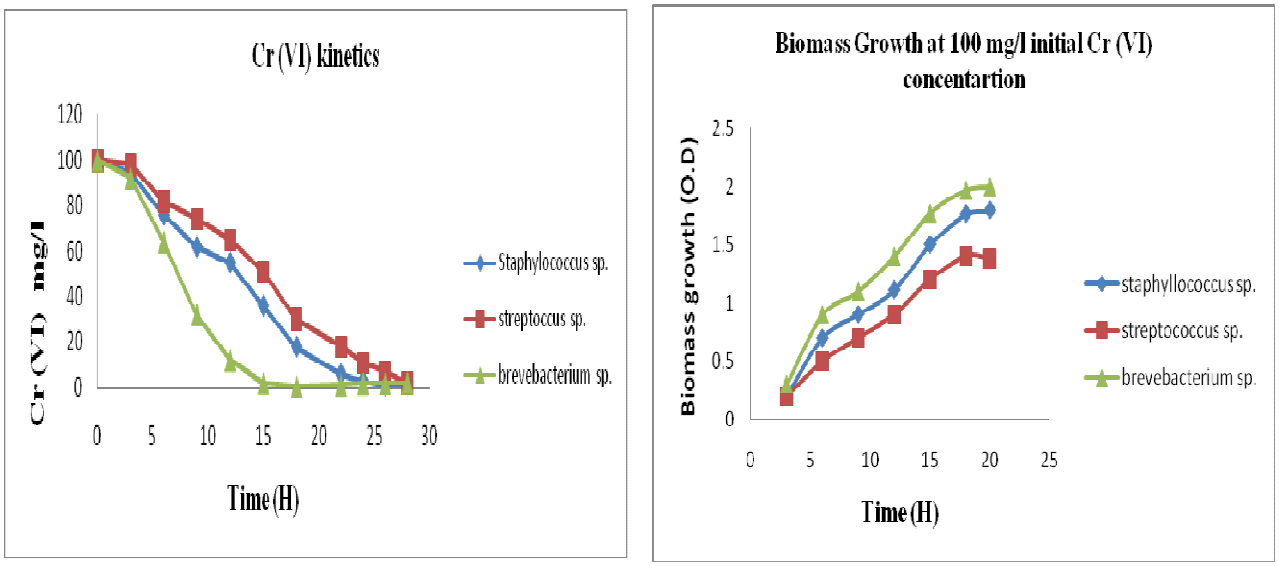


Fig-7(a) & (b): Kinetics of Cr (VI) degradation and biomass production by the isolated strains at initial Cr (VI) concentration of 100 mg/l at 30^0C, pH-7 and 100 rpm

4.4 Characterization of the isolated strains:

Three isolates capable of growing at varying Cr (VI) concentration are identified as *Staphylococcus, Streptococcus* and *Brevibacterium sp* by their physiochemical & morphological parameters Table 7. All of the three isolates share some similar (Indole Production, Citrate utilizing, MR-VP reaction, Starch utilizing, Growth in aerobic condition) and dissimilar (Gram staining, Colony morphology, Motility, H_2S production and catalyses activity) characteristics. The single strain capable of growing at 250µl/ml was identified as *Brevibacterium casei* (GenBank Accession Number: EU781952) based on nucleotides homology and phylogenetic analysis (16S rRNA sequencing).

Table-7: Morphological & Physiochemical characteristics of the chromium resistant *Staphylococcus* and *Streptococcus bacterial strain*

	Characteristics	**Staphylococcus. sp**	**Brevebacterium sp**	**Streptococcus. sp**
1.Morphological	Gram staining	+Ve	-Ve	+Ve
	Motility	-Ve	+Ve	-Ve
	Colony Morphology	Coccus in rod shape	Circular convex	Spherical, chain
	Colour	Creamish White	white	Brown
2. Biochemical	Indole Production	-Ve	-Ve	-Ve
	Citrate utilizing	-Ve	-Ve	-Ve
	VP reaction	-Ve	-Ve	-Ve
	MR reaction	+Ve	+Ve	+Ve
	Oxidase activity	-Ve	+Ve	+Ve
	H_2S production	-Ve	-Ve	+Ve
	Starch hydrolysis	-Ve	-Ve	-Ve
	Catalayse activity	+Ve	+Ve	-Ve
3. Physiological	Growth in aerobic condition	+Ve	+Ve	+Ve
	Temperature	$30^0C \pm 2$	$30^0C \pm 2$	$34^0C \pm 2$
	Growth pH	7 ± 0.2	7 ± 0.2	7 ± 0.2

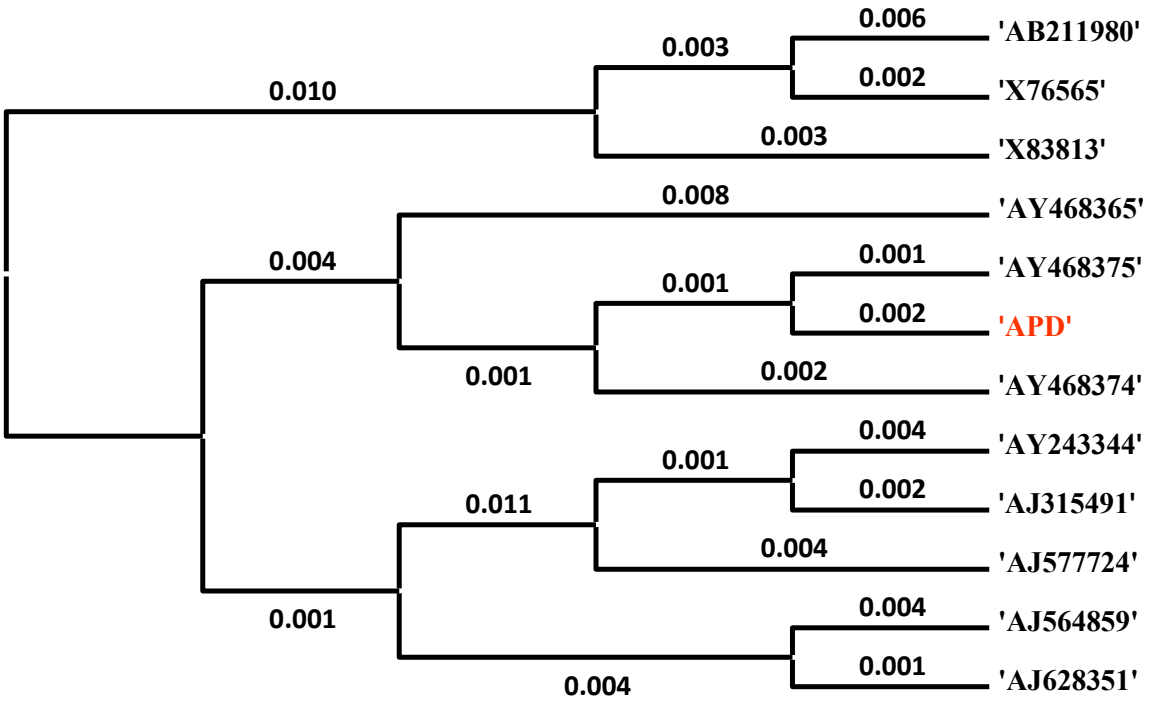
4.5 Bacterial Identification by 16S rRNA, Sequence Alignment and phylogenetics:

Genomic DNA was isolated from the pure culture pellet. Using consensus primers, the ~1.5 kb 16S rDNA fragment was amplified using high-fidelity PCR Polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homologs for the microbe. Based on nucleotides homology and phylogenetic analysis the microbe (Sample: **APD**) was detected to be *Brevibacterium casei.* Nearest homolog species was found to be *Brevibacterium sanguinis.* The final contiguous sequence of 1490 base pairs was used to search both the Ribosomal Database Project http://rdp.cme.msu.edu/ and Genbank http://www.ncbi.nlm.nih.gov/ databases as described in Table 8. Phylogenetic tree was made in MEGA 3.1 software using Neighbor Joining method as shown in Table 9. For comparing the inferred rRNA sequences (or those of any other appropriate molecule) it is possible to estimate the historical branching order of the species, and also the total amount of sequence change. A partial nucleotide sequence of 689 base pair was submitted at NCBI genbank with accession number EU781952.The 16s rDNA sequence has hypervariable regions, where sequences have diverged over evolutionary time. These are often flanked by strongly-conserved regions. Primers are designed to bind to conserved regions and amplify variable regions. The DNA sequence of the16S rDNA gene has been determined for an extremely large number of species are available over the internet through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the Ribosomal Database Project (www.cme.msu.edu/RDP/html/index.html). These sites also provide search algorithms to compare new sequences to their database.

Table 8: Alignment View using combination of NCBI Genbank and RDP database

Alignment View	ID	Alignment results	Sequence description
	APD	0.93	Studied sample
	AY468375	0.94	*Brevibacterium casei* strain 3Tg
	AY468374	0.93	*Brevibacterium casei* strain 3s (a)
	AY468365	0.89	*Brevibacterium casei* strain FM1A
	AY243344	0.91	*Brevibacterium antiquum* st. VKM Ac-2118
	AJ577724	0.92	*Brevibacterium antarcticum* type st. DVS 5a2
	AB211980	0.92	*Brevibacterium linens*
	X83813	1.00	*Brevibacterium iodinum*
	AJ315491	0.95	*Brevibacterium linens* strain SB1
	X76565	0.99	*Brevibacterium epidermidis* st. NCDO 2286
	AJ628351	0.90	*Brevibacterium sanguinis* strain CF 52
	AJ564859	0.91	*Brevibacterium sanguinis* type strain CF63T

Table 9: Phylogenetic Tree made in MEGA 3.1 Software using Neighbor joining method



4.6 Minimum inhibitory concentration (MIC):

Comparison of isolates MICs indicated that all strains isolated from the plates with $K_2Cr_2O_7$were resistant to Cr (VI), but isolates exhibited different levels of resistance (Fig 7). The strains identified as *Staphylococcus and Streptococcus* showed 200 and 100 mg/l Cr (VI) respectively. The isolated strain *Brevebacterium casei*, was the most resistant of the tested strains. The strain tolerated 250 mg/l Cr (VI) of chromate; therefore, this strain was defined as Cr (VI)-sensitive. The MIC of the selected *Brevebacterium casei* strain showed high-level resistance against potassium chromate in nutrient agar 8000mg/l as illustrated in Fig 8. A comparison of Cr uptake by the parental strains and their cured derivatives revealed that plasmids in these strains express high level resistance to chromate by exerting stringent control on the accumulation of Cr.

Literature review indicates that very limited strains have shown such high level resistance to Cr (VI). Rehman et al., 2008 assessed the ability of *Bacillus* sp.ev3 to reduce hexavalent chromium into its trivalent form. *Bacillus* sp.ev3 could tolerate Cr^{6+} (4800 µg/mL), Pb^{2+} (800 µg/mL), Cu^{2+} (200 µg/mL), Cd^{2+} (50 µg/mL), Zn^{2+} (400 µg/mL), Ni^{2+} (4000 µg/mL) and Hg^{2+} (50 µg/mL).

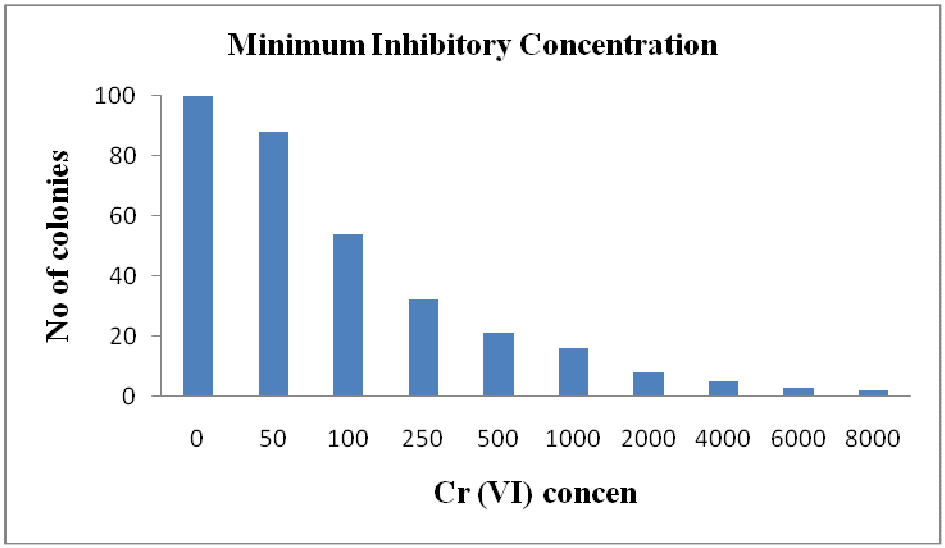


Fig-8: Minimum inhibitory concentration of the selected strain *B. casei*

4.7 Antibiotics Disk Sensitivity Test:

Strain isolated from same soil shows different susceptibilities to drugs. The chromate resistant isolate was tested for their sensitivity to 5 commonly used antibiotics such as chloramphenicol, ampicillin, streptomycin, tetracycline and kanamycin to access its degree of sensitivity. Isolate strain appeared to be most susceptible being inhibited by all antibiotics. The strain was resistant to paramycin and chloromphenicol, while it is intermediate to Kanamycin and has susceptible response to Gentamycin and Streptomycin as described in Table 10 and Fig 9. Similar results have been suggested by De Souza et al, 2006. He reported the bacterial isolates (Pseudomonas, Flavobacterium, Micrococcus, Alcaligenes, Aeromonas, Vibrio and Acinetobacter) isolated from Antarctic marine waters polluted with concentrate heavy metals showed varying degrees of resistance to antibiotics (chloramphenicol, ampicillin, streptomycin, tetracycline and kanamycin) and metals. Depending on the antibiotics the isolates showed different percentage of resistance. Multiple drug and metal-resistance were observed. High incidence of resistance to both antibiotics and metals were common among the pigmented bacterial isolates. Increased resistance decreased the ability of bacteria to express enzymes. Bezverbnaya et al., 2005 reported that in plasmids these heavy metal resistance genes may be linked with genes of antibiotics resistance and genes responsible for pathogenic property. Thus individual bacterial strains have developed capacity to survive under toxicological stress which is sometimes hazardous to humans and animals. Hence it is essential to study the metal resistant bacteria which are being formed in response to selective anthropogenic pollution.

Table 10: Antibiotic sensitivity profile of Cr-resistant bacteria

Antibiotics	Diameter of Inhibition Zone (cm)
Gentamycin (10 µg)	2.7 cm
Kanamycin (15 µg)	2 cm
Streptomycin (10 µg)	3 cm
Paramycin (10 µg)	1 cm
Chloromphenicol (30µg)	1 cm

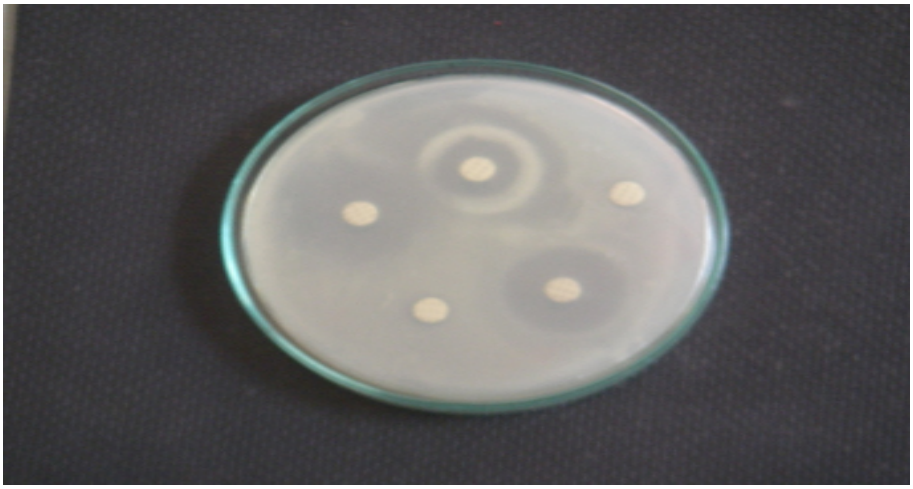


Fig- 9: Illustrates the diameter of inhibition zone for different antibiotic sensitivity profile of Cr-resistant bacteria

4.8 Resistance to other heavy metals:

The selected Chromium-resistant strain was tested for its resistance to other heavy metals such as Ni, Cu, Cr, Zn, Mn, Co, Pb, and Fe .Relative growth of isolate in different metal-containing media is shown in Fig-10. It was evident that isolate was resistant to all metals at low concentrations ranging from (1-15) mg/L, but at higher concentrations beyond 25 mg/L only it could resist Ni and Fe. It shows that the strain is incapable to grow in presence of high concentration of other heavy metals. Cr (VI) reduction by the selected strain was significantly

stimulated by the presence of Ni, Co and Fe. Presence of lead (Pb) was significantly inhibitory for Cr (VI) reduction, while Mn and Zn did not affect the reduction process. Sultan et al ., 2006 reported that Ochrobacterium intermedium strain STCr-5 significantly reduces Cr (VI) in presence of Cu, Co, Mn, Ni and Zn. He suggested that Ni and Cu resulted in more than 40 % increase in Cr (VI) reduction where as Pb did not affect the Cr (VI) reduction process.

Thacker et al., 2007, reported gram negative strain of Brucella sp., isolated from Cr(VI) contaminated sites was tested for its tolerance to other heavy metals that showed high degree of resistance to different heavy metals in the order Hg^{2+} > Co^{2+} > $Cr6^{+}$ > Ni^{2+} > Pb2+ >Zn^{2+}. It indicates that the bacterial sp. can be used to degrade the following metals at their optimized conditions. Viti et al. (2003) have compared the MIC of the chromium resistant isolates to various heavy metals and reported that different isolates exhibited different level of metal tolerance. Contaminated habitats are generally characterized by the co-existence of a large number of toxic cations and, therefore, it is necessary to study the multiple metal resistances of micro-organisms. Tolerance to other metals has an added advantage of withstanding the presence of different metallic ions while performing the desired activity. Sultan and Hasnain (2005) have shown that gram positive chromate resistant isolate exhibited tolerance against salts of Ba2+, Cd2+, Cu2+, Fe3+, Mn2+, Ni2+ and Pb2+ but showed sensitivity to Co2+, Hg2+ and Zn2+. Many genes conferring antibiotic resistance are located on mobile genetic elements, some of which are easily exchanged among phylogenetically distant bacteria. Many of these mobile genetic elements encode resistance to multiple antibiotics, heavy metals and other toxic compounds. Therefore, it is likely that selective pressure by one of such compounds indirectly selects for other resistances, and preliminary studies was raised significantly in heavy metal contaminated environments (Branco et al., 2005).

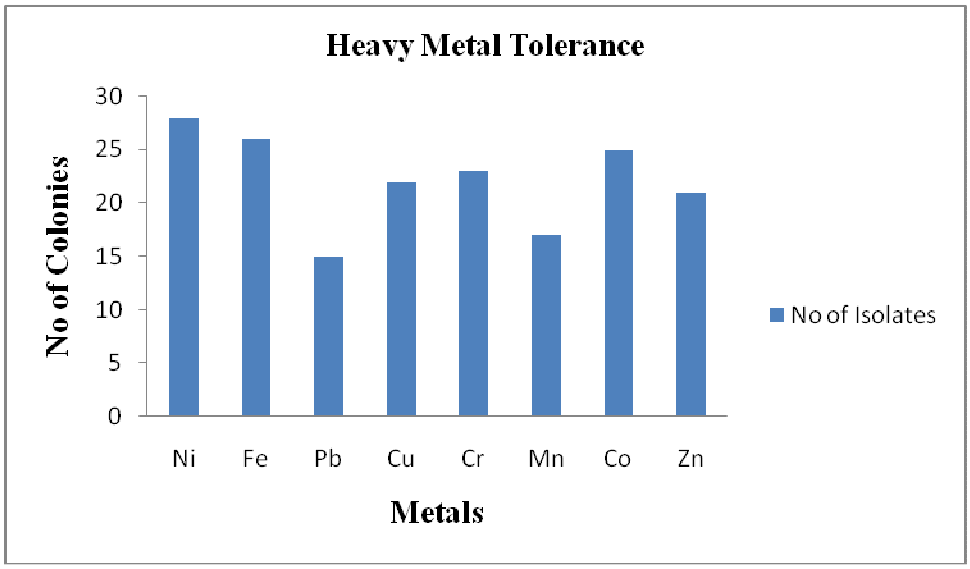


Fig-10: Heavy Metal Resistance studies and Relative growth of isolate in different metal-containing Nutrient Agar media

4.9 Growth Medium Optimization:

The Biomass growth studies were carried out using various organic and inorganic nitrogen sources (Ammonium chloride, Ammonium sulphate, Urea, Ammonium nitrate, Yeast extract, Beef extract and Peptone) and various carbon sources (Dextrose, Starch, Glycerol, Maltose, Sucrose, and soluble) in the form of saccharides and disaccharides. Growth of *B. casei* varied according to the nitrogen source used (Table 11). The biomass was greater when grown in the presence of organic rather than inorganic nitrogen sources. The highest productivity of *B. casei* was obtained when peptone was used in the media followed by Beef extract, and Ammonium nitrate (Fig- 11).

The effect of carbon sources on growth of *B. casei* was studied in presence of peptone that indicated highest productivity of *B. casei*. Final biomass was higher in the presence of organic nitrogen for all carbon sources tested (Table 12). It can be observed that significant reduction of Cr (VI) to 0.5 mg/l is obtained at maximum growth of biomass (5.7×10^9 cfu m/l). Significant

interactions were found between the carbon and nitrogen sources. The highest productivity of the strain was obtained when Dextrose was used as a carbon source in the media (Fig-12). It is inferred that Dextrose being monosaccharide was easily degraded by the organism. Similarly Rama Krishna et al. in 2005 suggested the effect of various electron donors on Cr (VI) reduction using *Ganoderm lucidum* (a wood rooting fungus) in a bioreactor–reduction system. Among five electron donors (peptone, acetate, dextrose, molasses and sewage) screened, peptone showed maximum Cr (VI) reduction followed by molasses. Shaili Srivastava et al., 2007 evaluated the potential of Aspergillus sp. for removal of chromium in shake flask culture in presence of carbon and nitrogen source. He reported maximum chromium could be removed using sodium acetate (0.2%) and yeast extract (0.1%).

Table -11: Growth of *Brevibacterium casei* (cfu m/l) in media containing different synthetic nitrogen sources

Carbon Source	Nitrogen source	Mean (cfu m/l)	Cr(VI) reduction mg/l	pH_0 (Initial)	pH_f (Final)
None	Peptone	3.7×10^9	5	6.6	7.3
	Beef extract	3.4×10^9	.30	6.7	5.1
	Yeast extract	2.4×10^9	6.60	6.8	5.6
	Ammonium nitrate	3.2×10^5	3.58	5.2	5.4
	Urea	2.6×10^5	2.70	7.7	8.0
	Ammonium chloride	3.1×10^5	9.40	6.2	6.7
	Ammonium sulphate	3.1×10^5	8.98	5.2	6.2

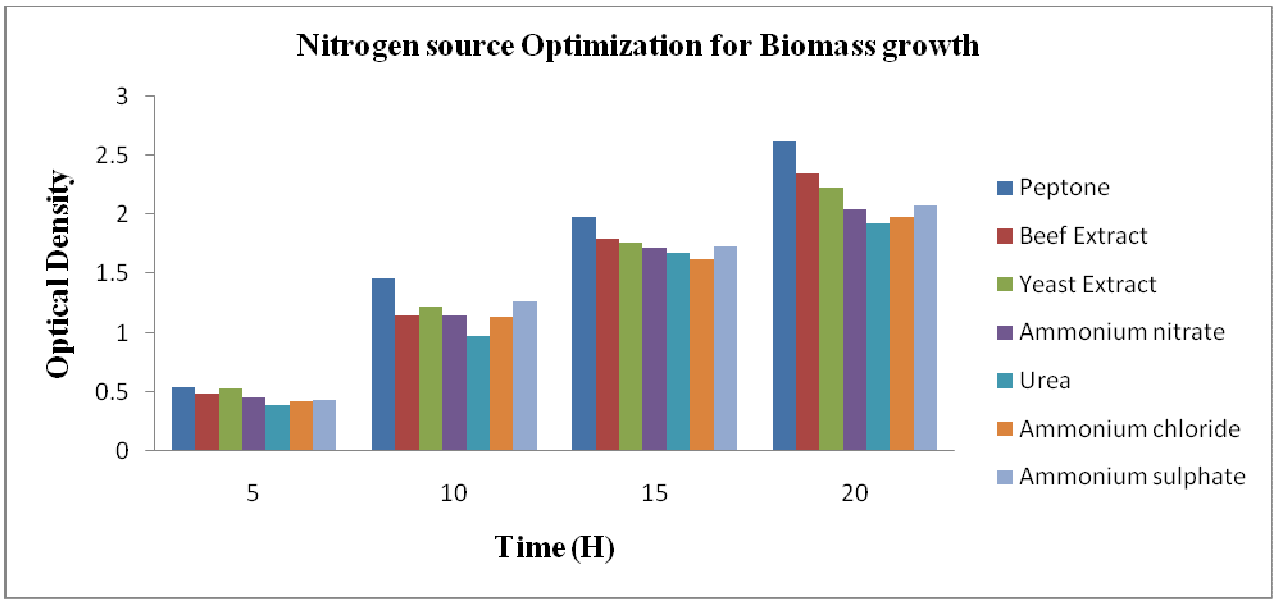


Fig-11: Effect of Nitrogen source on Biomass growth

Table -12: Growth of *Brevibacterium casei* in various synthetic carbon sources with Beef extract as nitrogen sources

Nitrogen source	Carbon Source	Mean (cfu m/l)	Cr(VI) reduction mg/l	pH_0 (Initial)	pH_f (Final)
Peptone	Dextrose	5.7×10^9	.50	6.6	5.4
	Starch	2.5×10^7	2.40	6.1	5.2
	Sucrose	5.0×10^9	0.80	6.5	5.3
	maltose	3.3×10^9	1.3	6.7	5.6
	Glycerol	3.4×10^7	.70	6.8	5.1
	Starch Soluble	3.7×10^7	1.58	6.6	5.2

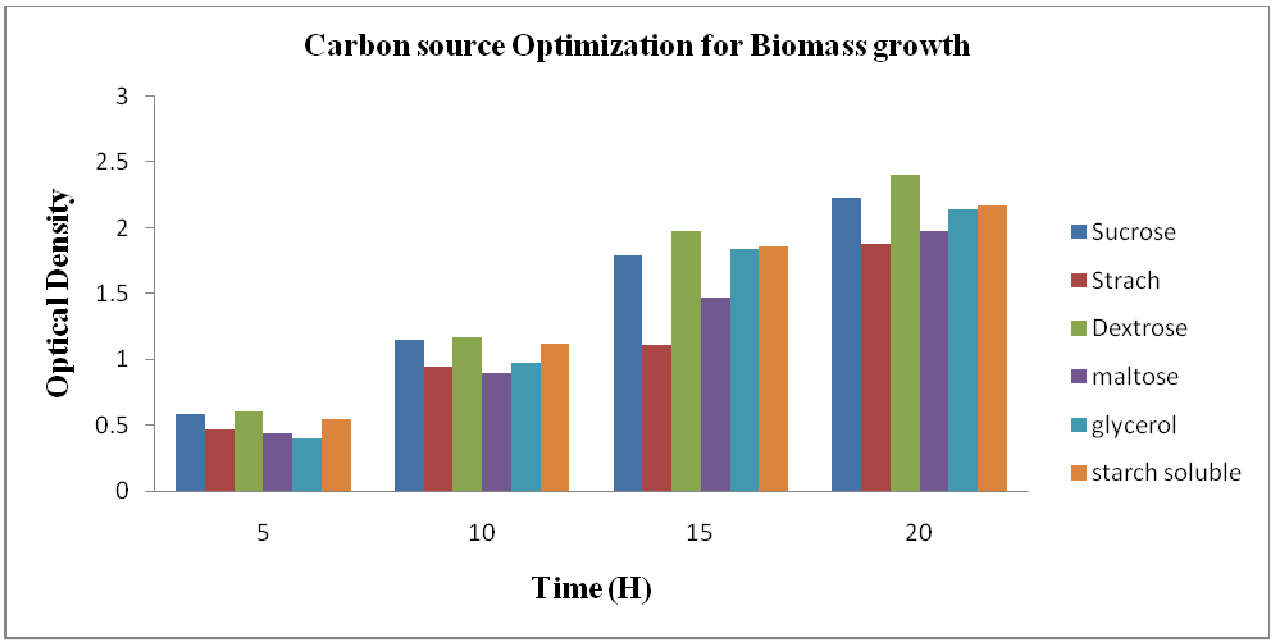


Fig-12: Effect of Carbon source on Biomass growth

4.10 Optimization of process parameters:

Cr (VI) degradation is significantly affected by various process parameters such as initial pH, incubation temperature, initial Cr (VI) concentration, inoculum volume and inoculum age etc. The study was conducted using M9 minimal salt media (Na_2HPO_4-$7H_2O$, KH_2PO_4, NaCl and NH_4Cl).

4.11 Effect of initial pH:

Fig-13 shows the effect of pH on Cr (VI) reduction by the bacterial strain *Brevebacterium.casei.* Cultures were initially supplied with 50 mg/L of Cr (VI) with innoculum volume of 1 ml. The chromate reduction study was carried out using freshly prepared overnight culture incubated at 30^0C with shaking at 200rpm. The cultures were harvested after 12 hours incubation period. Using *Brevebacteriun casei* strain Cr (VI) reduction occurred at a pH range of 4-9 but an optimum reduction was observed at pH 7. pH was adjusted with 1M NaOH and 1M HCl. It was observed from the experiment that the extreme pH (4 and 9) restricted bacterial growth and Cr

(VI) reduction. pH and Cr (VI) reduction relationship was not surprising because chromate (CrO_4^{2-}) is the dominant Cr(VI) species in an aqueous environment at pH 6.5 to 9.0 (McLean and Beveridge, 2001). However, since Cr (VI) reduction is enzyme-mediated, variation in pH will affect the degree of ionization of the enzyme, changing the protein's conformation and affecting the enzyme activity (Farrell and Ranallo, 2000). At optimum ph-7 Cr (VI) degraded from 50mg/l to 0.78mg/l reducing Cr (VI) by 98%. Optimal pH for growth of Cr (VI)-resistant bacteria was evidenced at 7.0 to 7.8 (Losi et al., 1994a), but Cr (VI) forms are soluble over a wide pH range and generally mobile in soil–water systems). Wang et al. in 1990 suggested that Cr (VI) reduction by *Enterobacter cloacae* occurred at pH 6.5 to 8.5 and was strongly inhibited at pH 5.0 and 9.0. Laxman et al., 2007 also reported optimum pH 6-7 for Reduction of hexavalent chromium by *Streptomyces griseus*. Again Donati et al., 2003 suggested that Cultures at pH 6.0 and 7.0 showed lag phases shorter than that at pH 5.0. At pH 6.0 cultures had the highest free bacterial populations and the highest chromium reduction values.

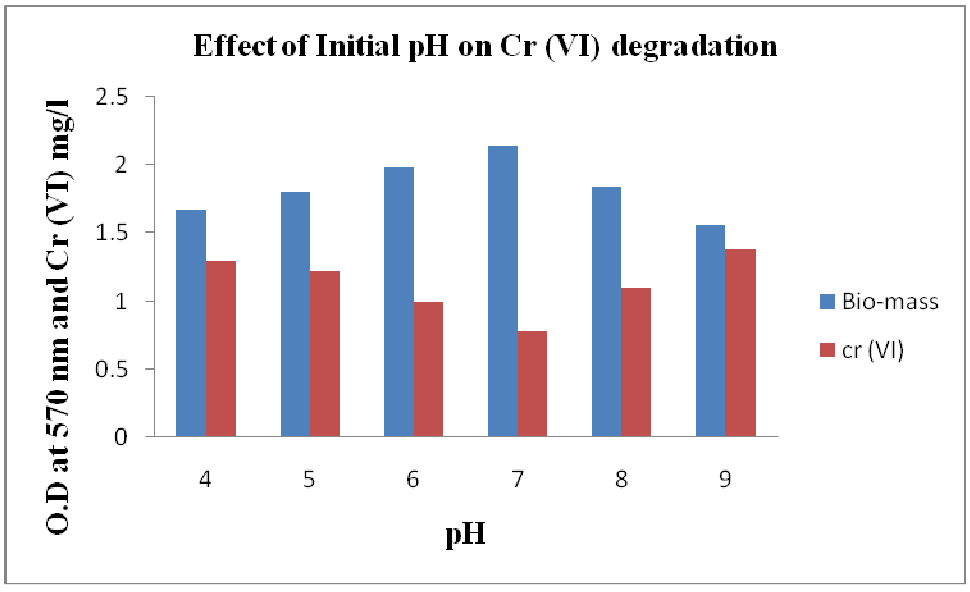


Fig-13: Effect of initial pH on Cr (VI) kinetics at initial Cr (VI) concentration-50mg/l, Temp- 30^{0}C, agitation-200rpm, inoculum volume-1 ml

4. 12 Effect of Temperature:

The Cr (VI) reduction capability by bacterial strain is greatly influenced by incubation temperature. Strain *Brevebacterium casei* exhibited good reduction over the temperature range 26–34^0 C with maximum at 30^{0}C [Fig-14]. Effect of temperature on chromium reduction using overnight grown culture was studied at pH 7 and 50 mg/l Cr (VI) concentration. It is demonstrated that maximum Cr (VI) degradation occurred at 30^{0}C reducing Cr (VI) from 50 mg/l to 0.6 mg/l. Losi et al., 1994 reported an optimal temperature of 30–37^{0}C for Cr (VI) reduction. Frankenberger, et al in 2003 again reported that optimum Cr (VI) reduction was observed at 30^{0}C and Cr (VI) reduction was severely affected by temperatures above 30^{0}C. In 2007 Laxman et al., suggested optimum temperature for Cr (VI) removal as 28^{0}C. At 50^{0}C, the cells started lysing when incubated beyond 24 h and hence there was increase in chromium concentration in the filtrate. Thus it can be concluded that high temperatures (30^{0}C–40^{0}C) severely reduced bacterial growth and chromate reduction due to loss of viability or metabolic activity of cells on prolonged incubation. Cr (VI) reduction was severely affected below 30^{0}C, because it decrease the lag time associated with building up a critical biomass concentration needed for Cr (VI) kinetics. Sumeet et al., 2002 also observed a critical temperature as 26^{0}C below which the rate of cell growth was inhibited.

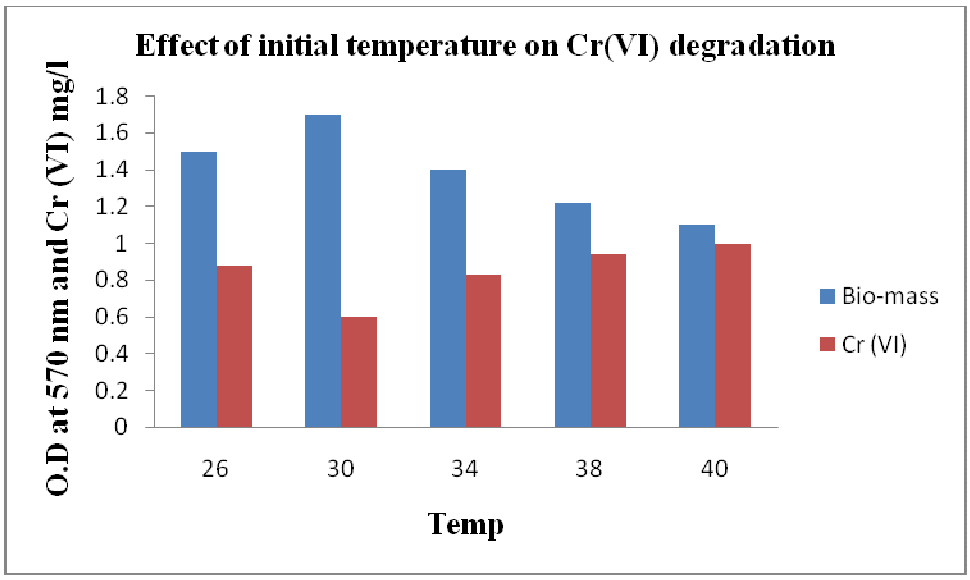


Fig-14: Effect of initial temperature on Cr (VI) degradation kinetics at initial Cr (VI) concentration-50mg/l, temp- 30^{0}C, agitation-200rpm, inoculum volume-1ml

4.13 Effect of inoculum volume and inoculum age on biomass growth and Cr (VI) reduction:

It is essential to determine the volume of the inoculums and age of the inoculum in order to achieve maximum Cr (VI) reduction. The study was conducted using fixed concentration of Cr (VI) at 50 mg/l of 100ml volume, initial pH-7, incubation temperature -30^{0}C and speed 200 rpm using a culture flask in an incubator shaker. Lowest Cr(VI) degradation (45 mg/L) were observed with 0.5ml inoculum volume where as highest Cr(VI) degradation (92 mg/L) was observed at 1.5ml inoculum volume as illustrated in Fig 15. Similarly at lowest inoculums volume 0.5ml, minimum biomass growth was achieved and it gradually increased with increasing inoculum volume to 1.5ml. At 2 ml inoculum volume no significant variation in cell growth was observed (Fig. 15). This may be due to over populated culture and fixed amount of nutrient with which the organism starts liberating proteolytic enzyme, enhancing self consumption (Sarkar et al., 1998).

The selection of inoculums age was accomplished in the medium where four flasks were inoculated with 1.5 ml cultures of different ages and were incubated at 30^0C in the shaking condition. The flasks were then inoculated with 6h, 12h, 18h, and 24h old culture, and were incubated at 30^0C, at 150 rpm for 12 hours to get the maximum Cr (VI) reduction activity. Cr (VI) reduction activity was maximum in the 12 hour old cultures as shown in Fig 16. Cr (VI) reduction was found to increase up to the inoculums age of 12h, but there was no significant difference in Cr (VI) reduction observed. Wang and Shih (1999) suggested that the inocula of poststationary phase contain biological metabolites that repress the expression of the enzyme encoding gene. Or possibly, older inoculums potentially containing spores could not revert to the vegetative cell cycle, which consequently limited the cell growth and Cr (VI) reduction (Hornbaek et al., 2004). Therefore, the inoculum at the age of 12 h was applied for Cr (VI) reduction.

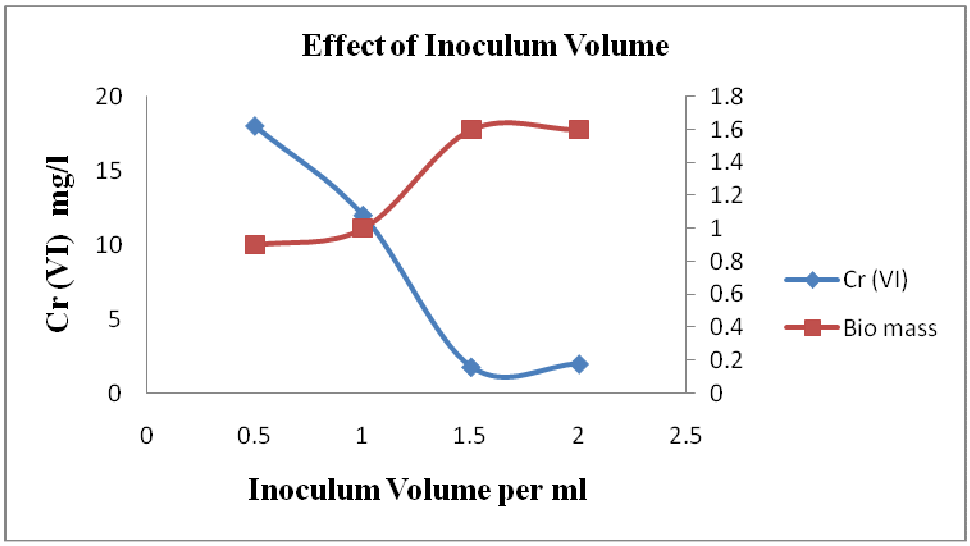


Fig-15: Effect of inoculum volume on biomass growth and Cr (VI) reduction

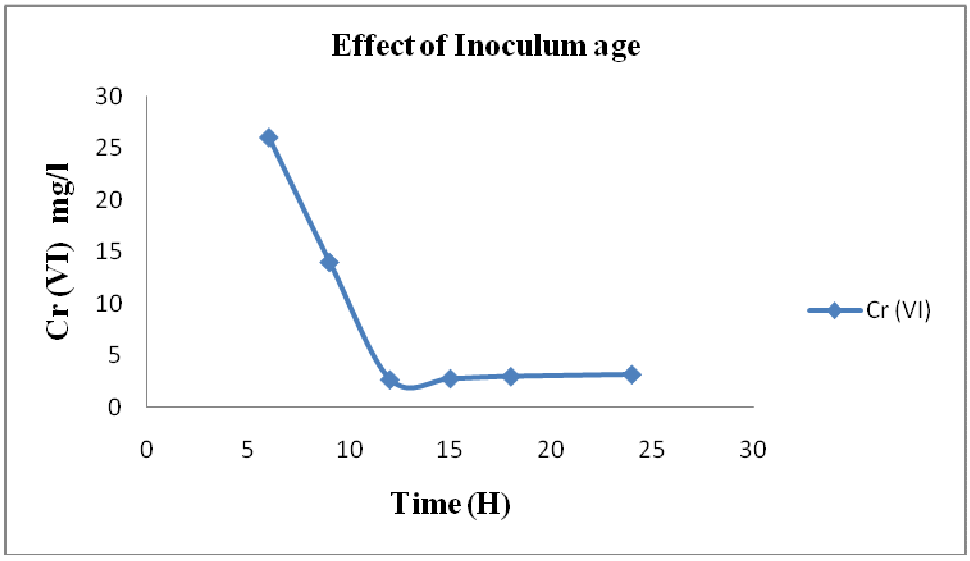


Fig-16: Effect of inoculum age on Cr (VI) reduction

4.14 Cr (VI) kinetics with inoculum & without inoculum:

Cr (VI) kinetics with inoculums& without inoculums for Cr (VI) initial Concentration of 100 mg/l and 50 mg/l respectively were carried out at optimum parameters (Inoculums age- 12 hrs, Inoculums Volume- 1.5ml, Cr (VI) – 50mg/l & 100mg/l, Initial $_{p}$H- 7, Temperature- 30^{0}C) to study the effect of bacterial culture on Cr (VI) reduction. Complete degradation of Cr (VI) at 100 mg /l in the inoculated flask occurred within 15-18 hours, while for 50mg/l it took 12-14 hours (Fig-12). There is no appreciable change observed for both the concentrations (50 mg/l and 100 mg/l) in absence of bacterial cells which indicates that abiotic Cr (VI) degradation is negligible. Similar behavior was reported by various researchers for the Cr (VI) degradation study (Levankumar et al., 2009 and Ligy Philip et al., 2004).

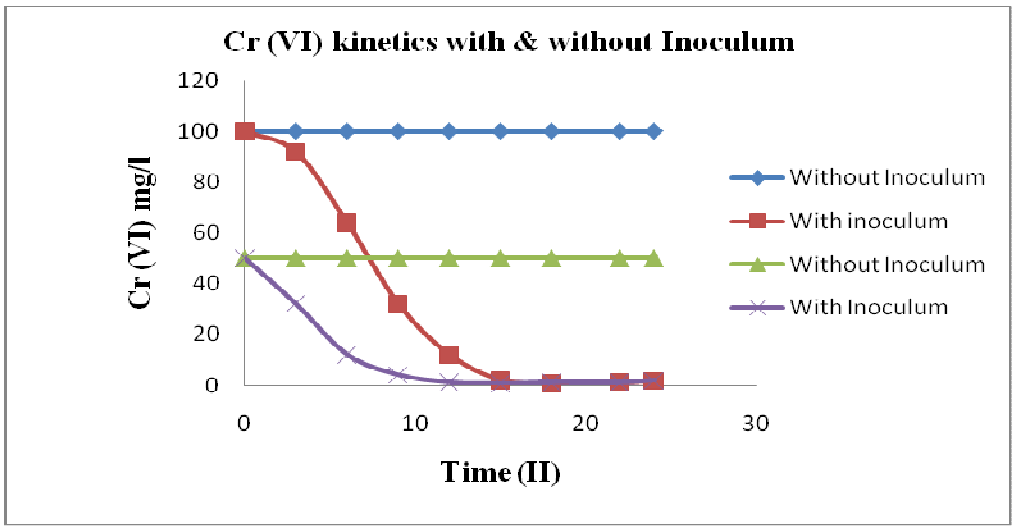


Fig-17: Cr (VI) kinetics with & without inoculum at inoculum age- 24 hrs, inoculum volume- 1.5ml, Cr (VI) – 50 & 100mg/l, initial $_{p}$H- 7, temperature- 30^{0}C

4.15 Growth kinetics for *B. casei* at its optimum condition:

All microbial species show different growth kinetics. The isolated species B. casei is subjected to grow at its optimum condition in a batch reactor (Fig-18 a). About 1.5 ml of inoculum was introduced into the batch reactor at initial solution pH-7, Temperature- 30 ^{o}C and at 200 rpm. At initial Cr(VI) concentration 50 mg/L the species showed different phases in growth behavior. Initially the growth rate was slow for five hour and then it showed significant growth in its log phase till 18 hours. Death phase started thereafter not indicating stationary phase. The above study was continuously monitored for 28 hours. The low lag phase indicates the species took some time to acclimatized then it grew exponentially. Initially the death rate was low that suggested the species could have shown reduced growth rate due to insufficient media and nutrients. Calibration plot for the bacterial strain was represented in Fig-18 b. Table-13 shows the cell density estimated at different time period for the isolated species (B.casei) at Cr(VI) concentration 50 mg/L at its optimum growth conditions.

Michael-Menten kinetics is used to describe the kinetics of various biological species. This kinetic model is relevant to situations where very simple kinetics can be assumed. The Michaelis–Menten equation relates the initial reaction rate μ to the substrate concentration [S]. The equation can be represented as:

$$\mu = \frac{\mu \max(S)}{S + Km} \qquad (1)$$

Fig – 18(c), shows the Michaelis–Menten plot between μ Versus S at initial Cr (VI) concentration 50 mg/L. The Km and μmax values are estimated as 1.018 and 6.06 g/L/hr respectively.

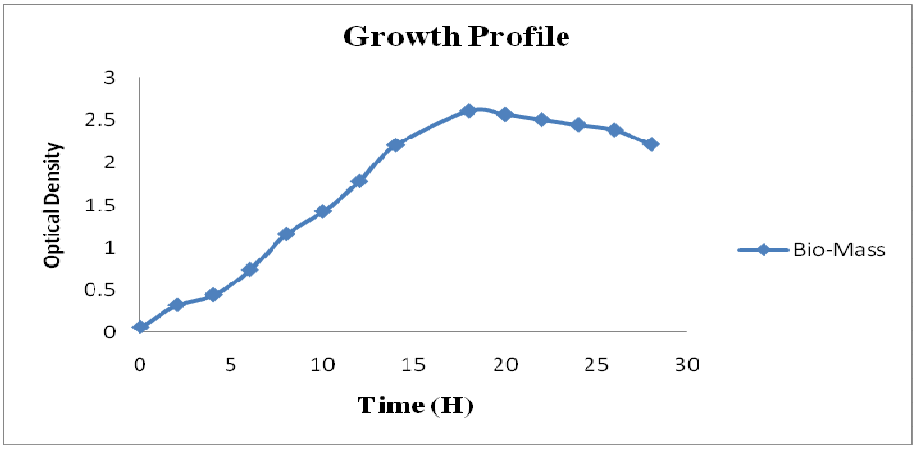


Fig:18 (a) Growth Profile of the isolated strain (*B.casei sp.)*

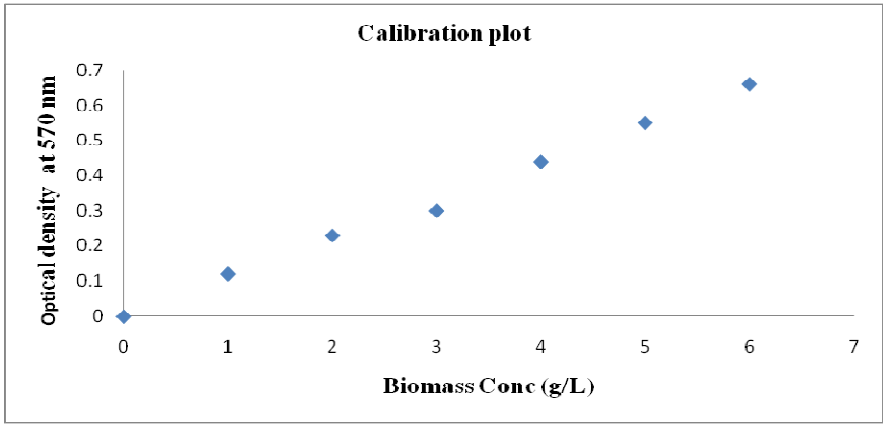


Fig: 18 (b) Calibration plots for biomass (*B.casei sp.)*

Table: 13 Biomass production and cell density at Cr (VI) concentration 50 mg/L

Time (H)	Cell Biomass (g/L)	Optical Density 570 (nm)
0	0	0
3	0.197	0.3
6	0.431	0.6
9	0.735	1.12
12	1.421	1.76
15	1.864	2
18	2.133	2.11
20	2.144	2.07

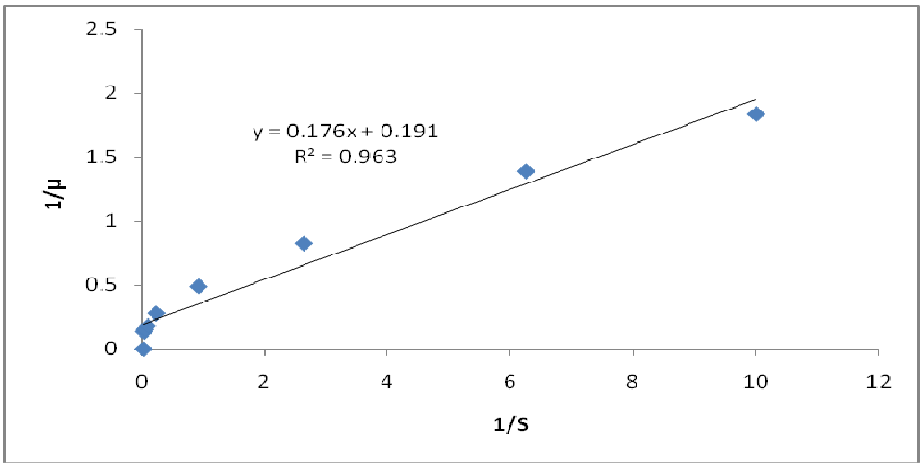


Fig:18(c) Michael-Menten plot for B.Casei at Cr(VI) concentration 50 mg/L

Cr (VI) reduction kinetics and bio-mass growth at varying Chromium concentrations:

Microbial reduction of Cr (VI) has been shown to be profoundly influenced by the metal concentration. The optimum time required for maximum Cr (VI) removal was estimated within12 to15 hours at an initial Cr (VI) concentration of 10-50 mg/l. The maximum Cr (VI) removal and biomass growth by *Brevibacterium casei* was recorded during 12 hours of incubation (Fig.18 (d) and Fig.19). The percentage of Cr (VI) reduction initially increased and extended upto12 hours and after that there was only marginal increase. While increasing the concentration of Cr (VI) from 10mg/l to 50mg/l, the percentage Cr (VI) reduction increased from 93% to 100%. The rate of Cr (VI) reduction was observed in two phases, an initial phase of faster degradation followed by the phase of slower degradation. The initial faster uptake might be due to the availability of abundant Cr (VI) species and empty metal binding sites of the microbes. The slower phase may be attributed to saturation of metal binding sites. This has been earlier reported by Garnham et al, 1992. The growth of microbial population reduces with increasing concentration of Cr (VI). Konopka et al, in 1999 confirmed that the microbial biomass generation decreased as the concentration of heavy metal increased. Bridge et al, in 1999 also confirmed that the microorganisms release a diverse range of specific and nonspecific metal binding compounds in response to high levels of toxic metals which can ameliorate the effect of toxic metals and mediate the uptake process. According to Vasanthy et al, in 2004 the growth or production of biomass increased with incubation period and reached the maximum at equilibrium time and then remained constant. He reported that *Bacillus* sp. was effective in Cr (VI) removal up to 83.4% at 10 ppm and 79.1% at 50 ppm concentration after 72 hours of incubation. Parameswari et al., in 2009 reported that the optimum time for maximum metal (Cr and Ni)

removal by *A. chroococcum, Bacillus* sp. and *P. fluorescens,* respectively was found to be 72 hours at an initial metal concentration of 25 ppm.

However with increase in Cr (VI) concentration from 10 mg/l to 50 mg /l there is increase in biomass growth by 0.7 optimal density. During the Cr (VI) degradation studies it was observed that up to 10-12 hours there is an elevation in bio mass growth and then after it follows equilibrium. The growth of the B.casei reached stationary phase within 12 hours, indicates complete utilization of media constituents in 12 hours of incubation.

Brevebacterium casei was able to substantially reduce Cr (VI) at higher concentrations (100mg/l to 800mg/l) illustrated in Fig 20. The strain demonstrated complete reduction up to 400mg/l Cr (VI) but very little degradation is observed with higher initial Cr (VI) concentrations (600mg/l and 800mg/l). Complete reduction of 100mg/l Cr (VI) was achieved within 12 hours and that of 200mg/l was achieved after 22 hours. With initial Cr (VI) concentration of 400mg/l, 600 mg/l and 800mg/l the Cr (VI) reduction was observed as 100%, 24% and 21 % respectively after 48 hours incubation. These results also showed that isolated microbial consortium was able to sustain a Cr (VI) concentration in the range of 400 mg/l without much adverse effect. This is an important observation especially when in situ bioremediation is contemplated. The microbes are able to reduce/ remediate Cr (VI) even at higher concentrations (< 400 mg/l) though it takes a long time. It can be concluded that at lower initial Cr (VI) concentrations, the cell yield may be less due to the inhibition effect. *Arthrobacter* sp. and *Bacillus* sp. reduced 30µg/ml of Cr (VI) during 46 h incubation (Megharag et al., 2003). *O. intermedium* CrT-1 completely reduced 100 µg Cr (VI) ml−1 in 72 h while *Microbacterium* sp. MP30 completely reduced 20 µgml−1 Cr (VI) within 72 h (Pattanapipitpaisal et al., 2001). *Pseudomonas* sp. strain RNP4 showed complete reduction of Cr (VI) at initial concentration of 100 µg Cr (VI) ml−1 in 72 h and 74%

reduction at initial Cr (VI) concentration of 300 µg/ml in 96 h (Rajkumar et al., 2005). Strain *B. casei* thus seems to be more efficient than all of these reported strains. However, rates of Cr (VI) reduction decreased over time with all Cr (VI) concentrations. It was probably due to Cr (VI) toxicity towards biological activity. With increasing time interval there is a distinct increase in biomass growth for all reported Cr (VI) concentration.

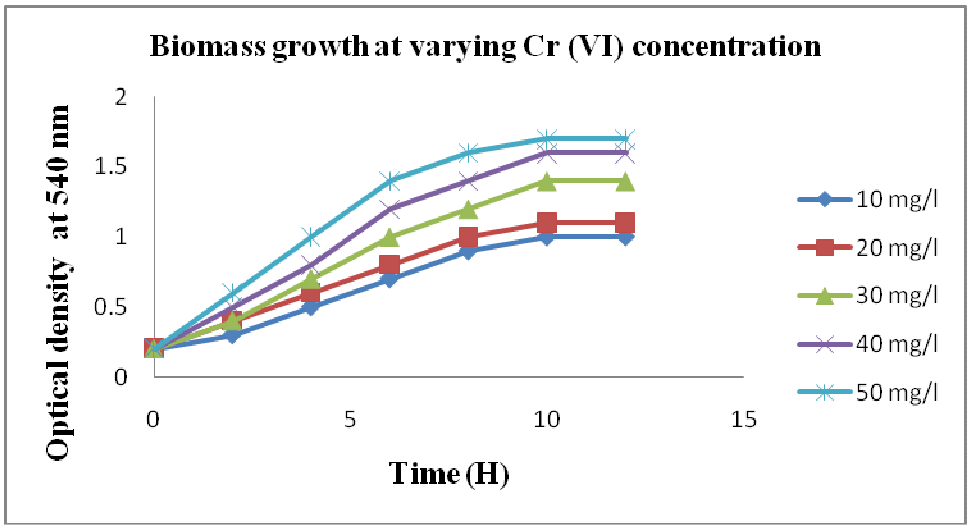


Fig-18 (d): Biomass growth at varying Cr (VI) concentration using optimum conditions: initial pH-7, temp-300C, agitation-200rpm, inoculum volume-1.5 ml, inoculum age-12 hours

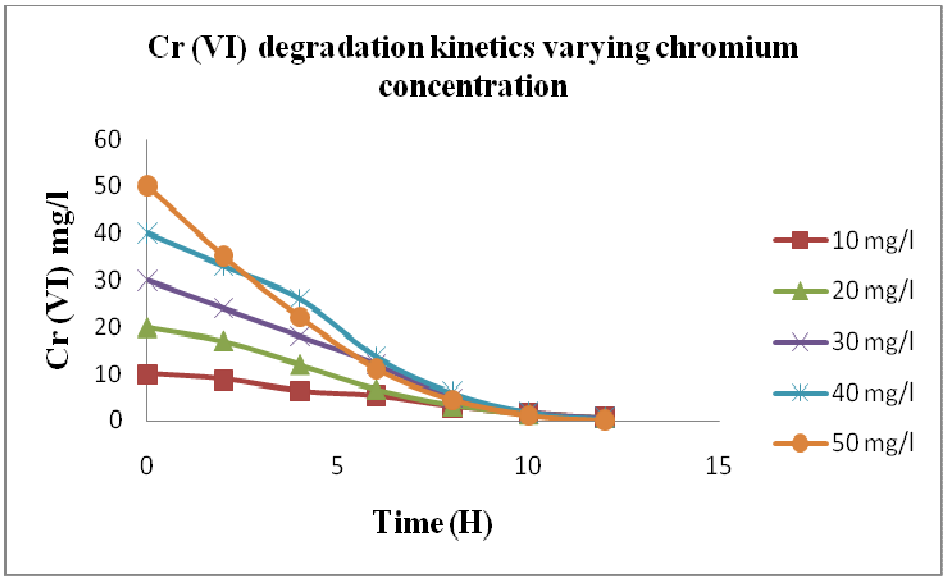


Fig-19: Cr (VI) reduction kinetics at varying Cr (VI) concentration using optimum conditions: initial pH-7, temp-300C, agitation-200rpm, inoculum volume-1.5 ml, inoculum age-12 hours

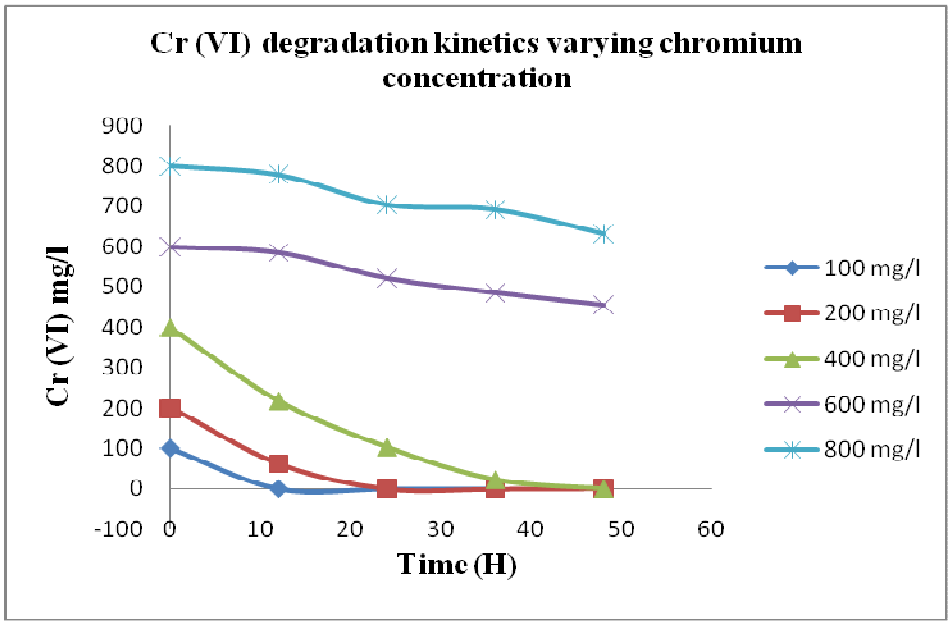


Fig-20: Cr (VI) reduction kinetics at higher Cr (VI) concentration using optimum conditions: initial pH-7, temp-300C, agitation-200rpm, inoculum volume-1.5 ml, inoculum age-12 hours

4.16 Batch degradation of hexavalent chromium through microbial fermentation:

Cr (VI) degradation study using Brevebacterium casei was conducted in a fermenter at its optimum process condition pH-7, temperature-30^{0}C, and inoculum volume- 1.5 ml/100ml in batch volume 3 litres. The Cr (VI) resistant bacterium degraded Cr (VI) in the fermenter at its optimum condition to below detection limit within 8-10 hrs (Fig-21). The Dissolved oxygen content reduced completely from 100 during this duration. As compared to Fig-19 it can be concluded that without pH control the Cr (VI) degradation characteristics of the solution decreased and could attain at a prolonged duration of 12 hours for complete degradation. The

same species could reduce 98% Cr (VI) from the Sukinda waste water in presence of other heavy metals within 18-20 hrs (Fig-22). The prolonged time needed for treatment of industrial effluent could be attributed to presence of heavy metals (Ni, Co, Al, Si, Fe and Cu) in their complex form. The strain was capable of degrading Cr (VI) along with other heavy metals effectively as shown in Table-13 .Similar result was suggested by. Rama Krishna in 2005 who reported Cr (VI) reduction above 80% could be achieved in the bioreactor with an initial concentration of Cr (VI) 50 mg/L at an hydraulic retention time (HRT) of 8 h. Thakur et al., 2007 documented that Acinetobacter sp. isolated from pulp and paper mill consortium removed higher amount (85%) of chromate Cr (VI) under aerobic conditions. It indicates that our species has a better degrading potential that could reduce 100% of Cr (VI) in the bioreactor within 8 hours.

Our study on microbial reduction of metals has led to identification of an indigenous microorganism *Brevebacterium casei* that has the capability to reduce Cr (VI) below its permissible limit. Our investigation of microbial chromium reduction has opened up exciting possibilities of further research on heavy metal pollution and its microbial remediation in water and soils using the same species. Another spin-off of this work is the possibility of using *Brevebacterium casei* as a means to extract metals from ores.

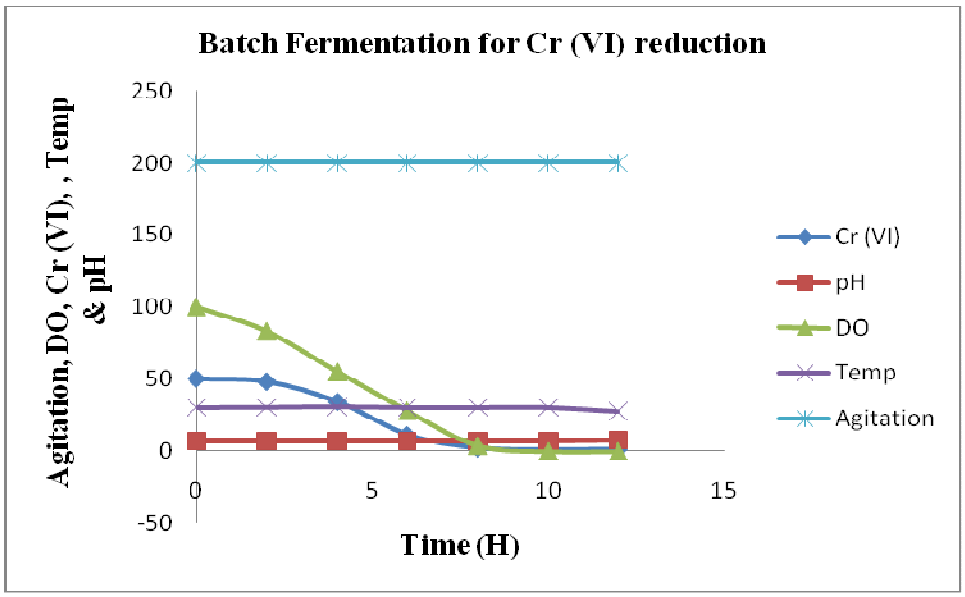


Fig-21: Cr (VI) kinetics with Cr (VI) – 100 mg/l, pH-7, DO-100, Temperature- 30^0C, Agitation- 200 rpm

Fig-22: Experimental set up (fermenter)

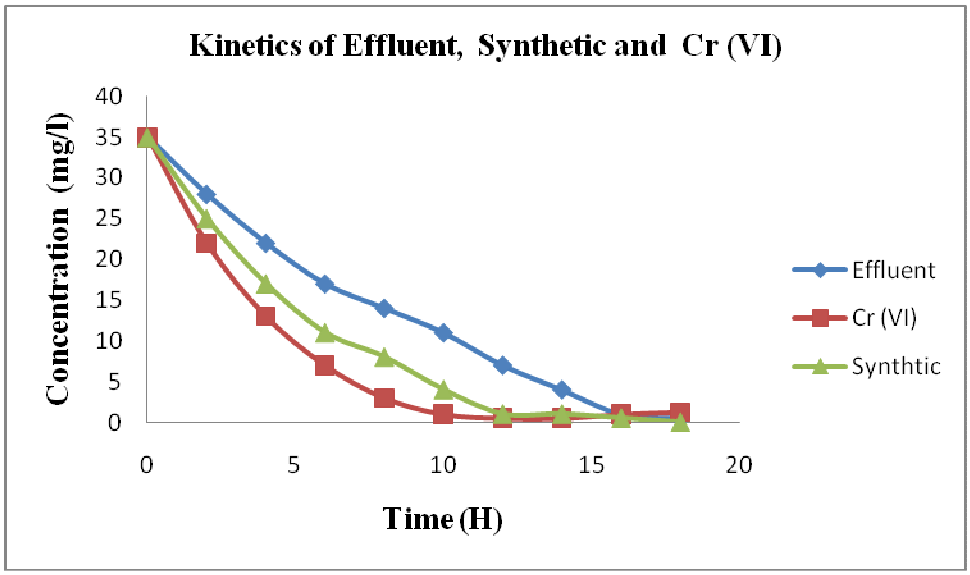


Fig-23: Cr (VI) degradation kinetics from the Sukinda waste effluent and synthetically prepared effluent in the lab.

4.17 Treatment of Sukinda mine waste water:

The heavy metal content of the untreated and treated effluent is presented in Table 13. The bioreduction technique using the indigenous strain *Brevibacterium casei,* reduction of Cr(VI) to its permissible limit (0.1) which is below the statutory limit of Indian Standards IS: 2296 and IS: 2490, could be accomplished. Significant reduction of other heavy metals present in the effluent also could be reduced. Nickel, Copper and silicon could achieve their permissible limits. Rehman et al., 2008 assessed the ability of *Bacillus* sp.ev3 to reduce hexavalent chromium into its trivalent form. *Bacillus* sp.ev3 could tolerate Cr^{6+} (4800 µg/mL), Pb^{2+} (800 µg/mL), Cu^{2+} (200 µg/mL), Cd^{2+} (50 µg/mL), Zn^{2+} (400 µg/mL), Ni^{2+} (4000 µg/mL) and Hg^{2+} (50 µg/mL). *Bacillus* sp.ev3 could reduce 91% of chromium from the medium after 96 h and was also capable to reduce 84% chromium from the industrial effluents after 144 h.

Table 13: Average concentration of treated and untreated effluent

Element	Untreated Effluent (mg/l)	Treated Effluent (mg/l)	Permissible Limits (mg/l)
Copper (Cu)	3.25	1.33	3.0
Iron (Fe)	36	2.79	3.0
Aluminum (Al)	24	3.65	5
Silicon (Si)	8	0.44	5
Cobalt (Co)	14	1.28	1.5
Chromium (Cr)	35	0.00	0.1
Nickel (Ni)	22	1.09	2.5

5. CONCLUSIONS AND RECCOMENDATION

Hexavalent chromium is a highly toxic pollutant introduced into natural water due to discharge of industrial waste water. Waste water from mining operation is one of the main sources to Cr (VI) contamination. Daily wagers residing in the premises of Sukinda mines of Jajpur District in Orissa state have fallen prey to Cr (VI) contamination in their water bodies that has affected their health drastically. It is our primary objective to decontaminate the water in this area in order to provide a means to obtain safe drinking water to the dwellers. Hence the present investigation has examined the presence of indigenous organisms from the Cr (VI) contaminated soil in sukinda area. It identifies an organism that has high resistance to Cr (VI) and has significant capability to completely degrade Cr (VI) in the water bodies of Sukinda area. The influence of

various culture conditions on cell growth such as carbon and nitrogen source, volume of inoculum and age of inoculum are presented. This data helps to optimize the maximum growth condition for the identified species in Cr (VI) environment. Moreover, the effect of various operating conditions such as initial pH of the medium, Temperature, initial concentration of Cr (VI) on Cr (VI) degradation helps to estimate the hydraulic resilience time of the species in the reactor to completely detoxify the water.

Sample Collection and Analysis:

- Soil sample collected shows presence of 2-5.9 mg/g Cr(VI)
- Water analysis also indicates high level of Cr (VI) in the range 36mg/l-44 mg/l which is many folds higher than its permissible limit (0.05 mg/l).
- Presence of other heavy metals such as Ni, Fe, Si, Co, Cu, Mn in the water samples is documented.
- Three Cr (VI) resistant species have been isolated from the soil samples namely *Staphylococcus, Streptococcus and Brevibacterium.*

Identification and characterization of the highly tolerant species

- Maximum Cr (VI) tolerance up to 8000mg/l is evidenced by *Brevebacterium sp*.
- 16S rRNA sequencing studies identified the species as *Brevebacterium casei.*
- It was reported through antibiotic study that the strain was resistant to paramycin and chloromphenicol, while it was intermediate to Kanamycin and had susceptible response to Gentamycin and Streptomycin.
- Heavy metal resistance study indicate that *Brevebacterium casei* followed resistance in this sequence >Ni>Fe>Cr>Co>Cu>Zn>Mn>Pb.

Optimization of the culture growth condition

- High productivity of *Brevebacterium casei* is accomplished in presence of Peptone as nitrogen source
- Similarly in presence of Dextrose as sole Carbon source maximum biomass growth could be obtained.

Optimization of process parameters

- Effect of initial pH of the solution was studied and optimum pH was obtained at pH-7 that reduced Cr (VI) from 50 mg/l to 0.78 mg/l.
- Influence of incubation temperature suggests an optimum temperature at 30^0C where maximum degradation occurred reducing Cr (VI) concentration from 50 mg/l to 0.6 mg/l.
- Influence of inoculum volume indicates maximum Cr (VI) degradation 99% using 1.5 ml volume of the inoculum.
- Influence of inoculum age showed 100% Cr (VI) reduction is permissible using 12 hour culture.
- There is no appreciable change observed for both the concentrations (50 mg/l and 100 mg/l) in absence of bacterial cells which indicates that abiotic Cr (VI) degradation is negligible.
- Optimum time required for complete degradation of Cr (VI) at initial concentration 50 mg/l using *Brevebacterium casei* at its optimum process condition is reported as 12 hours.

- *Brevebacterium casei* has the capability to degrade Cr (VI) completely at higher concentration up to 400 mg/l. beyond this value only 25% reduction could be observed up till initial Cr (VI) concentration 800 mg/l.
- At constant pH through fermenter complete degradation of Cr (VI) from 50 mg/l was accomplished within 10 hours.
- Treatment of Sukinda mines waste water using *Brevebacterium casei* reduced 99% Cr (VI) in the effluent within 18 hours.
- Synthetically prepared waste water presented complete degradation within 12 hours indicating the inhibitory effect of other heavy metals in its complex form present in the sample water.

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