Studies on Bioreduction of Cr(VI) using Environmentally Significant Microorganisms

Thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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Dedicated to:

My Beloved Parents

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.....CERTIFICATE.....

This is to certify that the thesis entitled "*Studies on Bioreduction of Cr(VI) using Environmentally significant microorganisms*" being submitted by **S. Murugavelh** for the award of PhD degree has been carried out under my guidance and supervision. The work documented in this thesis has not been submitted to any other University or Institute for the award of any degree or diploma.

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Abstract

Cr(VI) bioreduction was studied with fungi and bacteria. Initially *Phanerochaete chrysosporium* a white rot fungi was tested for bioreduction of Cr(VI). *P. chrysosporium* successfully reduced 10 mg L^{-1} of Cr(VI) at pH 6 in 160 h. In order to ease the separation of cells *P. chrysosporium* was entrapped in Ca-alginate and three other matrices. A maximum of 98.5 % reduction of Cr(VI) at an initial Cr(VI) concentration of 10 mg L^{-1} was obtained with Ca-alginate immobilized cells at pH 5. Cr(VI) reduction by *P. chrysosporium* involves charge based interaction with the amide and amine groups of the chitin of cell wall and intracellular reduction by metabolism dependant process. With the aim to reduce the time taken for the Cr(VI) bioreduction, *Halomonas sp*, a halophillic bacteria was applied for bioreduction of Cr(VI). A modified growth media for the *Halomonas sp* was developed. *Halomonas sp* survived in a highly saline environment containing 20 g L^{-1} of NaCl. Free cells of *Halomonas sp* successfully reduced up to 50 mg L^{-1} Cr(VI) within 48 h. The cell free extracts of *Halomonas sp* successfully reduced 60 mg L^{-1} Cr(VI) in less than 6 h duration. NADPH was found to be the suitable electron donor for the Cr(VI) bioreduction with the cell free extracts of *Halomonas sp*. The Cr(VI) reduction of immobilized cells of *Halomonas sp* was also studied. Free cells of the *Halomonas sp* were more efficient than the immobilized cells. Isolation of bacteria capable of reducing Cr(VI) was carried out from soil contaminated with Cr(VI). The isolated culture have the advantage as they are naturally acclimatized to Cr(VI) due to their long exposure to Cr(VI) in the environment. The 16s rDNA study showed that the organism was *Bacillus cereus*. The isolated *Bacillus cereus* was successful in reducing Cr(VI) at an initial concentration up to 50 mg L^{-1} at pH 6. The CFE of the *Bacillus cereus* successfully reduced 60 mg L^{-1} within 6 h in the presence of NADPH as electron donor. Packed bed reactor study was conducted in order to provide better aeration to *Halomonas sp*. The reactor was operated in batch mode and continuous mode. The HRT of 24 h resulted in complete reduction of 20 mg L^{-1} of Cr(VI). A maximum COD reduction of 84.1% was obtained.

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Nomenclatures

ABBREVIATIONS

BIONJ	Bio neighbor joining method
BLAST	Basic Local Alignment Search Tool
BSA	Bovin serum albumin
CFE	Cell free extracts
COD	Chemical oxygen demand
Cr	Chromium
Cr(III)	Trivalent Chromium
Cr(VI)	Hexavalent Chromium
DNS	Dinitro salilic acid
DO	Dissolved oxygen
DPC	Diphenylcarbazide
EDX	Energy-dispersive X-ray spectroscopy
FTIR	Fourier transform infrared spectroscopy
KCN	Potassium cyanide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaN_3	Sodium azide

OD	Optical density
P. chrysosporium	*Phanerochaete chrysosporium*
PBS	Phosphate buffer saline
PCP	Pentachlorophenol
rDNA	Ribosomal DNA
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
USEPA	United States Environmental Protection Agency
UV	Ultra Violet Visible Spectroscopy
YEPG	Yeast Extract, Peptone and Glucose Media

Notations

S	Substrate Concentration (g L^{-1})
K'	Cr(VI) Reduction Coefficient (h^{-1})
K_i	Rate Coefficient of Equation (4)
X_{oxi}	Fraction of the Organic Compound in the Oxidized Cells (g L^{-1})
Y_{cr}	Chromium Reduction Capacity (mg L^{-1})
μ_{max}	Maximum Specific Growth Rate (h^{-1})
X_{max}	Maximum Biomass Concentration (g L^{-1})
K_s	Substrate Coefficient (mg L^{-1})
X	Active Biomass (g L^{-1})

K_m	Specific Chromium Reduction Rate (mg Cr(VI) $cell^{-1}$ h^{-1})
K_c	Half Velocity Constant (mg Cr(VI) L^{-1})
R_c	Maximum Cr(VI) Reduction Capacity of the Cells (mg Cr(VI) $cell^{-1}$)
S_1	Supernatant of the Sonicate at 12000 rpm
S_2	Supernatant of the Sonicate at 24000 rpm

Chapter I

Introduction and Literature Survey

1.1. Introduction

Water is one of the most important natural resources. It is essential for all forms of life. Every day it was contaminated by various anthropogenic activities such as rapid growth of population, urbanization and industrialization that ultimately make the environment polluted. Since recent years, for irrigation purposes sewage waters have been used. There are greater concerns about heavy metal contamination in the receiving water system and land [1]. Heavy metals are metals with density above 5 g cm^{-3}. Most of the heavy metals are transition elements with incompletely filled *d* orbitals. The occurrence of these toxic heavy metals in the soil is of geogenic or anthropogenic origin. Heavy metals from the point of origin and other sources can be transported to distant environments. High levels of heavy metals can damage soil fertility and may affect productivity. Heavy metals in the environment may also change plant diversity.

Vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, lead are the most common heavy metals. Vanadium exists as trivalent oxyanion vandate. Vanadium is widely used in steel production, ceramics and fusion reactors. Vanadium affects the enzyme profile, lipid metabolism, reproduction and other metabolic actions in animals and humans. Due to poor absorption from the gastrointestinal tract, the metal is not very toxic for human beings. Vanadium has beneficial effects like it was used as antiseptic for a spirochetocide and as a tonic [2].

Manganese exists in various oxidation states from Mn(II) to Mn(VII). It is widely used in steel production, dye industries, alkaline batteries production etc. Prolonged exposure of Mn leads to mitochondrial dysfunction, oxidative stress, and affects the nasal epithelium. Manganese is less toxic to humans and animals. Manganese has beneficiary

functions such as, (*i*) it acts as an activator of the gluconeogenic enzymes pyruvate carboxylase and isocitrate dehydrogenase, (*ii*) it is involved in protecting mitochondria1 membranes through superoxide dismutase; and (*iii*) it activates glycosyl transferase, which is involved in mucopolysaccharide synthesis [3].

Iron is the only macro bioelement among the heavy metals. Iron has a wide range of industrial application. It is used as a catalyst, in reduction of nitrobenzene to aniline and as flocculating agent in water treatment. Iron based proteins are available in all organisms. The transfer of oxygen is conducted by the Fe group of the hemoglobin [4]. Ferric iron is used in iron containing enzymes, such as peroxidase, catalase, and cytochrome-*c*. Iron is an important metal in the biological system. Excessive ingestion of iron may lead to gastrointestinal ulcerations, dehydration, hypovolemia, anemia and liver failure.

Cobalt is mainly found in Co^{2+} state. Cobalt is widely used as catalysts, in paint industries, in spectroscopy and in nuclear weapon production. Cobalt is of medium toxicity but cobalt exposure may cause lung diseases in humans [5].

Nickel is released to the atmosphere by wind blown dust, volcanoes, combustion of fuel oil, municipal incineration, and industries involved in nickel refining, steel production, and other nickel alloy production. Nickel is an essential trace element in animals. Nickel deficiency leads to abnormal cellular morphology, decreases in lipid levels. The dietary amount of Ni is not regulated in humans. Increased intake of Ni may lead to dermatitis, hyper sensitivity, chronic bronchitis, emphysema, pulmonary fibrosis, and impaired lung function [6].

Copper is available in +1 and +2 oxidation states. It is widely used in electrical wires, electroplating, wood preservation etc. Copper has a significant role in the metabolism of the living organisms. Copper is responsible for the uptake of electrons from cytochrome c and for the delivery of heme. Copper (hemocyanin) acts as oxygen carrier in crabs and mollusks [7]. Excess of Cu in humans can lead to excessive cholesterol, arthritis, hair loss and anemia [8].

Zinc exists as a divalent cation. Zinc is widely used in galvanization process, production of brass and bronze. Zinc is also used in paint industries, as a catalyst and wood preservation. Zinc has a major role in metabolism of animals and humans. Zinc is a component in variety of enzymes and DNA binding proteins [9]. Excess consumption of Zn leads to urinary complications, damages to kidney and liver.

Lead is not a transition element. It mainly occurs in two oxidation states +2 and +4. Lead is used as an additive in fuel industries. It is also used in paint industry. It has wide application as electrodes in electrolysis process. It is toxic to plant, animals and humans. Lead acts on the central nervous system and affects the blood pressure and reproduction in humans [10]. Lead poisoning is lethal, it leads to ineffective heme synthesis and causes anemia.

Chromium is available in hexavalent and trivalent form. It is widely used in paint, tannery and wood preservation industry. It is a carcinogenic component. Chromium toxicity can lead to cancer in lung, liver etc. Prolonged exposure to chromium leads irritation of skin and eyes. The beneficiary effects of chromium is less when compared with other heavy metals such as iron, copper and zinc which play a major role in metabolic activity. Chromium enters the body through sulfate uptake system [5]. There is no natural pathway available for the excretion of chromium in humans and animals. Chromium remains persistent in the nature and causes severe damages to plants and animals. Chromium is highly soluble in water and it enters the water body easily. Chromium remains persistent for long time in the environment if not removed immediately. Moreover the amount of industrial wastewater containing chromium is huge in comparison to other heavy metal effluents. The toxic effects of chromium are more severe and therefore it is essential to treat the chromium containing wastewater before being discharged to ecosystem.

1.2. Chromium

Chromium is the 24th element in the periodic table and 21st most abundant element in the earth's crust. It has an average atomic weight of 52. Chromium has the symbol Cr and atomic number 24. It is a steely gray, lustrous, hard metal and has a high melting point. It is odorless, tasteless, and malleable metal. Chromium is widely used metal in industries due to its high corrosion resistance and hardness. Chromium exists in nine valence states, from −2 to +6 [11]. Out of the nine oxidation states only trivalent chromium (Cr(III)) and hexavalent chromium (Cr(VI)) are ecologically important because they are the most stable in the environment.

Fig. 1 (a) Chromium ore

Fig. 1 (b) Chromium in metallic form

Cr(VI) is the third abundant pollutant as a result of anthropogenic activities. It has the ground state electronic configuration $1s^2\ 2s^2 2p^6\ 3s^2 3p^6\ 3d^5\ 4s^1$. The chromate has two tetra hedra linked through a corner oxygen. The peak for absorption of Cr(VI) was obtained at 350 – 450 nm wavelength. Cr(VI) is readily soluble in water and has higher toxicity when compared with trivalent chromium Cr(III). Due to its toxicity United States Environmental Protection Agency (USEPA) has regulated the minimum permissible limit of Cr(VI) in surface water at 0.05 mg L^{-1}. Cr(III) has lower toxicity when compared

with Cr(VI). It has an electronic configuration of $1s^2$ $2s^2 2p^6$ $3s^2 3p^6$ $3d^3$. Cr(III) shows absorption peak at 404 and 570 nm. Cr(III) exists in natural waters in hydrolyzed form. Cr(III) complexes adsorbs on colloidal matter [12]. The strong impact of Cr(VI) on human health has increased the demand for reducing the toxicity of Cr(VI).

1.2.1. Biological significance of chromium

Chromium is present in many of the food stuffs and gets entry in to the body through dietary intake [13]. Chromium is an essential micronutrient required for the growth of many microorganisms. It is an essential micronutrient in the human body as it combines with various enzymes to transform sugar, protein and fat. It plays a major role in the maintenance of normal glucose, cholesterol and fatty acid metabolism [14, 15]. Chromium is an essential element required for normal carbohydrate and lipid metabolism in humans. Chromium deficiency leads to elevated blood glucose, insulin, cholesterol and triglycerides, and decreased high density lipoproteins (HDL) level in numerous occasions [16].

1.2.2. Industrial applications of chromium

Chromium is used in stainless steel manufacturing. It has high resistance to corrosion, wear, temperature and decay, as well as strength, hardness, permanence, hygiene and colour [12]. Cr(III) forms complexes with basic oxygen and/or nitrogen atoms of protein. This property of Cr(III) was used in leather tanning process. The inertness of the trivalent oxide of chromium gives the benefit of usage of chromium as corrosion inhibitors and agents for anodizing and plating [13]. Chromium is also used in synthetic dye industry for their oxidizing property.

Cr(VI) compounds are widely used as corrosion inhibitors, in the manufacture of pigments, in metal finishing and chrome plating and in wood preservatives. Chromium compounds are used in production of wood preservatives (52%), leather tanning (13%), metals finishing (13%), pigments (12%), refractories (linings for high-temperature industrial furnaces) (3%), and other uses (7%). Chromium(VI) compounds also are used

in textile dyeing processes, printing inks, drilling muds, pyrotechnics, water treatment, and chemical synthesis.

The major industrial source of Cr(VI) emissions includes chemical manufacturing industry, e.g., dyes for paints, rubber, and plastic products, metal finishing industry, 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.

1.2.3. Chromium toxicity

Hexavalent chromium causes lung cancer, chromate ulcer, perforation of nasal septum and kidney damage in humans and it is also toxic to other organisms as well. It has a high tendency to bind with oxygen [13, 17]. The discharge of Cr(VI) to surface water was regulated to below 0.05 mg L^{-1}. Strong exposure of Cr(VI) causes cancer in digestive tract and lungs and may cause epigastric pain, nausea, vomiting severe diarrhea and hemorrhage. Chromium in its trivalent form is an essential micronutrient for many microorganisms, relatively insoluble in water and 100 times less toxic than the hexavalent form [18].

1.2.4. Cr(VI) regulations

Presence of Cr(VI) more than the standard limit in the water bodies causes many adverse effects to human beings, animals and plants. Hence stringent regulations have been imposed by various regulatory organizations all over the world. According to the World Health Organization (WHO) drinking water guidelines, the maximum allowable limit for hexavalent chromium is set at 0.05 mg L^{-1} [19]. According to Safe Drinking Water Act, the total chromium allowed is 0.1 mg L^{-1}. Maximum permissible level of chromium in bottled water is 0.1 mg L^{-1}. Specific color additives may contain chromium at levels no greater than 50 mg L^{-1}. 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 m^{-3} (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 Cr(VI)) (for chromic acid & chromates listing). Recommended Exposure Limit (time-weighted-average workday) is restricted to 0.001 mg m^{-3} (for chromic acid & chromates and chromyl chloride listings).

The strong impact of Cr(VI) on the earth has increased the necessity to neutralize the toxic Cr(VI) to less toxic Cr(III). Currently most of the Cr(VI) sites are treated by pump and treat or dig and treat methods which involves pumping and digging out the contaminated material.

1.2.5. Conventional methods for heavy metal removal from industrial effluents

Various conventional methods to reduce Cr(VI) from the wastewater stream includes physical and chemical methods such as ion exchange, filtration, precipitation, electrochemical treatment, chemical reduction, adsorption, membrane technologies and evaporation recovery [20,21].

1.2.5.1 Electrochemical precipitation

This method utilizes an electrical potential to maximize the removal of heavy metal from contaminated wastewater over the conventional chemical precipitation method. It is the most common method for removing toxic heavy metals up to parts per million (ppm) levels from water. Using this process Cr(VI) concentration could be removed from 3,860 mg L^{-1} to less than 0.2 mg L^{-1} [22].

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.

1.2.5.2. 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. About 99.4% removal of Cr(VI) was achieved in the studies [23].

The 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 aqueous 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 highly sensitive to pH of the solution.

1.2.5.3. 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. The membrane was used for the separation of Cr(VI) from aqueous solution. Removal of Cr(VI) with different nanofiltration composite polyamide membranes while varying concentration and pH of the membrane feed solution was reported [24]. Two membranes were used for this investigation: one, a high rejection membrane and the other, a low rejection membrane. 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 of this technique are economically expensive, incomplete metal removal, high reagent and energy requirements, and generation of toxic sludge or other waste products that require disposal.

The drawback of these Conventional treatment methods for Cr(VI) contaminated soil and groundwater include high energy expenditure in the process, use of expensive and toxic chemical reductants as well as inefficient removal of low concentrations of Cr(VI) in wastewater [25]. These methods are also relatively expensive and sometimes generate secondary wastes that require subsequent disposal. Biological treatment of wastewater is a more attractive option, in that, the technology is relatively cheap and environmentally compatible [26].

1.2.5.4. Biosorption

Biosorption of chromium from aqueous solutions is relatively a new process that has proven to be very promising in the removal of even low amount of contaminants from aqueous effluents. Adsorbent materials derived from low-cost agricultural/forest wastes and dead biomasses can be used for the effective removal and recovery of chromium ions from wastewater streams. 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 surface, and surface adsorption [27, 28]. Readily available materials such as fish scales have also shown great potential as biosorbent to remove Cr(VI) from aqueous solution at various temperature [29]. The drawback with the use of biosorbents is that they require pretreatment methods. Considering the huge volume of the wastewater discharged by the industries, the amount of biosorbent required will be higher.

1.2.5.5. Metal-microbe interaction and bioreduction

Microorganisms can play an important role in the detoxification and removal of hexavalent chromium from the polluted sites. The ability of some microorganisms to interact with different Cr forms makes them attractive in the context of environmental biotechnology [30].

Microbial detoxification is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. As such, it is relatively low-cost, which generally have a high public acceptance and can often be carried out easily. Significant advances in the understanding of microbe-metal interactions have been made in recent years. Many of these microbe-metal interactions have potential application for the remediation of metal-contaminated environments and waste stream [31]. Microbial detoxification strategies for metals are yet to be significantly applied to large-scale environmental restoration efforts. Engineering aspects of the microbial detoxification are yet to be completely exploited.

Microorganisms have the ability to develop resistance to Cr(VI), microorganisms cannot degrade metals but can render them to less toxic form through the metabolic activities. Many microbes are reported for their ability to reduce Cr(VI). It includes bacteria, fungi, algae, actinomycetes etc. Microbial reduction of Cr(VI) can be attained directly by microbial metabolism or by various metabolites such as enzymes. Cr(VI) reduction can be achieved with soluble reductase produced during the reduction studies. The production of the soluble reductase by the bacteria is a cometabolism process and hence does not generate biochemical energy to support growth.

Bacteria and fungi posses great ability to reduce Cr(VI) in wastewater. Fungal reduction of Cr(VI) is a biphasic process. It involves metabolism independent and metabolism dependant process [32]. Variety of genera of bacteria are reported for the ability to reduce Cr(III). Bacterial cell proteins have shown better results in Cr(VI) reduction [33].

Microbes present in the Cr(VI) contaminated sites have the ability to survive under the toxic load of the metal [34-36]. Microbes in the contaminated sites have

developed resistance through various mechanisms [37, 38]. One such is the production of enzymes which reduces the toxicity of the Cr(VI) for the better survival. Organisms which are capable of withstanding Cr(VI) toxicity can be isolated and used for the treatment of Cr(VI). Many researcher have reported the use of microbes isolated from contaminated sites for the reduction of Cr(VI) [33,34, 36,37].

In industrial or technical operations the immobilized microbial cells provide additional advantages over free cells. Immobilized cells are easy to separate and have less clogging effect during continuous operation [38]. Natural polymers such as alginate, chitosan, chitin, and cellulose are mostly used as the matrix for the immobilization of microbial cells via entrapment technique. These polymers are also known to bind metal ions strongly [39]. Immobilized fungal cells are found to be far more stable than fungal free cells during metal removal from wastewater [38]. The use of non living and inactive biomass for large scale utilization is not practicable. The native forms of fungal cells suffer from low mechanical strength and smaller particle size and difficulty in separation from liquid stream [40]. The choice of immobilization matrix is an important factor in the application of immobilized cells in wastewater studies. The polymer matrix determines the mechanical strength and the chemical resistance of the microbial cells. The most extensively investigated biopolymer in the bioremediation studies is sodium alginate [41].

In attached growth system the microbe capable of reducing Cr(VI) was packed in a matrix forming a biofilm. The Cr(VI) solution was fed at an controlled hydraulic retention rate and the physical such as pH and temperature are continuously monitored. The fixed bed reactors provide more advantages over suspended system as the biomass concentration can be maintained in the reactor.

1.3. Literature Review

Metals form a large proportion of the pollution load of different contaminants present in the environment. Risks associated with pollution of water by heavy metals for several years are an unavoidable phenomenon. Chromium is a common waste product

generated from industrial process like electroplating, metal finishing, textile dyeing and tanning. Though chromium exists in nine valence states ranging from −2 to +6, Cr(III) and Cr(VI) are of major environmental significance because of their stability in the natural environment. 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. Trivalent chromium is relatively less toxic and less mobile. The chromate anion is highly soluble and therefore can overcome the cellular permeability barrier, entering via sulphate transport pathways since it bears structural similarity with SO_4^{2-}.

Chromium remediation is an environmental challenge. Conventionally, hexavalent chromium containing industrial effluent is treated by physico-chemical methods such as reduction, precipitation, ion exchange, reverses osmosis and electro-dialysis. Conventional methods are encountered with certain major disadvantages such as high energy requirements, incomplete removal, and generation of toxic waste sludge [42]. The maximum achievable chromate removal efficiency by conventional methods is not sufficient to attain the desired treated effluent quality standard for disposal by the industries [14].

Microorganisms can play an important role in the detoxification and removal of hexavalent chromium from the water and polluted sites. The ability of some microorganisms to interact with different Cr forms makes them attractive in the context of environmental biotechnology.

1.3.1 Bioreduction of Cr(VI)

Bioreduction has developed from the laboratory to a fully commercialized technology over the last 20 years. A successful bioreduction scheme relies on the management of 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. The search for Cr^{6+} reducing microorganisms in both aerobic and anaerobic condition is studied vigorously.

1.3.2. Bioreduction using bacteria

Das and Chandra (1990) [43] reported the chromium reduction in *Streptomyces griseorubiginosus*. Incubation of the chromate with different cell fractions in the presence of NADH and NADPH resulted in a decrease of Cr^{6+} in the reaction mixture. The level of Cr^{6+} was reduced by 82.7 % by a particulate cell fraction in the presence of NADH. The reducing enzyme was associated with this cell fraction. The enzyme was constitutive and reduced Cr^{6+} to Cr^{3+}.

Ishibashi et al (1990) [18] have studied reduction of chromate by chromium resistant species of *Pseudomonas and E. coli.* The cell free extracts of both the organisms were tested for chromate reduction. The cell-free enzyme required NADH or NADPH as an electron donor for the reduction of chromate. The optimum pH for chromium reduction by cell free enzymes was found to be around 6.5 and 7.5. Kinetics of the chromium reduction by enzymatic method showed 10-40 µM chromate reduction.

Bader et al (1999) [44] have studied the chromium removal by aerobic and anaerobic organisms. They have reported that 33% removal of chromium was attained within 21 days of time. This study gave an essential information that microbial chromium reducing organisms are wide spread in soil and water polluted with chromium.

Bae et al (2000) [45] have reported that glucose is the best electron donor for chromium reduction by *E. coli*. Various range of pH was tested with a temperature of 37 °C and a hydraulic retention time of 20 h, Cr(VI) reducing efficiency was 100% to 84% while the influent Cr(VI) concentration was in the range of 10 to 40 mg L^{-1}.

Laxman and More (2002) [34] have studied the reduction of hexavalent chromium to trivalent chromium using an actinomycetes species *Streptomyces griseous.* Their study revealed that chromium reduction was higher when medium was spiked with chromium 24 h after inoculation. Batch flask study revealed that actinomycetes species

was able to reduce Cr^{6+} to Cr^{3+} in 24-48 h of time, pH 6-7. This study revealed the potential of *Streptomyces* in bioremediation of chromium.

Megharaj et al (2003) [46] tested *Arthrobacter* sp. and *Bacillus sp* for their ability to reduce chromium. Both the strains were isolated form chromium contaminated soil. Shake flask study was conducted to compare the performance of both the bacterial cultures with the chromium concentration of 100 mg mL^{-1} on a minimal salts agar medium supplemented with 0.5% glucose, but only *Arthrobacter* could grow in liquid medium at this concentration. *Arthrobacter sp* was able to reduce 50 µg mL^{-1} and *Bacillus* species reduced 20 µg mL^{-1}. The results showed that *Arthrobacter sp* is a suitable strain for bioremediation of chromium.

Poopal and Laxman (2008) [47] studied the reduction of Cr(VI) to Cr(III) using thirteen different strains of actinomycetes. The authors have reported the ability of the organism to utilize various carbon sources viz., glucose, sucrose, glycerol, ethanol, glycine, sodium salts of acetic acid, citric acid and tartaric acid. The advantage with the application of *Streptomyces griseusis* is that it completely reduced Cr(VI) in 24 h. The organism was able to reduce the chromium by producing an enzyme chromate reductase. This study revealed that the metabolites produced by organisms can also be used for bioremediation as cell free extracts.

Cordoba et al (2008) [48] have reported chromium reduction by *Arthrobacter* sp. and *Bacillus sp* in a batch operated packed columns. *Arthrobacter Cr47* was used for the chromate reduction study. Glucose was used as sole electron donor and the system was able to remove Cr(VI) from wastewater containing 30 mg L^{-1} of chromium. Packed bed reactors were also tested for chromium reduction with the same native organism. The recirculated packed bed reactors resulted near complete removal of chromium.

Poopal and Laxman (2009) [26] studied the chromium reduction using free and immobilized cells of *Streptomyces griseus*. Immobilized cells completely reduced 25 mg L^{-1} chromium in less than 24 h. The effectiveness of immobilized cells to reduce

chromium was checked by batch flask studies. Beads (3–5 mm in diameter) were washed 3 times with 200 mL sterile distilled water and added aseptically to 50 mL medium containing 25 mg L^{-1} Cr^{6+} in a 250 mL flask. Flasks were incubated at 28 °C with shaking at180 rpm. Study for the reuse of the immobilized cells *was* also conducted. The results showed that the immobilized cells can be used for 3-4 times.

Liu et al (2010) [49] studied the Cr(VI) reduction by resting cells of *E. coli*. Over 97.5 % of Cr(VI) at an initial concentration of 100 mg L^{-1} was reduced within 4 h. The study showed that addition of glucose enhanced the reduction of Cr(VI). Presence of other metal ions drastically affected the direct reduction of Cr(VI).

Contreas et al (2011) [42] reported the importance of the nutrient composition on Cr(VI) reduction by microbes. A mathematical model was proposed to describe the transient concentration of Cr(VI). The model effectively predicted the operating condition for the Cr(VI) reduction.

1.3.3. Bioreduction using cultures isolated from contaminated sites

Camargo et al. (2004) [50] have isolated *Arthrobacter crystallopoietes* from soil contaminated with dichromate and cell free extract of actinomycetes was studied for the reduction of hexavalent chromium. Both cell free extract and intact cells were tested for chromium reduction. The K_M and V_{max} were found to be 2.61 µM and 0.0142 µmol µg mL^{-1} proteins for intact cells and K_M of 1.78 µM and a V_{max} of 0.096 µmol min^{-1} mg^{-1} proteins. The study signifies that the cell free extracts can also be used for chromium reduction.

He et al (2009) [51] isolated *Ochrobactrum* sp from chromium landfills. The results showed that the *Ochrobactrum* sp. strain CSCr-3 was tolerant to very high concentration of Cr(VI) (800 mg L^{-1}) and capable of reducing different forms of Cr(VI) (chromate and dichromate), under a wide range of temperatures (25–40 °C) and pH (7–11) with optimum at 35 °C and initial pH 10. Higher rates of Cr(VI) reduction were observed with higher initial cell and Cr(VI) concentrations ranging from 100-800 mg L^{-1}.

Nanchariah et al (2010) [52] studied the bioreduction potential of mixed consortia of the microbes isolated from chromium contaminated sites. The bioreduction experiments were conducted with synthetic minimal media. The organism reduced 0.2 mM Cr(VI) at an estimated reduction rate of 0.17 mM day^{-1} g^{-1}.

Castillo et al (2010) [53**]** studied the Cr(VI) reduction using bacterial consortia isolated from alkaline industries effluent. The Cr(VI) reduction was better at neutral pH rather than under *in situ* conditions (alkaline pH with carbonate). 16S rDNA studies revealed that the strain was characterized as *Pseudomonas fluorescens* and *Enterobacter aerogene.* The study revealed that the Cr(VI) bioreduction can be carried out at neutral pH.

Anyanwu and Nwachukwu (2011) [54] studied the resistance of soil bacteria towards the toxicity of Cr(VI). It was observed that the minimum inhibitory concentration of Cr(VI) was found to be ranged between 200 to 600 µg mL^{-1}. Most of the bacterial isolates from the soil were resistant to very high concentration of Cr(VI). It was proposed that the resistance ability of the isolates can be used for the decontamination of metal polluted sites.

Focardi et al (2012) [55] reported the Cr(VI) reduction by bacteria isolated from polluted marine sediments. The 16S r DNA study revealed that the isolated strain was *Halomonas sp*. TA-04. The free cells and Cell free extracts were able to reduce Cr(VI) effectively. It was observed that the growth was inhibited by the presence of 4.0 mM of Cr(VI) in the growth media.

Ibrahim et al (2012) [56] have studied the Cr(VI) reduction by bacterial cultures isolated from lake. The 16S rDNA sequence showed that the culture was *Bacillus sp*. The isolated culture exhibited 92% percentage of Cr(VI) reduction at an initial Cr(VI) concentration of 30 mg L^{-1}. The organism was able to reduce Cr(VI) in a wide range of NaCl (0 to 20%), indicating the halo tolerance nature of this alkaliphilic bacterial strain. Addition of glucose as an electron donor to the culture medium led to significant increase of both growth and chromate reduction by this *Bacillus* sp.

Oztruk et al (2012) [57] studied the Cr(VI) reduction by *Pseudomonas sp*. Three *Pseudomonas sp*. isolates (*P. aeruginosa 78, P. aeruginosa 99, and P. stutzeri T3*) were investigated for their ability to reduce 10 mg L^{-1} of Cr(VI). The percentage reductions obtained with all the three isolates were similar. It was proposed that the organism produced rhamnolipids which was playing a significant role in the Cr(VI) reduction process.

1.3.4. Bioreduction using fungi

Zakaria et. al (2007) [58] studied chromium reduction by *Acinetobacter haemolyticus*. A laboratory scale reactor was used for the study with *Acinetobacter haemolyticus* immobilized on wood husk. Pineapple wastewater was used as the carbon source. Near complete removal of chromium was obtained at a chromium concentration of 8.37 mg L^{-1} in a period of three days. This study suggested the usage of economical nutrients for remediation purpose.

Alonso et al (2009) [59] showed that chromium reduction by *Aspergillus tubingensis* responds well to chromium reduction in the presence of citrate. Similar study was conducted with lactate as the additive but no increase in the chromium reduction was observed. The study makes a significant point on usage of additives such as citrate, tartarate, salicilate as stimulators for chromium reduction.

Sanghi et al. (2009) [60], studied the reduction of hexavalent chromium using *Coriolus versicolor* fungus. Their study gave an indication that chromium reduction was possible at an acidic pH 2. The initial chromium concentration was also a major factor in the effective reduction of chromium. 50 mg L^{-1} was found to be the optimum concentration of chromium.

Caravelli and Zaritsky (2009) [61] evaluated the capacity of *Sphaerptilus natans* to reduce Cr(VI) in a continuous system limited in carbon and energy. *S. natans* exhibited

resistance to chromium at an initial concentration of 78 mg L^{-1}. High concentration of biomass produced the highest performance of the process of Cr(VI) reduction.

Nacorda et al (2010) [62] tested the bioreduction capacity of *Chlorella vulgaris*. The growth of the microalgae was significantly inhibited at concentrations higher than 0.1 mg L^{-1}. The cells of *C. vulgaris* removed 62% (4.70 µg 100/mL) of the metal from the medium with 0.1 mg L^{-1} Cr(VI). The study reported that a higher cell density was needed for higher percentage reduction of Cr(VI).

Sharma and Adholeya (2011) [63] studied the Cr(VI) reduction by *Paecillomyces lilacinus* isolated from tannery effluent. Complete reduction of Cr(VI) was obtained with an initial Cr(VI) concentration of 50 mg L^{-1}. Cane sugar was optimized as the best suitable carbon source. The fungi showed resistance towards pH change as it was able to reduce Cr(VI) under a broad pH range of 5.5 to 8. The results indicated that the Cr(VI) reduction can be carried at neutral to basic pH range.

Shugaba et al (2012) [64] studied the Cr(VI) reduction by *Aspergillus niger* and *Aspergillus parasiticus.* The cultures reported 96.3 % and 91.6 % reduction of Cr(VI) respectively at an initial Cr(VI) concentration of 20 mg L^{-1}. Incubation of cell-free extracts of both fungi with NADH at 30 °C for 2 h showed Cr(VI) reduction of 68.0% and 55.5% for *A. niger* and *A. parasiticus*, respectively. These findings suggested that uptake and metabolic reduction may be the process by which the two fungi were able to tolerate the toxic effects of hexavalent chromium.

1.3.5. Bioreduction using various reactors

Shen and Wang (1995) [65]**,** studied the reduction of Cr^{6+} in a two-stage bioreactor system using *E. coli*. The first reactor was operated under continuous condition with continuous supply of oxygen and nutrients. The effluent from the first reactor was mixed with chromium stream (0.24 mL min^{-1}). The second reactor was operated under up flow anaerobic mode. Near complete removal of chromium was reported with an influent chromium concentration of 1.54 mg L^{-1}.

Chirwa and Wang (1997) [1] studied the removal of chromium in a packed bed reactor using *Bacillus sp*. Chromium removal was tested with a range of influent chromium concentration (10-200 mg L^{-1}). The hydraulic detention time was maintained at 6-24 h, near complete removal of chromium was reported.

Philip et al (1998) [35] reported the biotransformation of chromium in a laboratory scale once fed batch reactor with immobilized *Bacillus coagulants.* Complete removal of Cr(VI) was achieved in the reactor with an influent concentration of 26 mg L^{-1} of chromium and a hydraulic retention time of 24 h. The pH of the bioreactor was monitored regularly and the pH was found to be in the range of 7.2 – 8.3.

Costley (2001) [66] studied Cr(VI) reduction in a rotating biological contactor (RBC) which represents a viable means for the secondary treatment of both municipal and industrial wastewaters by exploiting the advantages of both fixed film and suspended growth systems. Although RBCs entail high initial capital costs, their low costs of operation and maintenance as well as their simple process control, increase their economic viability. Metals are removed by biosorption onto the microbial biofilm and the metal-loaded biomass may either be periodically removed for controlled disposal or suitably treated to recover sorbed metals such that the biofilm may be re-used in multiple cycles.

Dermou et al (2005) [67] studied the application of trickle filters in the biological removal of chromium. Indigenous bacteria from industrial sludge were enriched and used as inoculum for the filter. Sodium acetate was used as carbon source and it was found to inhibit chromate reduction at high concentrations. Three different operating modes were used to investigate the optimal performance and efficiency of the filter, i.e. batch, continuous and SBR with recirculation. The latter one was found to achieve removal rates up to 530 g Cr (VI) mg L^{-1} d^{-1}, while aeration was taking place naturally without the use of any external mechanical means. The low operating cost combined with the high hexavalent chromium reduction rates indicated that this technology may offer a feasible solution to a very serious environmental problem.

Singh and Philip (2009) [68] experimented hexavalent chromium reduction in aerobic and anaerobic soil bioreactors. Though aerobic reactors gave better results in shake flask studies, they have tested the efficiency of soil microbes isolated from TCCL premises located at Ranipet in the bioreactors under anaerobic condition. The soil bioreactor was able to reduce entire Cr (VI) and neutral pH played a key role in reduction of chromium.

Elangovan and Philip (2009) [69] studied the performance of aerobic suspended growth system, aerobic attached growth system and anoxic attached growth system with respect to Cr(VI) reduction using *Arthrobacter rhombi RE*. The aerobic suspended growth system was able to achieve 95% Cr(VI) reduction at a HRT of 24 h. However aerobic attached growth system produced 98% of chromium reduction. Aerobic attached growth performed better in treating industrial wastewater compared to aerobic suspended and anoxic attached growth system.

1.4. Objective of the study

The initial step in the current study was to collect details related to chromium, chromium contamination and its impact on the environment and the methods used for the treatment of Cr(VI) from the literatures. Based on the literature survey it was observed that there was enough scope for further studies on Cr(VI) reduction using different microbes. One white rot fungi (*Phanerochaete chrysosporium*) and one halophillic bacteria (*Halomonas sp*) was selected for Cr(VI) reduction studies. Bioreduction of Cr(VI) using microorganisms isolated from chromium contaminated soil is also planned along with performance of packed bed reactor to reduce Cr(VI) using bacterial culture.

To achieve the goal of Cr(VI) bioreduction from aqueous solution the current work was carried out with the following objectives,

- To study the Cr(VI) bioreduction capacity of live and active *Phanerochaete chrysosporium* and to optimize the physical parameters such as pH, temperature, substrate concentration, initial Cr(VI) concentration etc.
- Cr(VI) reduction using the immobilized live cells of *Phanerochaete chrysosporium*, optimization of substrate concentration, pH, temperature, biomass loading rate, initial Cr(VI) concentration and best suitable matrix.
- Evaluating the possible mechanism of Cr(VI) removal by *Phanerochaete chrysosporium* using various methods such as FTIR, SEM and TEM.
- To study the bioreduction capacity of free cells, cell free extracts (CFE) of *Halomonas sp,* the role of electron donors on the percentage reduction of Cr(VI), inhibitory effect of metabolic inhibitors on Cr(VI) bioreduction.
- To study the Cr(VI) reduction by immobilized cells of *Halomonas sp*, optimization of substrate concentration, pH, temperature, biomass loading rate, initial Cr(VI) concentration and best suitable matrix.
- Isolation, identification and characterization of Cr(VI) reducing microorganism from chromium contaminated soils, 16SrDNA sequencing, biochemical characterization, phylogenetic analysis, optimization of media and physical parameters such as pH and temperature. Evaluating the role of CFE of isolated culture on bioreduction is also planned.
- Packed bed reactor study using *Halomonas sp* with the optimized media. Evaluation of performance of the reactor under batch and continuous mode along with optimization of HRT, biomass (suspended and attached), DO and COD.

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Chapter II

Bioreduction of Cr(VI) by Live and Active *Phanerochaete chrysosporium*

2.1. Introduction and Literature Survey

In recent years application of biotechnology in control and removal of heavy metals is gaining importance because of its various advantages over the other conventional physico-chemical treatment methods. Microbial cells have the ability to develop resistance to virtually all toxic heavy metals [1]. Biological reduction of Cr(VI) to Cr(III) represents the detoxification process of Cr(VI)-polluted waters [2].

Critical review of literature revealed that many microbes were able to reduce Cr(VI) under both aerobic and anaerobic conditions. Studies have shown that certain bacteria and fungal species can detoxify Cr(VI) compounds by reducing them to relatively less harmful Cr(III) [1,2,3]. Bader et al. [4] reported Cr(VI) reduction by live microbes under aerobic and anaerobic condition.

The white rot fungus *Phanerochaete chrysosporium* is a filamentous fungus possessing good potential for removal of heavy metal (Fig. 2.1). The fungal cell wall outer layer is composed of glucans, mannans and with inner layer of crystalline chitin. The reduction of Cr by fungal cells depends on the structural organization of entire protein-carbohydrate complex [5]. Previous studies reported on the literature on chromium removal using *P. chrysosporium* were restricted to the use of the fungus as inactive and dead biosorbent [6].

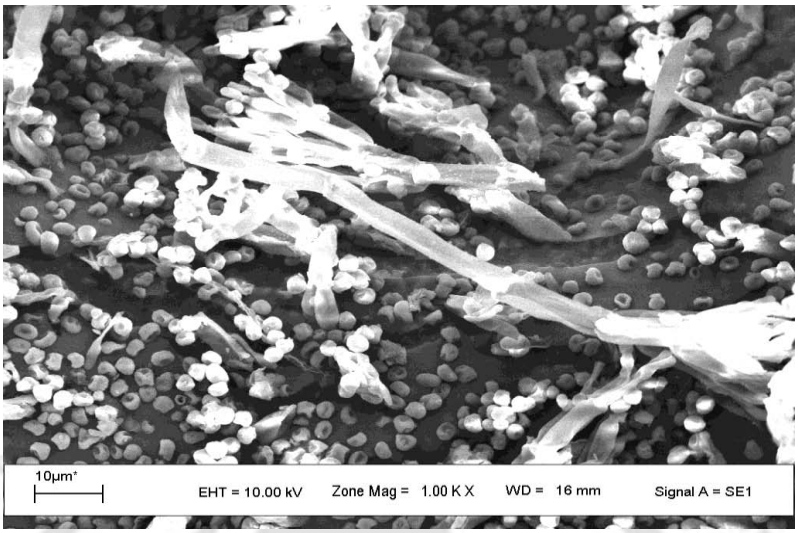

Fig. 2.1. SEM image of *Phanerochaete chrysosporium*

In this chapter the potential of live and active *Phanerochaete chryosporium*, a white rot fungi to remove lower concentration of Cr(VI) from aqueous solutions was reported. The significance of the medium pH on the growth of the *P. chryosporium* and bioremoval of Cr(VI) was studied. Substrate inhibition on the growth of *P. chrysosporium* was evaluated. The optimum biomass concentration required for the growth and reduction of Cr(VI) was reported. The minimum inhibitory concentration of Cr(VI) was also reported. A mathematical expression for the bioreduction of Cr(VI) considering the organic compounds in the cells was proposed. Also the mechanism of Cr(VI) removal by *P. chrysosporium* with the support from Fourier transform infra red spectroscopy, scanning electron and transmission electron microscopic investigations is reported.

2.2. Materials and Methods

2.2.1 Microorganism and culture conditions

Phanerochaete chrysosporium (MTCC 787) was obtained from the Institute of Microbial Technology, Chandigarh, India and cultivated at 25 ºC in a liquid medium at pH 6 with agitation of 150 rpm on a rotary shaker. The fungus was maintained on Malt Extract Agar–Blakeslee medium. Subcultures of the parent culture were made on every 15 days of duration and stored at 4 ºC.

2.2.2 Media

The general growth media for growth of the fungi *P. chrysosporium* consisted of Malt Extract 20 g L^{-1}, Dextrose 20 g L^{-1}, and Peptone 1 g in 1 L of distilled water. The pH of the medium was maintained at 6 using 0.1N HCl. The media for bioreduction contained malt extract 20 g L^{-1}, dextrose 20 g L^{-1}, and peptone 1 g spiked with different concentration of chromium in 1 L of distilled water. All media were autoclaved at 15 kPa and 120 ºC for 20 min.

2.2.3 Preparation of stock solution

The bioremoval capacity of living *P. chrysosporium* was investigated using aqueous solutions. A Cr(VI) stock solution was prepared by dissolving 2.8269 g of $K_2Cr_2O_7$ in 1000 mL deionized water, shaking it at 150 rpm for 15 min to get complete dissolution. Cr spiked medium was prepared by diluting this solution to required concentration in the growth media.

2.2.4 Bioremoval experiments

The factors affecting the growth and metal bioremoval of *P. chrysosporium* was investigated in 250 mL conical flasks containing 100 mL of Cr spiked medium for 164 h. The bioreduction medium containing Cr(VI) ranging from 10 mg L^{-1} to 40 mg L^{-1} were incubated in incubator shaker (Daihan LabTech Co Ltd, Model LSI 3016–R) at 25 ºC and 150 rpm. Previously inoculums were carried out aseptically. Samples were taken

aseptically at regular time intervals of 8 h and centrifuged at 10000 X g for 15 min and the biomass was separated, weighed, and the supernatant was used for metal analysis. The precipitated samples were used to determine the dry weight of the biomass and its concentration. Bioremoval efficiency was expressed as the percentage change in the concentration of bioremoved chromium at time intervals to the initial Cr(VI) concentration by the growing *P. chrysosporium.* Bioreduction was reported as the change in the concentration of Cr(VI) to Cr(III) at regular time intervals.

Experiments were also conducted to study the optimum concentration of dextrose required for the growth *P. chrysosporium.* The dextrose concentration was varied between 10 g L^{-1} to 50 g L^{-1} at pH 6 and 25 ºC in the absence of Cr(VI). Samples were drawn at regular time intervals and centrifuged at 10000 X g to measure the biomass concentration. An important aspect of wastewater treatment for removal of metals is the pH of the solution. Experiments were conducted to optimize the pH of the bioremoval process. The medium was prepared by varying the pH of the medium between 1 to 6 for different initial concentration of Cr(VI) at 25 ºC. The solution pH was measured using a pH meter (Sartorius AG 37070 Goettingen, Germany). The concentration of chromium was measured spectrophotometrically using diphenyl carbazide (DPC) at 540 nm using an UV spectrophotometer (Perkin Elmer, Model Lambda 35). Interference of growth was eliminated using calibration curves obtained with the same medium. The biomass concentration was determined after drying the organism overnight at room temperature. *P. chrysosporium* was grown on metal free medium and was used for comparing its growth during bioremoval. Two parallel experiments were conducted for each experimental condition and the arithmetic mean was used for data evaluation.

2.2.5. Analytical method

The total chromium concentration was measured using atomic absorption spectrophotometer (Varian, Model AA240FS). The Cr(VI) concentration was measured by DPC method using UV-spectrophotometer (Perkin Elmer, Model Lambda 35).

FTIR analysis

Infrared spectra (Perkin Elmer, Model Spectrum one FTIR) of chromium free and chromium accumulated cells of *P. chrysosporium* were recorded in the region 4000–450 cm^{-1}. The samples were pressed into spectroscopic quality KBr pellet with a sample/KBr ratio about 1/100.

Scanning electron microscopy and energy dispersive x-ray analysis

Scanning electron microscope (LEO, Model 1430VP) was used to determine the morphology of the fungi *P. chrysosporium* before and after chromium accumulation. The supernatant after bioaccumulation experiment was treated with 2.5 % glutaraldehyde for 1-2 h. It was then washed with Millipore water followed by treatment with 0.1% phosphate buffer saline (PBS) for 1 h. The samples were dehydrated before mounting on the sample holder.

Transmission Electron microscopy and energy dispersive X- ray analysis

TEM (JEOLJEM 2100) analysis was performed to study the chromium concentration in the cytoplasm. The sample for the TEM analysis was prepared by fixing the centrifuged sample in the TEM grid with 2.5 % glutaraldelyde followed by treatment with 0.1% PBS. The samples were then washed with different concentration of ethanol for dehydration.

2.3. Mathematical Model for Bioreduction of Cr(VI)

The objective of the present study was to develop a kinetic model for the reduction of Cr(VI) by live and active *P. chrysosporium* . It was observed that Cr(VI) was near completely reduced to Cr(III). Based on this a model was proposed

$$Cr(VI) + Microbialcells \rightarrow Microbialcells_{(oxidized)} + Cr(III) \quad (1)$$

A part of the organic component present in the microbial cells are responsible for the

reduction of Cr(VI). Considering that the organic components present in the media and microbial cells are primary factor in the reduction, a theoretical model was proposed.

$$Cr(VI) + S \rightarrow S_{(oxidized)} + Cr(III) \quad (2)$$

The above equation was purely an empirical equation as it cannot be used to predict the mechanism. The organic compounds present in the cells cannot be completely defined. Based on the assumption that the reduction of Cr(VI) by a single organic compound present in the cell is of first order and can be expressed as,

$$\frac{d[Cr(VI)]}{dt} = -K'[S][Cr(VI)] \quad (3)$$

where *K'* is the Cr(VI) reduction coefficient (h^{-1}).

It was expected there are more number of organic compounds available in the oxidized cells and all the reduction reaction occur parallel. Therefore the overall reduction rate of Cr(VI) is the sum of the individual reactions.

$$R = \frac{d[Cr(VI)]}{dt} = \left(-\sum_{i=1}^{n} K_i[S_i][Cr(VI)]\right) \quad (4)$$

where K_i and S_i are the rate coefficients and the concentration of organic compounds in the oxidized cells. The above equation can be written in terms of equivalent weight fraction of the i^{th} compound and the total organic compound.

$$\frac{d[Cr(VI)]}{dt} = \left(-\sum_{i=1}^{n} K_i f_i\right)[S^*][Cr(VI)] \quad (5)$$

where $f_i = \frac{[S_i]}{[S^*]}$,

*S** is the total organic compound present in the cell.

We define a new rate coefficient similar to the rate coefficient in equation (3)

$$K = \left(-\sum_{i=1}^{n} K_i f_i\right)$$

Now the equation can be written in terms of the new rate constant

$$\frac{d[Cr(VI)]}{dt} = -K\ [S][Cr(VI)] \tag{6}$$

The reduction rate of Cr(VI) was found to decrease as the Cr(VI) in the media was depleted and the organic compounds in the media was used for metabolic activities. It was therefore essential to consider the oxidation of the organic compounds that was responsible for Cr(VI) reduction.

For any time instant the concentration of the organic compound that reduce Cr(VI) is

$$[S^*] = [S_i]\left((1 - X_{oxi})\right) \tag{7}$$

where X_{oxi} is the fraction of the organic compound in the cells which are oxidized during reduction of Cr(VI).

$$X_{oxi} = \frac{([Cr(VI)]_0 - [Cr(VI)])}{S}, \tag{8}$$

Combining equations (3) and (6)

$$\frac{d[Cr(VI)]}{dt} = -K\ [Cr(VI)]\left([S] - [Cr(VI)]_0 + [Cr(VI)]\right) \tag{9}$$

Rearranging the variables,

$$\left(\frac{1}{[Cr(VI)]} - \frac{1}{[S] - [Cr(VI)]_0 + [Cr(VI)]}\right) d[Cr(VI)] = -K\ \left([S] - [Cr(VI)]_0\right) dt \tag{10}$$

Integrating the above equation (10)

$$Y_{Cr} = \ln\left(\frac{[Cr(VI)]_0([S]-[Cr(VI)]_0+[Cr(VI)])}{S[Cr(VI)]}\right) \quad (11)$$

where Y_{cr} is the Cr reduction capacity. The rate constant of the Cr reduction can be obtained from

$$m = K\left([S]-[Cr(VI)]_0\right) \quad (12)$$

2.4. Results and Discussion

The effect of chromium ions on the growth of *P. chrysosporium* and bioremoval of Cr(VI) was studied and the results obtained were presented in this section. In the first section (2.4.1), the effects of various physical parameters such as pH and dextrose concentration on the growth of *P. chrysosporium* was reported. In the second section (2.4.2), substrate consumption and in third section (2.4.3), bioreduction potential of *P. chrysosporium* was reported.

2.4.1 Effect of physical parameters on the growth of *P. chrysosporium*

In order to study the effect of carbon source on the growth of *P. chrysosporium* batch tests were conducted using dextrose as the sole carbon source. The effect of dextrose on growth rate of *P. chrysosporium* was studied in a metal free as well as Cr spiked media containing dextrose concentration in the range of 10 g L^{-1} to 50 g L^{-1}. From Fig. 2.2, it can be seen that the specific growth rate increased from 0.07 h^{-1} to 0.152 h^{-1} for an increase in dextrose concentration from 10 g L^{-1} to 20 g L^{-1} for metal free media.

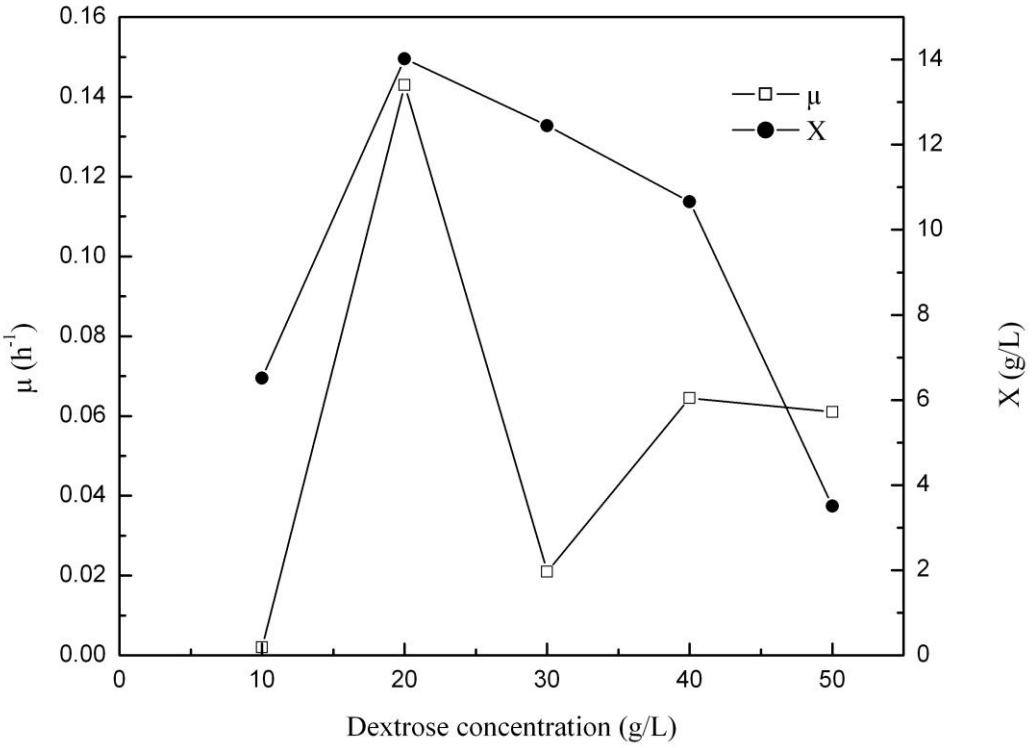

Fig. 2.2. Effect of initial dextrose concentration on the growth of *P. chrysosporium*

With an increase in dextrose concentration to 30 g L^{-1}, the specific growth rate decreased. This decrease in specific growth rate with increasing dextrose concentration beyond 20 g L^{-1} was due to the substrate inhibition on the growth of *P. chrysosporium*. However, it can be seen from Fig. 1 that there was an increase in the specific growth rate with further increase in dextrose concentration beyond 30 g L^{-1}. This increase in specific growth rate was probably due to the acclimatization of the organisms towards the increased substrate concentration. Similar pattern of fungal growth was reported by Congeevaram et al. [7]. The maximum biomass concentration of *P. chrysosporium* was obtained at an initial dextrose concentration of 20 g L^{-1} (Fig. 2.2). The inhibitory effect of dextrose on the maximum biomass concentration beyond 20 g L^{-1} was clearly depicted in Fig. 2.2.

The pH of the solution plays an important role on the growth as well as bioremoval of Cr(VI) by *P. chrysosporium.* The solution pH affects the solubility of metal ions and ionization state of various functional groups present on the cell wall of the biomass [8-10]. The growth of the fungus was found to be dependent on the pH of the growth medium. Fig. 2.3 showed that a decrease in pH (6 to 1) to acidic conditions has decreased the specific growth rate. The maximum biomass concentration of *P. chrysosporium* was also affected by the change in the pH of the growth media. This may be attributed to an extended lag period of the fungi from 24 h to 48 h during which the fungi gets acclimatized under acidic condition. However, it can be seen from the figure that the specific growth rate marginally increased at pH 3.0, which may be attributed to the fact that the specific growth rate is a function of biomass concentration at a particular time interval [11]. A complete weight loss was obtained at pH ranging from 1 -3.

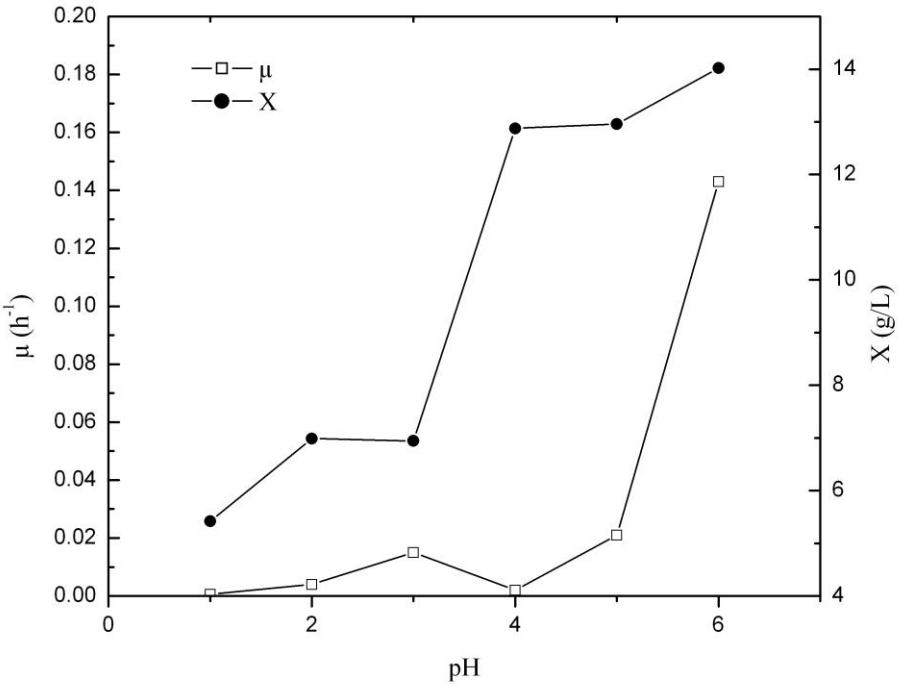

Fig. 2.3. Effect of pH on specific growth rate of *P.chrysosporium*

2.4.2. Substrate consumption

The glucose utilization by the organism in the presence of the Cr in the growth medium was monitored in order to study the different stages of growth during the bioreduction. The organism showed difference in the rate of dextrose consumption during the bioreduction of Cr. An initial lag of 48 h for the media containing 10 and 20 mg L^{-1} was observed. The dextrose consumption rate was drastically affected by the presence of Cr in the medium. The Cr free control culture was able to consume dextrose completely in 156 h (Fig. 2.4).

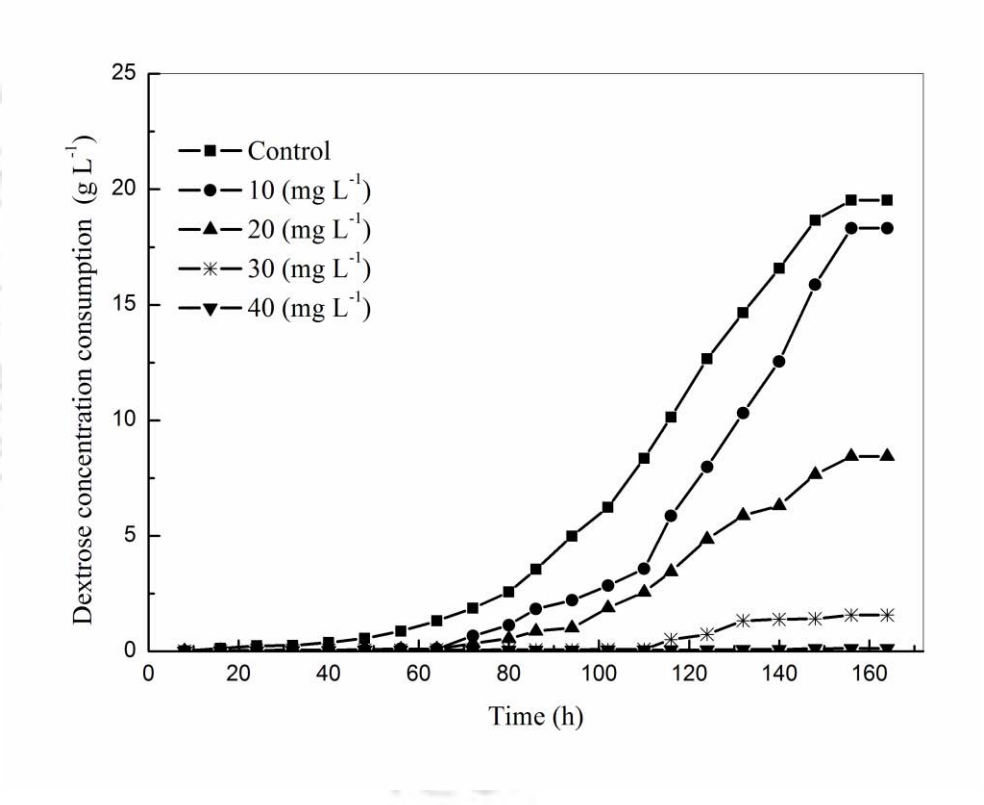

Fig. 2.4. Substrate consumption profile of *P.chrysosporium*

The substrate utilization capacity was found to decrease with increase in Cr concentration in the media. The bioremoval rate was also found to decrease with the presence of Cr in the growth media. It is evident that the Cr had an inhibitory effect on the growth and metabolic activity of the fungus. The substrate utilization is an important

factor in the bioreduction of Cr. The specific growth rate of the fungi was drastically affected by the presence of Cr in the growth media. A maximum specific growth rate of 0.275 h^{-1} was obtained for an initial Cr concentration of 10 mg L^{-1}. As the concentration of the Cr was further increased the specific growth rate was found to decrease and a minimum of 0.04 h^{-1} was reported for 40 mg L^{-1} of Cr in the growth media (Fig. 2.5).

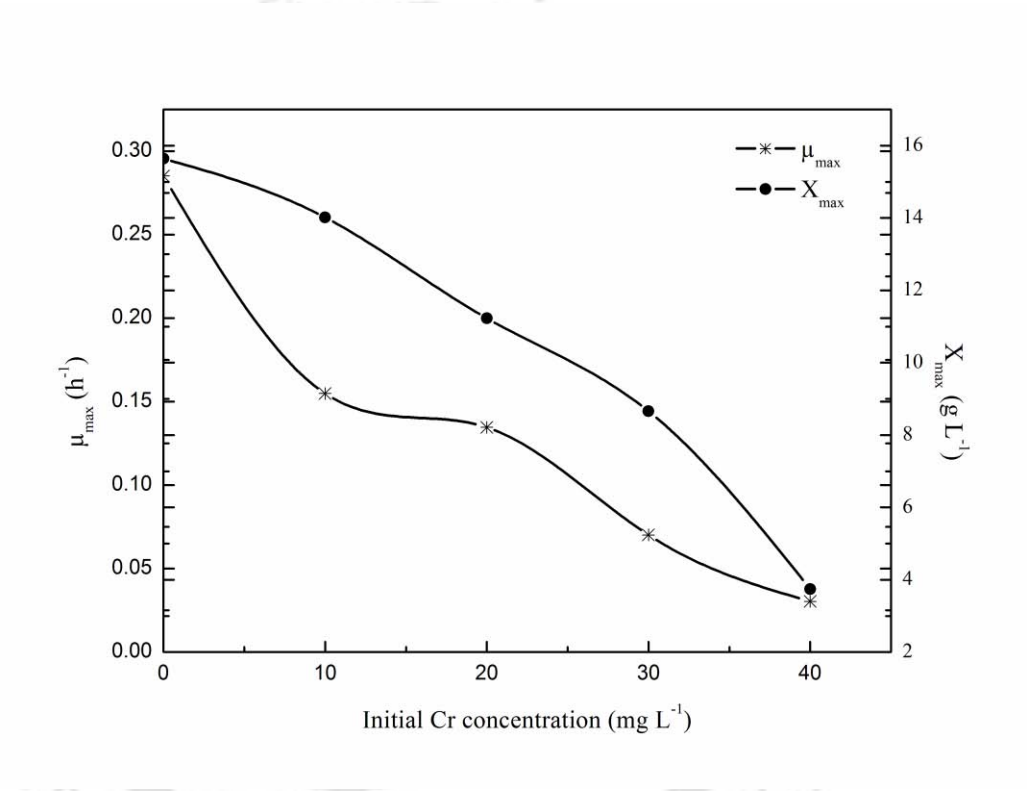

Fig. 2.5. Effect of intial chromium concentration on the growth of *P.chrysosporium*

2.4.3. Bioreduction of Cr(VI) by *P. chrysosporium*

The effect of initial Cr concentrations on the reduction rate Cr(VI) by metabolically active *P. chrysosporium* was studied over a concentration range from 10 – 40 mg L^{-1}. Fig. 2.6 showed that bioreduction occurred for all the concentration studied. A

maximum of 98.92 and 95.1 % of bioreduction occurred for 10 mg L^{-1} and 20 mg L^{-1} initial Cr(VI) concentration respectively.

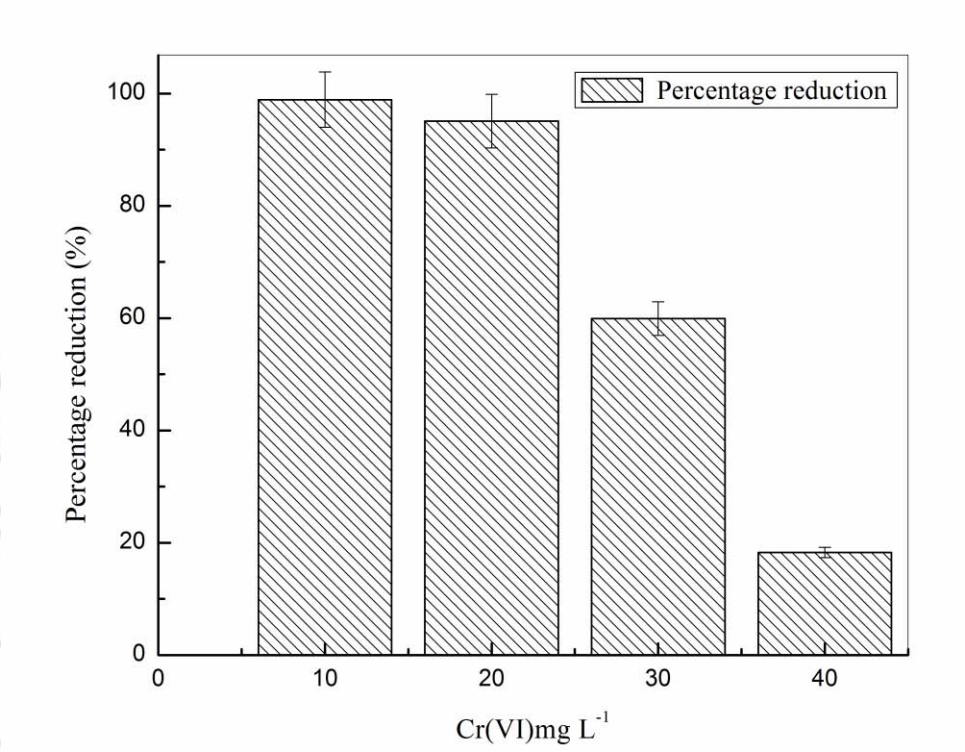

Fig. 2.6. Bioreduciton percentage of Cr(VI) by *P.chrysosporium*

Even though reduction of Cr(VI) was observed for 30 and 40 mg L^{-1} of initial chromium concentrations the results were not significant. The decrease in the bioreduction rate for concentrations above 20 mg L^{-1} was a clear indication of inhibitory effect of Cr on the metabolic activity of the white rot fungus. It can be noticed from the time profile (Fig. 2.7), that the concentration of the Cr(VI) decreased with time from a maximum concentration of 9.81 mg L^{-1} to a minimum of 0.19 mg L^{-1} for an initial concentration of 10 mg L^{-1} of Cr. The concentration of the Cr(III) was found to increase from 0.139 mg L^{-1} to 9.09 mg L^{-1} (Fig. 2.7).

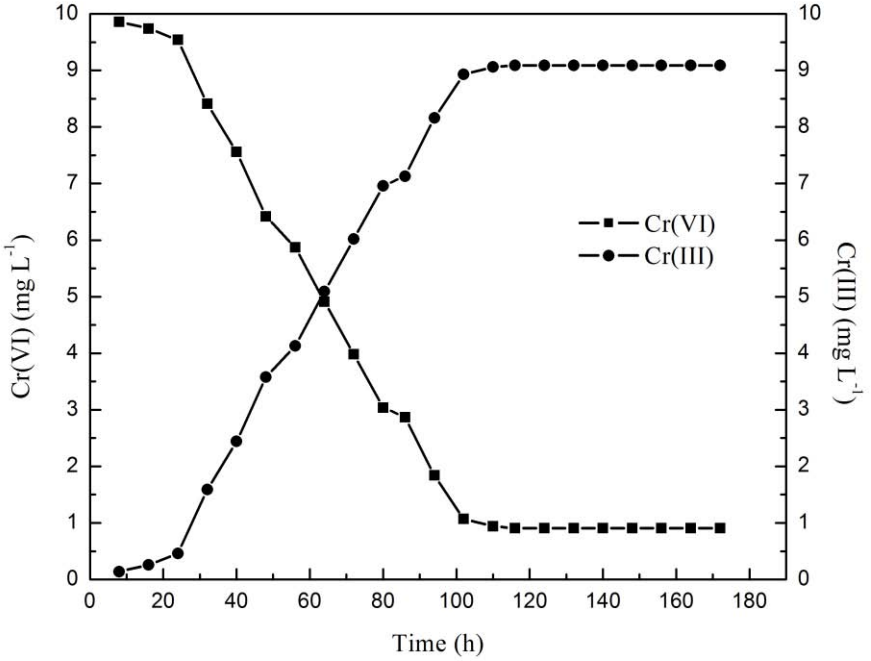

Fig. 2.7. Bioreduction of Cr(VI) at an initial concentation of 10 mg L^{-1}

The experimental Cr reduction factor, Y_{Cr} was plotted against time. The curves were linear and the predicted values were closer to the experimental values. The model predicted a maximum Cr reduction factor of 7.1 (R^2= 0.9347) obtained for 40 mg L^{-1} of initial Cr(VI) concentration. The Cr reduction factor was found to increase linearly with time indicating that the Cr reduction was dependant on the oxidation of the organic compounds present in the cells (Fig. 2.8).

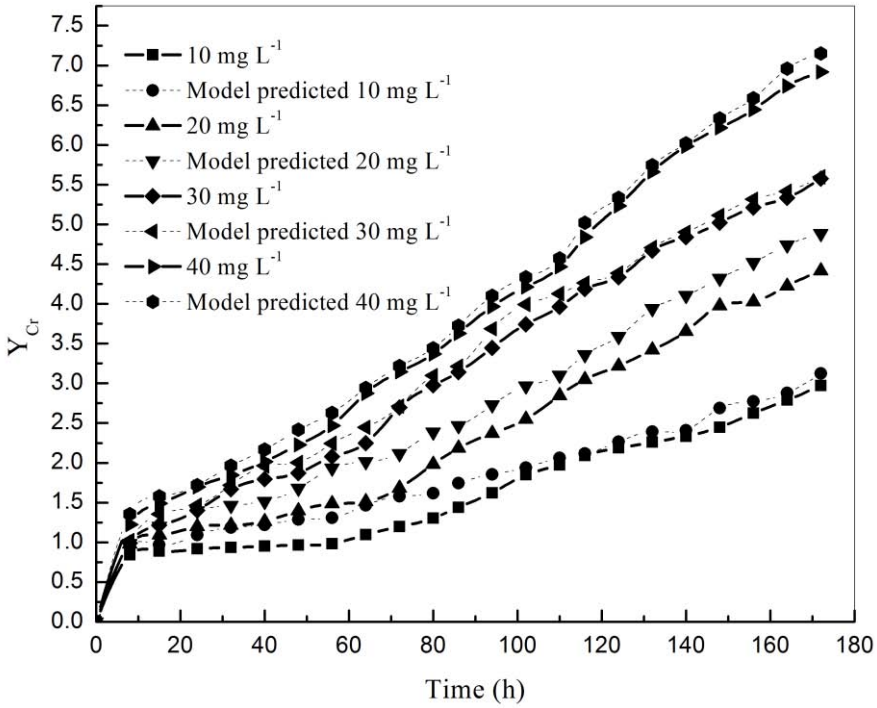

Fig. 2.8. Modeling of Cr(VI) reduction by *P.chrysosporium*

The kinetic parameter *K* is not a constant because it depends on the Cr(VI) and the organic compounds present in the cells. The kinetic constant was found to vary with increase in the initial metal concentration. A maximum rate constant of 0.2981 mg L^{-1} h^{-1} was reported for an initial Cr(VI) concentration of 40 mg L^{-1} (Table 2.1).

Table 2.1. Cr reduction coefficient for *P. chrysosporium* obtained by fitting Equation (12)

Cr(VI) concentration (mg L^{-1})	Initial biomass (mg L^{-1})	Overall rate coefficient (mg L^{-1} h^{-1})	R^2
10	687	0.1665	0.998
20	587	0.1731	0.994
30	568	0.193	0.996
40	190	0.2981	0.993

The rate coefficients obtained with different pH were presented in Table 2.1. A maximum of 0.437 mg L^{-1} h^{-1} was obtained for an initial Cr concentration of 10 mg L^{-1} at pH 6 (R^2= 0.993).

Table 2.2. Cr reduction coefficients at different pH

pH	Overall rate coefficient (mg L^{-1} h^{-1})	R^2
1	0.117	0.974
2	0.1367	0.938
3	0.191	0.978
4	0.2204	0.957
5	0.437	0.988
6	0.1665	0.993

The experimental data was fitted for the exponential phase of the growth curve and the kinetic parameters obtained were presented in Table 2.3. A maximum specific growth rate of 0.3541 h^{-1} was obtained for an initial Cr concentration of 10 mg L^{-1} (R^2=

0.9621). The maximum K_S of 0.449 mg L^{-1} (R^2= 0.947) was reported for Cr(VI) free culture.

Table 2.3. Growth parameters obtained with different concentration of Cr(VI)

Cr(VI) Concentration (mg L^{-1})	Initial Biomass Concentration X_0 (mg L^{-1})	μ_{max} (h^{-1})	K_S (mg L^{-1})	R^2
0	967	0.2457	0.449	0.962
10	687	0.3541	0.314	0.947
20	587	0.3031	0.258	0.981
30	568	0.1892	0.018	0.565
40	190	0.021	0.009	0.435

A milky white biomass was obtained at the end of bioremoval. Biochemical analysis of the biomass was done and the results are provided in the Table 2.4. A maximum of 51 µg mL^{-1} extracellular protein was obtained for an initial chromium concentration of 40 mg L^{-1}. A maximum of 10.42 µg mL^{-1} of total carbohydrate was also reported. The involvement of extracellular enzyme and the exoploymer is a possible reason for the bioreduction of Cr. The involvement of enzymes and transporter system in the intracellular accumulation of chromium needs further investigation.

Table 2.4. Concentration of extracellular protein and total carbohydrate

Cr (mg L^{-1})	Extracellular Protein (µg mL^{-1})	Total carbohydrate (µg mL^{-1})
10	37	8.14
20	37	8.42
30	42	8.93
40	51	10.42

2.4.4. Comparison of chromium removal by other microbes

Table 2.5. Comparison of chromium removal by live and active Microorganisms reported in literatures

Type of microorganism used	Type of reactor	Initial chromium concentration (mg L^{-1})	Percentage removed (%)	Optimum pH	References
Arthrobacter rhombi	Packed bed and airlift reactors	20	99.8	6	[12]
Indigenous bacteria	Trickle filters	30	Complete removal	7	[2]
E.coli	Shake flask	4.37	Complete removal	7	[13]
Natural microbes in soil	Soil bioreactor	50	97	6-8	[14]
Bacillus coagulans	Immobilized microbial bioreactor	26	Near complete removal	7	[15]
Bacillus sp	Packed bed reactor	10	Near complete removal	7	[16]
B. subtilis	Batch	10	88.6	2	[17]
Exiguobacterium sp., *Pantoea* sp., *Aeromonas* sp	Batch	100	70.2	2.5	[18]
P. chrysosporium	Batch flask study	10	98.92	6	Present work

The comparison of chromium removal capacities of other microorganisms as reported in literature are summarized in the Table 2.5. From the present study it was found that a maximum of 98.92 % bioreduction of Cr(VI) was obtained with P. *chrysosporium* at an initial chromium concentration of 10 mg L^{-1} at pH 6. Usage of live *P. chrysosporium* provides an advantage that the need of separate techniques for production of biomass and pretreatment of biomass as required in biosorption can be avoided. Bioreduction of Cr(VI) occurs at near neutral pH suggesting that no costly chemicals or external energy input are required for removal of chromium from wastewaters, however the inhibitory effect of dextrose and Cr(VI) may lead to cell inactivation and loss of efficiency in bioreduction.

2.4.5. Mechanism of Cr(VI) removal by *P. chrysosporium*

Surface binding of the Cr ions on the cell wall of P. chrysosporium

The functional groups of the fungal cells also play a vital role on the removal ofchromium. The FTIR spectra of both metal accumulated and metal free dry fungal biomass were analyzed at 4000- 450 cm^{-1} (Fig. 2.9). The fungal cell wall in general possesses anti parallel poly N acetyl glucosamine chain (α chitin) stabilized by hydrogen bonds. The FTIR spectra confirmed the presence of functional groups responsible for Cr binding (amino, carboxyl and phosphate groups). The metal accumulated fungal biomass (Fig. 2.9 b) showed variation in peak values in comparison with the metal free fungal biomass (Fig. 2.9 a). Intense peaks which are the characteristic of hydrogen are observed at the frequency in the range of 3500 – 3200 cm^{-1}. A narrow shift in the peak values from 3410 – 3374 cm^{-1} characteristic peaks for primary amines and amides was observed in the metal accumulated fungal biomass. This may be due to the OH, NH and acetamide groups of the chitin fractions of the fungal cell wall. A distinct peak at the 1742 cm^{-1} of the metal accumulated biomass can be attributed to the C=O stretching band of the amino acids. The fungal biomass shows strong amide I and amide II absorption bands at 1647

and 1550 cm^{-1} which was the characteristic for protein molecules. The IR spectra for the Cr accumulated biomass exhibited characteristic vibrations bands of chromate near 900 cm^{-1} [19]. An absorbance band of sulfate ions was observed at 616 cm^{-1} which inhibited the transport of Cr in to the cytoplasm. Apart from the amide groups the IR spectra showed the peaks for the polysaccharides at 1100 – 1000 cm^{-1}. The FTIR spectra clearly indicated the involvement of amines and amides of chitin as the functional groups responsible for the Cr uptake.

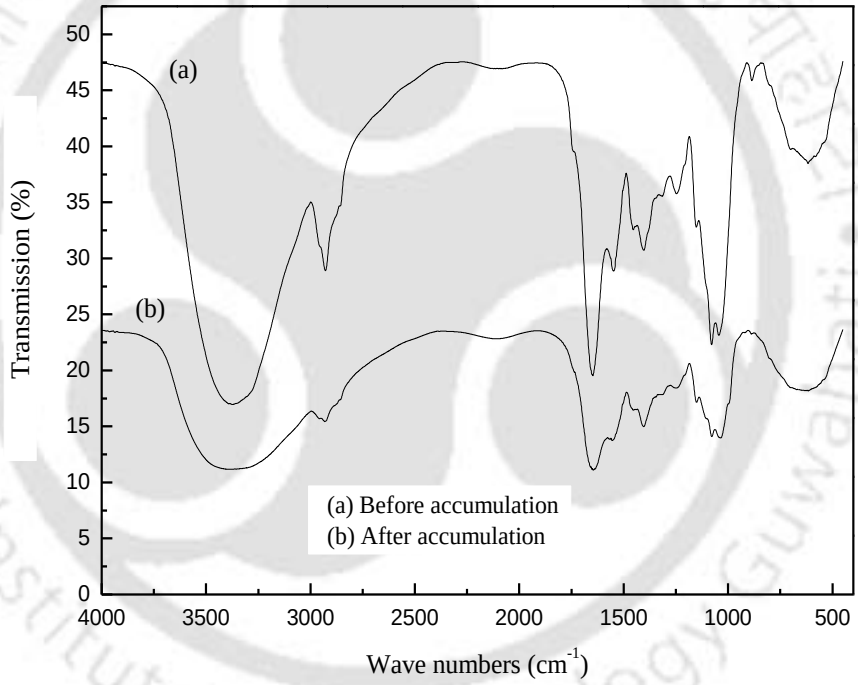

Fig. 2.9. FTIR spectra of the *P. chrysosporium* before and after Cr(VI) removal

Scanning electron microscopic study and Energy dispersive X-ray analysis

To understand the metal-microbe interaction SEM-EDX was performed. The morphology of *P. chrysosporium* was studied. The accumulation of Cr on to the fungal cells is clearly visible which further was confirmed by the EDX. It was observed that there was a significant change in the morphology of the fungi before and after treatment with the metal.

The fungal fruit bodies, basidiospores, responsible for the reproduction of fungi were found missing in the organism grown in chromium spiked medium. Clusters of filaments found in the culture grown in chromium free media was also found missing in the culture grown in Cr removal media. The morphology of the fungi was also found to vary. Fig. 2.10 (b), the morphological differences indicate that the Cr ions affected the reproductive metabolism of *P. chrysosporium.*

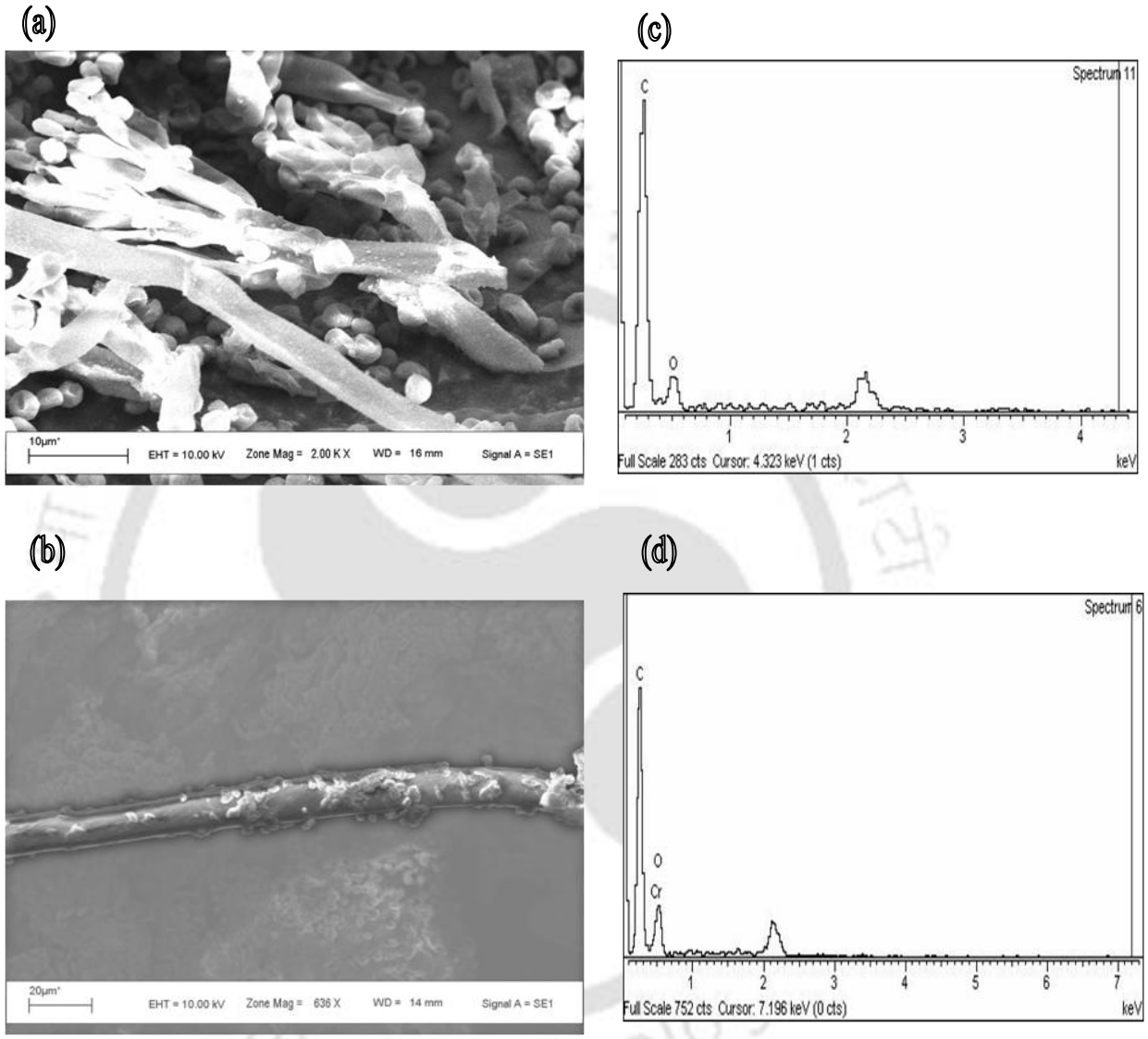

Fig. 2.10. SEM micrograph of *P. chrysosporium* (a) before chromium removal (b) after chromium removal; EDX spectra of *P. chrysosporium* (c) before chromium removal (d) after chromium removal

Intracellular accumulation of Cr

When extracellular concentration of the metal ion is higher than the intracellular metal concentration the metal ions can penetrate in to the cells through the cell wall by free diffusion [20]. Accordingly to understand the mechanism of the metal-microbe interaction it is important to locate the Cr inside the cells. TEM with EDAX was used as a tool to study the presence of the metal inside the cytoplasm [21]. It can be seen from the Fig 2.11 (b) that the Cr accumulated biomass exhibited dense granules in the cell wall and also in the cytoplasm. These dense granules were not observed in the Cr free control Fig 2.11 (a). Further the EDAX confirmed that the dense granules are composed of Cr. Since the Cr ions are found on the cell wall and in the cytoplasm, a two step mechanism was proposed. The first step is the binding of the Cr ions with the positively charged functional groups of the *P. chrysosporium*. The second step involves the transport of the Cr ions by the energy dependant transporter system. The transport of the Cr in to the cytoplasm through the different layers of the chitins and mannans occurs by the sulphate transporter system.

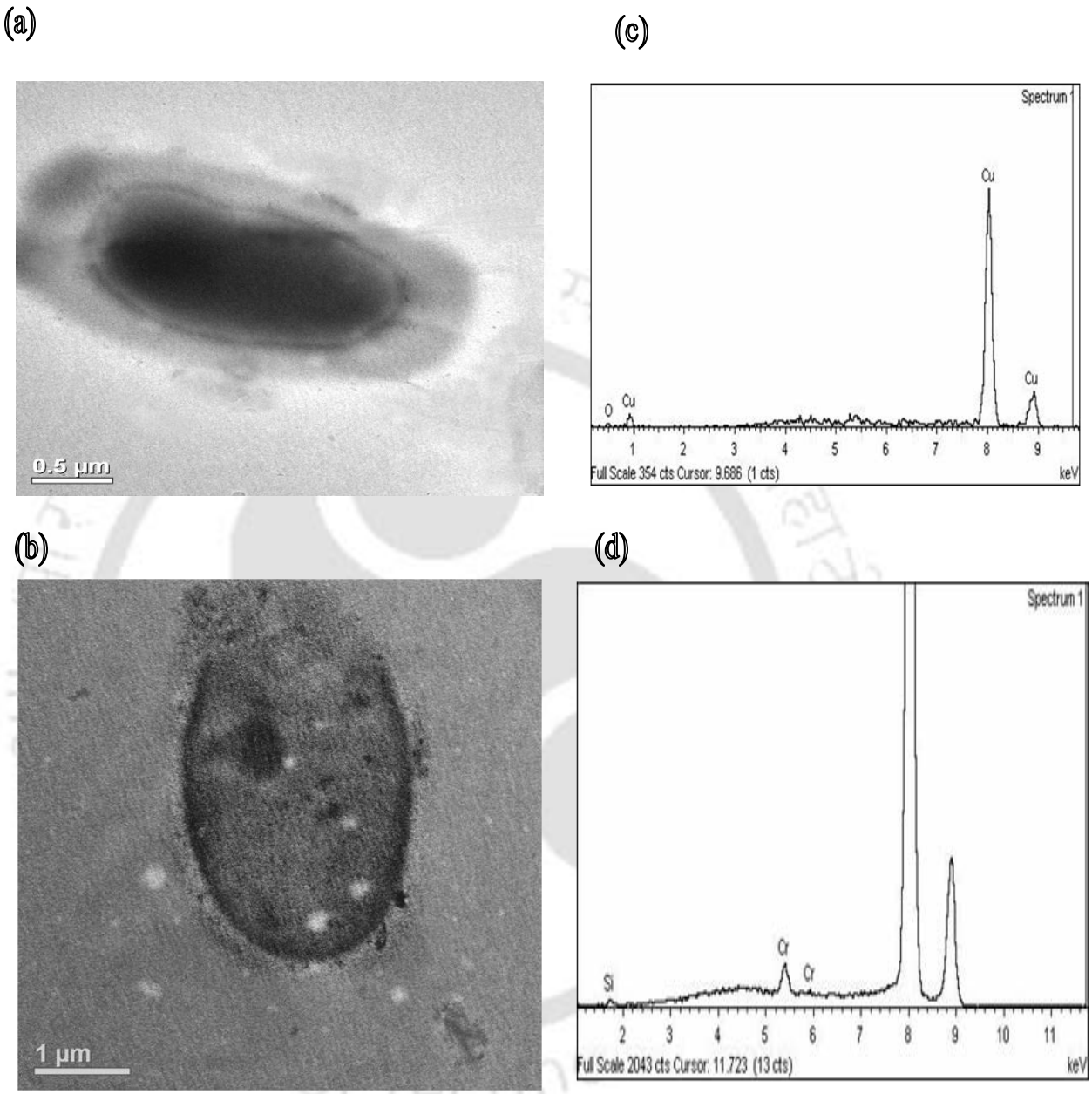

Fig. 2.11. TEM micrograph of *P. chrysosporium* (a) before chromium removal (b) after chromium removal; (c) EDX spectra of *P. chrysosporium* before chromium removal (d) after chromium removal

2.5. Conclusion

The aim of the present study was to find the chromium bioreduction capacity of *P. chrysosporium* during its growth. Bioreduction of chromium was dependant on the initial metal ion concentration, pH of the media and dextrose concentration. *P. chrysosporium* was capable of bioreduction of chromium at a concentration of 10 mg L^{-1} without showing any significant inhibitory effect on growth. The optimum pH for the growth and bioreduction was evaluated as 6.0. Bioreduction efficiency decreased from 98.29 to 18.25 % as Cr concentration was increased from 10 to 40 mg L^{-1}.

The uptake of Cr by live *P. chrysosporium* was found to be a two step process. The Cr ions first bind on to the surface of the cell wall by charge based interaction with the functional groups. The second step involves the intracellular accumulation by metabolism dependant process. The FTIR study revealed that the amide and amine groups of the chitin and the polysaccharides were the functional groups involved in the surface binding of the Cr with the cell wall. The scanning electron microscopy showed the surface binding of Cr ions on to *P. chrysosporium* cell wall. The TEM coupled with the EDX showed that the Cr ions were found in the cell wall as well as in the cytoplasm.

From the present study it was evident that *P. chrysosporium* can be suitably used for the bioreduction of lower concentration of chromium ions from water and wastewater in a greener way.

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Chapter III

Bioreduction of Cr(VI) using Live and Immobilized *Phanerochaete chrysosporium*

3.1. Introduction

The conventional methods for treatment of heavy metals require high capital cost and recurring expenses such as chemicals, which are not suitable for small scale industries [1]. Fungal biomass has certain advantage over bacterial biomass in respect of processing and handling [2]. Many fungal species have been extensively used for heavy metal biosorption and relatively few studies have been done with *Phanerocahete chrysosporium* for heavy metal reduction [3]. *P. chrysosporium* is a well known white rot fungus and a strong degrader of various xenobiotics. It could also be used for heavy metal removal from wastewater [4, 5]. In industrial or technical operations the immobilized microbial cells provide additional advantages over free cells. Immobilized cells are easy to separate and have less clogging effect during continuous operation. Immobilized cells have the ability to withstand higher concentrations of heavy metals [6]. Natural polymers such as alginate, chitosan, chitin, and cellulose are mostly used as the matrix for the immobilization of microbial cells via entrapment technique. These polymers are also known to bind metal ions strongly [7]. The use of non living and inactive biomass for large scale utilization is not practicable. The native forms of fungal cells suffer from low mechanical strength and smaller particle size and difficulty in separation from liquid stream [8]. Immobilized fungal cells are found to be far more stable than free fungal cells during metal removal from wastewater [5].

The choice of immobilization matrix is an important factor in the application of immobilized cells in wastewater studies. The polymer matrix determines the mechanical strength and the chemical resistance of the microbial cells. The most extensively

investigated biopolymer in the bioremediation studies is sodium alginate [9]. In this work the basidiospores of the white rot fungus *P. chrysosporium* was entrapped using Na-alginate and three other matrices. After growth the entrapped spores were studied for bioreduction of Cr from aqueous solution in a batch system. The mechanically stable matrix for immobilization of fungi was investigated. The effect of various physical parameters such as the temperature and pH on bioreduction was studied. Cr uptake capacity of the entrapped spores was reported.

3.2. Experimental

3.2.1. Microorganism and media

White rot basidiomycete, *P. chrysosporium* (MTCC 787), a white rot fungus was obtained from the Institute of Microbial Technology, Chandigarh, India. The culture was maintained by sub-culturing on malt dextrose agar slants. Spore suspensions for immobilization were freshly prepared from 7 day old culture, grown on malt dextrose agar slants at 25 ºC.

The general growth media for growth of the fungi *P. chrysosporium* consisted of malt extract 20 g L^{-1}, dextrose 20 g L^{-1}, and peptone 1 g in 1 L of distilled water. The pH of the medium was maintained at 6 using 0.1 N HCl. The media for bioreduction contained malt extract 20 g L^{-1}, dextrose 20 g L^{-1}, and peptone 1 g spiked with different concentration of chromium in 1 L of distilled water. All media were autoclaved at 15 kPa and 120 ºC for 20 min.

3.2.2. Chemicals

All reagents were of AR grade and procured from Merck, India. Cr(VI) stock solution (1000 mg L^{-1}) was prepared by dissolving $K_2Cr_2O_7$ salt in deionized water. For bioreduction experiments, concentrations ranging from 10 mg L^{-1} to 40 mg L^{-1} were

prepared. The chemicals used for the biopolymeric matrices are Sodium alginate (Loba chemie, India), Acryl amide (Merck, India) and Agar (Merck, India).

3.2.3 Immobilization of *P. chrysosporium* basidiospores

The entrapment of the basidiospores was done on various polymer matrices viz., Ca alginate, Acryl amide and Agar.

Entrapment in Sodium alginate

A 2% slurry of Ca alginate was prepared in hot water. After cooling, different concentration (2% to 10 % v/v) of the fungal spore suspensions (2.8 x 10^8 spores per mL) were added and stirred. The mixture was introduced in to a solution containing 0.1 M of $CaCl_2$ through a burette and stirred. The height of the burette was adjusted to get a uniform bead size of 4 mm every time. The beads were cured in $CaCl_2$ for 2 h and washed thrice with 200 mL of deionized water.

Preparation of Acryl amide gels

A (10% w/v) polyacryl amide gels was prepared with varying concentration of biomass (2% to 10 % v/v) and the polymerized gel was cut in to beads of equal size (3 mm). The gel beads were preserved at 4 ºC.

Preparation of agar

A 2% agar solution was prepared by boiling agar in hot water. The solution was allowed to cool for polymerization and (2% to 10%) of fungal spore suspension was added to the solution. The resultant polymer was cut in to equal sized beads of 3 mm. The agar beads were stored at 4 ºC before use.

3.2.4. Bioreduction studies

The immobilized beads were transferred to the growth media of 100 mL in a 250 mL flask and incubated in an orbital shaker (Daihan LabTech Co Ltd, Model LSI 3016-R) at 25 ºC for 120 h. The mycelia growth was monitored using a microscope. The

metabolically active immobilized fungus were transferred from growth media to the media spiked with (10 to 40 mg L^{-1}) of Cr. For all the experiments carried out with immobilized fungus, spore free matrices were used as control to study the effect of the matrices on bioreduction. The effect of media pH, temperature and initial Cr concentration on the bioreduction were also studied.

3.2.5. Analytical procedure

After bioreduction the supernatants were collected by centrifugation (Remi R-24) and analyzed for total chromium concentration using Atomic Absorption Spectrophotometer (Varian AA140). The Cr(VI) concentration was measured spectrophotmetrically (Perkin Elmer, Model Lambda 35) at 540 nm by complexation with 1,5 diphenyl carbazide .

3.3. Kinetics of Cr(VI) reduction

Shen and Wang (1994) [10], demonstrated that Cr(VI) reduction by enzymes can be expressed in the form of Monod equation (1). The model proposed by Shen and Wang considered the concentration of active cell mass and proposed the Cr(VI) reduction model.

$$-\frac{dCr}{dt} = \frac{K_m Cr}{K_c + Cr} X \qquad (1)$$

where Cr (mg L^{-1}) is the concentration of Cr(VI) at time t (h), X is the concentration of biomass (cells L^{-1}). K_m and K_c are the specific Cr(VI) reduction rate (mg Cr(VI) $cell^{-1}$ h^{-1}) and half velocity constant (mg Cr(VI) L^{-1}) respectively.

The concentration of the Cr(VI) was dependant on the concentration of the active biomass concentration, X (cells L^{-1}). It was assumed that the active cell mass concentration decreases with time due to the toxicity of the Cr(VI),

$$X = X_o - \frac{Cr_o - Cr}{R_c}$$

(2)

where, R_c is the maximum Cr(VI) reduction capacity of the cells (mg Cr(VI) cell^{-1}).

Replacing the term biomass with the active biomass concentration in Eq. (1),

(3)

$$-\frac{dCr}{dt} = \frac{K_m Cr}{K_c + Cr}\left(X_o - \frac{Cr_o - Cr}{R_c}\right)$$

Eq. (3) was integrated,

$$t = \frac{K_c}{K_m\left(\frac{Cr_o}{R_c} - X_o\right)}\ln\left[\frac{CrX_o}{Cr_o\left(X_o - \frac{Cr_o - Cr}{R_c}\right)}\right] + \frac{R_c}{K_m}\ln\left[\frac{X_o}{\left(X_o - \frac{Cr_o - Cr}{R_c}\right)}\right] \quad (4)$$

The above equation represents the Cr(VI) by the active biomass at various time intervals. The maximum amount of Cr(VI) that can be reduced was dependant on active biomass concentration.

3.4. Results and Discussion

The effect of the glucose, temperature and pH on the bioreduction of Cr with immobilized *P. chrysosporium* was studied and the results are discussed in this section. Various matrices for immobilization like Ca alginate, acryl amide and agar were investigated. The best suitable matrix with high mechanical stability and chemical resistance was optimized.

3.4.1. Glucose consumption during bioreduction of Cr(VI)

Glucose was tested as the sole carbon source for the growth and reduction of Cr(VI) by *P. chrysosporium.* The glucose concentration in the media was varied from 0 to 20 g L^{-1} and trace concentration of glucose was analyzed periodically using dinitro salilic acid method. It was observed that the glucose was completely utilized for an initial concentration of 10 and 20 mg L^{-1} of Cr by the fungus entrapped in Ca alginate (Fig. 3.1). This was a clear indication that the fungus was metabolically active throughout the study. Glucose provided the essential energy required for the metabolic activity of the fungi. The decrease in glucose consumption for concentration exceeding 20 mg L^{-1} was due to the fact that Cr acts as an inhibitor for the growth of the organism and there by affected the metabolic activity of the fungi.

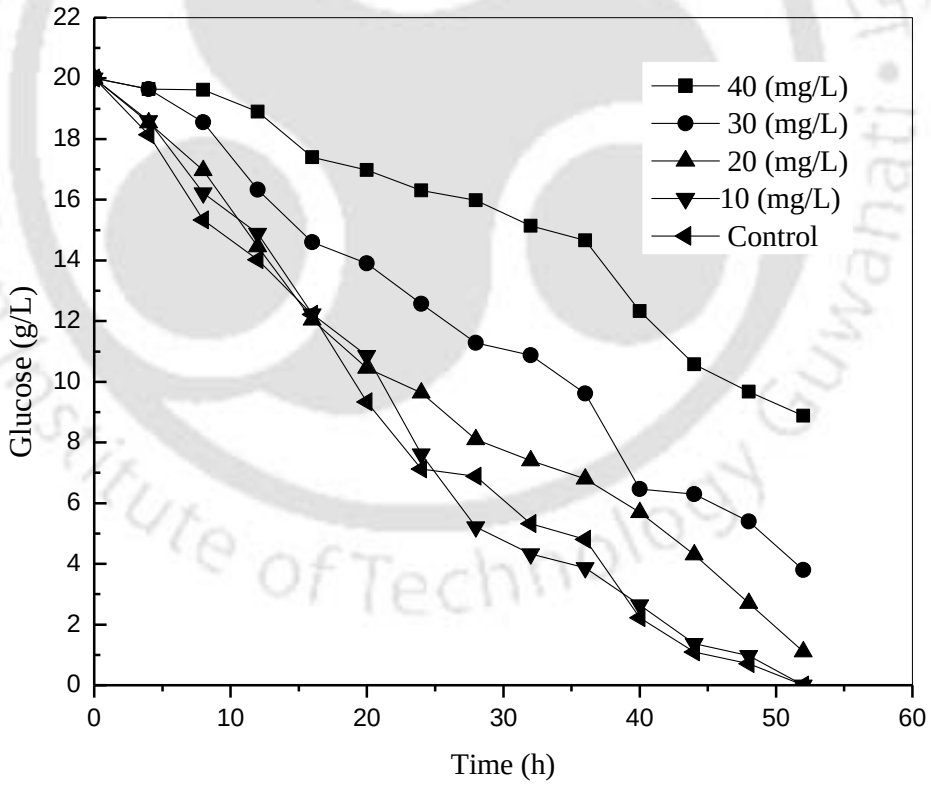

Fig. 3.1. Glucose consumption profile of immobilized *P. chrysosporium*

3.4.2. Effect of pH on bioreduction

The medium pH affects the solubility of the metal ions and the ionization state of the functional groups. pH of the media affects the activity of the biosorbents [11]. The bioreduction was studied at various pH ranging from 2 to 7. Maximum bioreduction was obtained with pH 5. A maximum of 24, 48, 78, 98.3, 92.58, 90.24% of bioreduction were reported for pH 2, 3, 4, 5, 6, 7 respectively with an initial Cr(VI) concentration of 10 mg L^{-1} (Fig. 3.2). The increase in bioreduction from pH 3 to 5 was due to the physico-chemical interaction between the metal ions with the charged groups of the matrix. As the pH was increased further the charge on the cell wall was neutralized and thereby the bioreduction capacity decreased. It was found that pH 5 was the optimum pH for the bioreduction using immobilized beads.

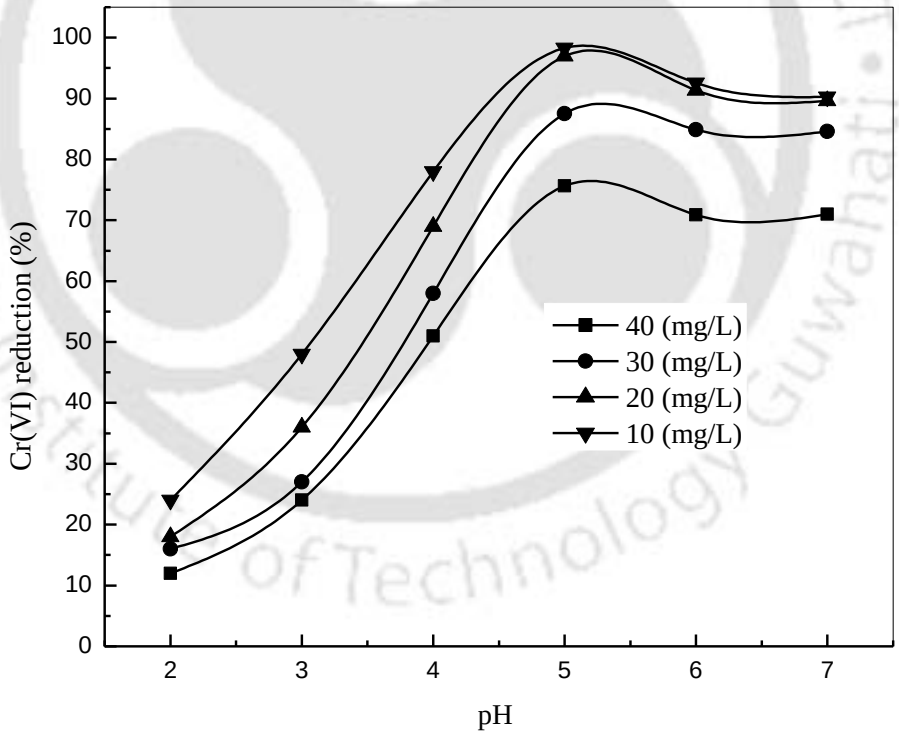

Fig 3.2. Effect of pH on bioreduction of Cr(VI)

3.4.3. Effect of time on bioreduction

The bioreduction of Cr was studied for an extended period of 52 h. It was observed that the bioreduction of the Cr increased with time. A maximum bioreduction of 98.3% was reported at 52 h for an initial Cr(VI) concentration of 10 mg L^{-1} with fungus entrapped in Ca alginate (Fig. 3.3). It was also observed that the Ca alginate was the best suitable matrix. The other two matrices reduced 92.6 and 90.3% of Cr(VI) at 52 h.

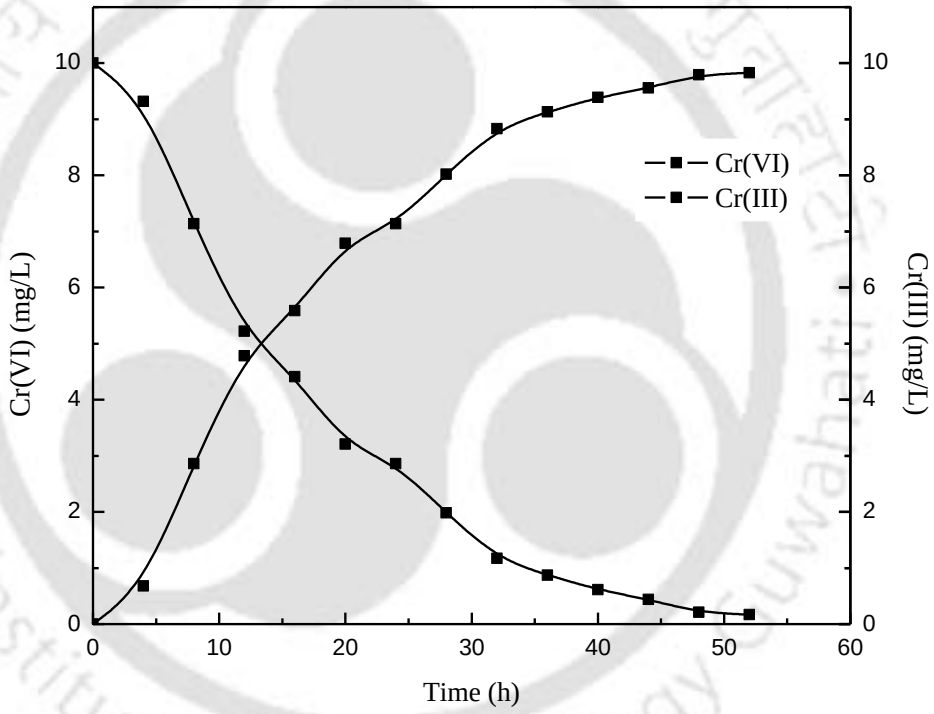

Fig. 3.3. Effect of time on bioreduction of Cr(VI)

3.4.4. Effect of temperature on bioreduction

The bioreduction of Cr(VI) was studied at various temperatures ranging from 10 ºC to 35 ºC. The bioreduction capacity of the organism was found to increase when the temperature increased from 10 ºC to 25 ºC. When the temperature was further increased there was no significant reduction. It was observed that 25 ºC was found to be the optimum temperature for the bioreduction. The maximum bioreduction obtained with 25 ºC was 98.3% (Fig. 3.4).

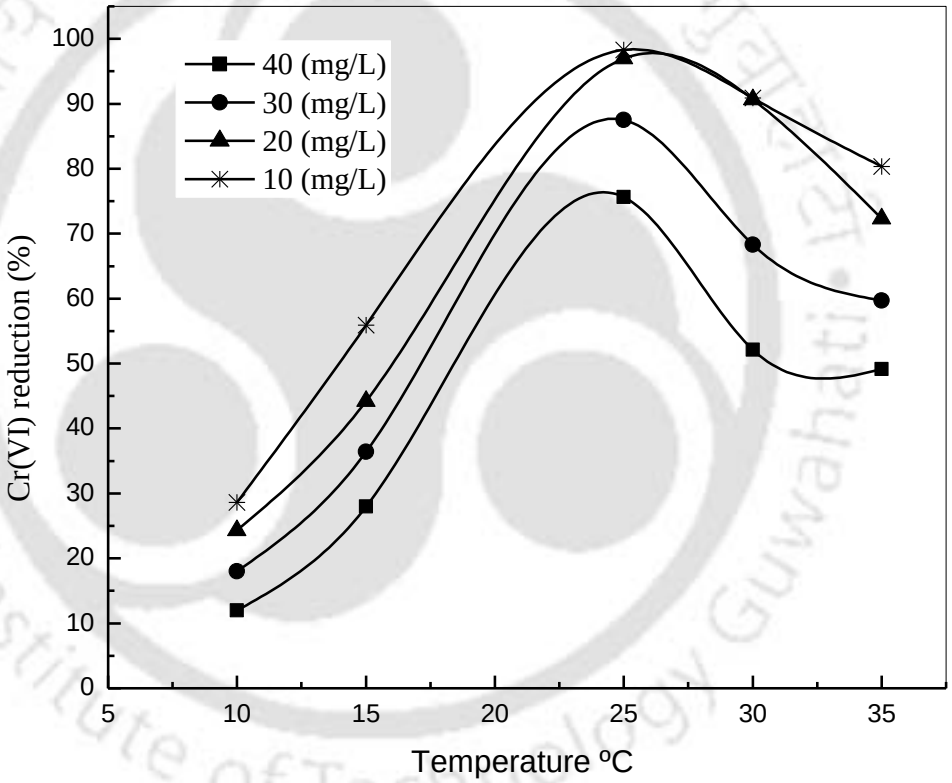

Fig. 3.4. Effect of Temperature on bioreduction of Cr(VI)

The decrease in the bioreduction with increase in temperature can be attributed to the fact that the organism was acclimatized to a specific temperature. The substrate

consumption was also dependant on the temperature. Even a small change in the operating temperature affected the metabolic activity of the fungi which in turn affected its bioreduction capacity.

3.4.5. Effect of inoculum size

The inoculums size was varied from 2 %(v/v) to 10 %(v/v). It was observed that the bioreduction was found to increase with the increase in the inoculums size. The increase in bioreduction with increase in inoculums dosage was due to the fact that bioreduction was dependant on the growth of the white rot fungi.

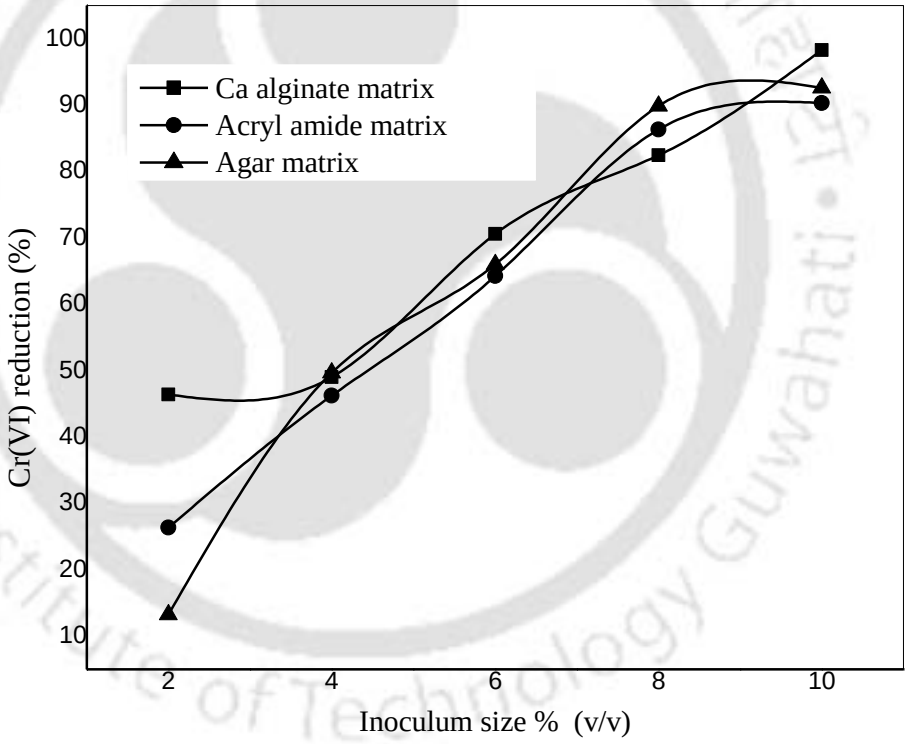

Fig. 3.5. Effect of Inoculum dosage on bioreduction of Cr(VI)

The number of viable colonies increased with increasing inoculums dosage. The bioreduction obtained reached a constant value for inoculums dosage exceeding 8 %(v/v) (Fig. 3.5). The optimum inoculums dosage for the other matrices such as acryl amide and agar were found to be 6% and 10 % respectively.

3.4.6 Effect of different matrices on percent reduction of Cr(VI)

A maximum of 98.3 percentage of reduction was reported with alginate matrix for an initial Cr(VI) concentration of 10 mg L^{-1}. The acryl amide and agar matrices yielded a Cr(VI) reduction percentage of 90.3 and 92 percentage respectively. The acryl amide matrix was found to disintegrate as the study was continued for a period of 52 h. The agar matrix was found to be soluble in the media causing difficulty in analyzing the Cr(VI) concentration. Table 3.1 showed the percentage reduction of Cr(VI) obtained for different matrices studied with different initial concentrations of Cr(VI) ranging from 10 mg L^{-1} to 40 mg L^{-1}.

Table 3.1. Percentage reduction of Cr(VI) using different matrices

Matrices	Percentage reduction (%)			
	10	20	30	40
Alginate	98.3	96.95	87.53	75.65
Acrylamide	90.3	68.4	46.6	29.2
Agar	92.6	89.3	56.13	54.9

3.4.7. Kinetics of Cr(VI) bioreduction

Fig. 3.6 represents the experimental and model fit of Cr(VI) reduction by immobilized *P. chrysosporium*. It was observed that the experimental and the model predictions are closer for all the concentrations studied. The model predicted a maximum reduction capacity of 30.2 mg L^{-1} for an initial Cr(VI) concentration of 40 mg L^{-1} at initial cell density of 2.08 X 10^{8} cells L^{-1} with alginate as the immobilization matrix (Table 3.2). The predicted value was closer to the experimental reduction capacity of 30.26 mg L^{-1}. The results indicated that the model studied fitted well for the Cr(VI) reduction by immobilized *P. chrysosporium.*

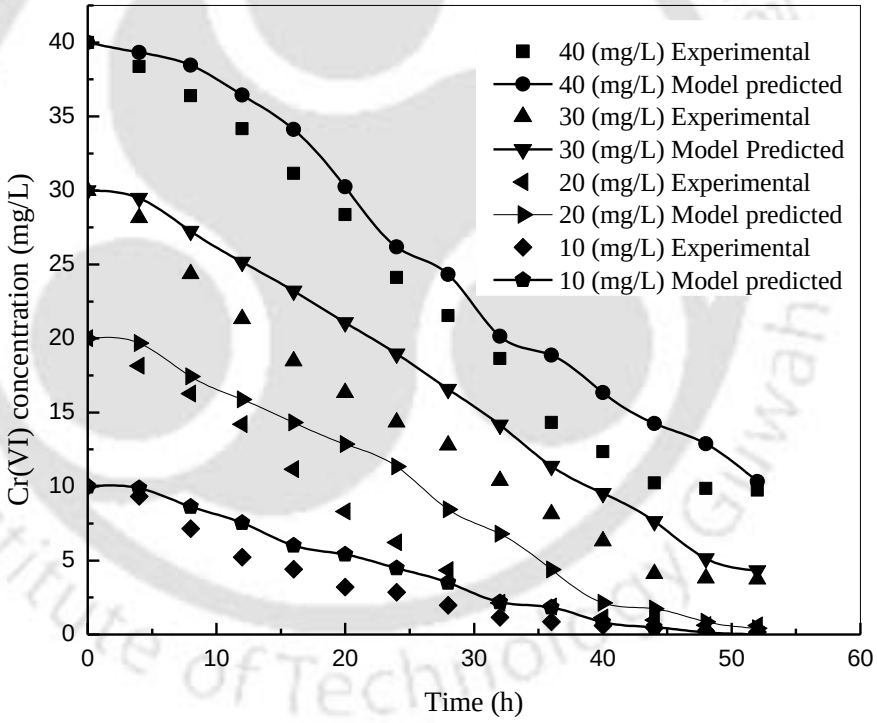

Fig. 3.6. Kinetic modeling of Cr(VI) bioreduction

Table 3.2. Kinetic parameters for Cr(VI) reduction (40 mg L^{-1}) using Ca alginate immobilized *P. chrysosporium*

Matrices	K_m (mgCr(VI)/cell/h)	K_c (mgCr(VI)/L)	R_c (mgCr(VI)/cell)	X_o (cells/L)	R_cX_o Model Predicted (mgCr(VI)/L)	R_cX_o Experimental (mgCr(VI)/L)
Alginate	3.63 X 10^{-9}	0.974	1.452 X 10^{-7}	2.08 X 10^{8}	30.201	30.26
Acrylamide	1.56 X 10^{-9}	1.374	6.245 X 10^{-8}	1.87 X 10^{8}	11.678	11.68
Agar	3.03 X 10^{-9}	1.24	1.215 X 10^{-7}	1.81 X 10^{8}	21.99	21.98

3.4.8. Comparison of of Cr(VI) reduction using immobilized microbes

Table 3.3. Comparison of of Cr(VI) reduction using immobilized microbes reported in literature

Type of microorganism used	Matrix	Initial chromium concentration (mg L^{-1})	Percentage reduced (%)	Optimum pH	References
B. coagulants	Acryl amide	26	100	7	Philip, et al., [12]
A. haemolyticus	Wood husk	15	97	7	Zakaria, et al., [13]
S. griseus	PVA - alginate	25	100	7	Poopal and Laxman, [14]
Pseudomonas Sp	Alginate	100	66.5	6-8	Murugesan and Maheswari [15]
Bacillus sp	Celite, amberlite, and Ca-alginate	2 – 8	98	7	Camagro et al., [16]
P. chrysosporium	Alginate, acryl amide, agar	10	98.3	5	Present study

The comparison of other immobilized microbes used for Cr(VI) reduction reported in literature was summarized in Table 3.3. From the present study it was observed that a maximum of 98.3% reduction of Cr(VI) was reported with an initial concentration of 10 mg L^{-1} of Cr(VI) at pH 5. Immobilization provides an advantage of separation of biomass. Usage of live microbes provides advantage over other process as no costly chemicals are required for the treatment of Cr(VI). Immobilized beads were able to reduce Cr(VI) under adverse conditions.

3.5. Conclusion

Bioreduction using the immobilized *P. chrysosporium* was dependant on the pH, temperature and metabolic activity of the organism. Glucose at 20 g L^{-1} was essential nutrient for the bioreduction and growth. A maximum of 98.3 % of bioreduction was reported for 10 mg L^{-1} of initial Cr(VI) concentration. Results indicated that higher cell concentrations (2 X 10^8) was necessary for significant reduction of Cr(VI). The Cr(VI) reduction rate was also affected by the initial Cr(VI) concentration. The optimum reduction was observed at pH 5 and 25 ºC. The Shen and Wang model fitted well for the experimental data. The model predicted a maximum reduction capacity of 30.2 mg L^{-1} for an initial Cr(VI) concentration of 40 mg L^{-1}. Ca alginate was found to be the best suitable matrix for the immobilization of the fungus.

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Chapter IV

Bioreduction of Cr(VI) by Free Cells and Cell Free Extracts of *Halomonas sp.*

4.1. Introduction

A wide range of bacteria are reported in literature for the reduction of Cr(VI). Shen and Wang (1994) [1] reported complete reduction of Cr(VI) at an initial concentration of 27 mg L^{-1} by *E.coli*. Wang and Shen [2] studied the reduction of Cr(VI) by *Bacillus sp*, *Pseudomonas fluorescens* LB 300 and reported 100% and 90% reduction respectively for an initial concentration of 27 mg L^{-1} of Cr(VI). Liu et al. (2006) [3] used *Bacillus sp* for the reduction of Cr(VI) in the presence of glucose as a substrate for growth, a maximum of 87.5% reduction of Cr(VI) was obtained with an initial Cr(VI) concentration of 40 mg L^{-1}. During the bioreduction process, Cr(VI) acts as an electron acceptor [4]. Microbial reduction of Cr(VI) can be attained directly by microbial metabolism or by bacterial metabolites such as enzymes Cr(VI) reduction can be achieved with soluble reductase produced during the reduction studies. The production of the soluble reductase by the bacteria is a cometabolism process and hence does not generate biochemical energy to support growth [2]. The reduction of Cr(VI) by the enzymes is not substrate specific for Cr(VI) [5]. Previous studies on Cr(VI) reduction using CFE extracts have reported NADH, NADPH as electron donors [6].

Halomonas is a halophilic bacterium which can survive under heavy load of salt concentration in the growth media. Since the leather tanning process involves usage of salt, *Halomonas sp* was selected for this study as it can grow in the presence of the NaCl.

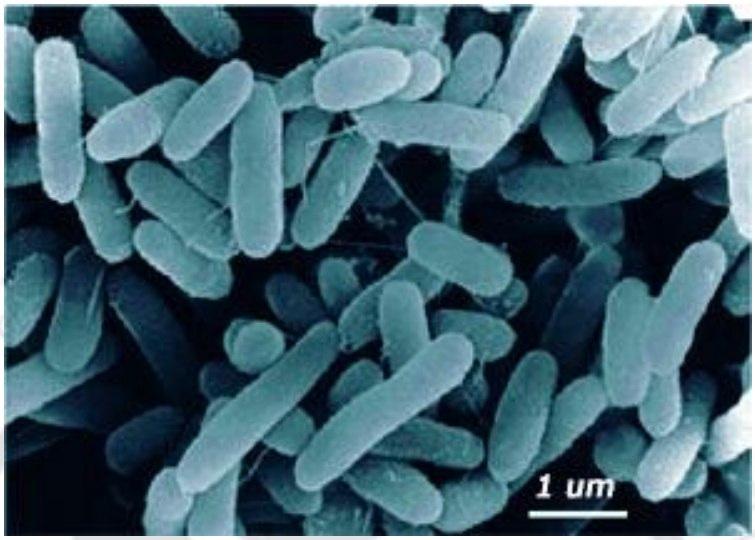

Fig. 4.1. SEM image of *Halomonas sp*. (Source: http://genome.jgi-psf.org/maraq/maraq.home.html)

In the present work the media composition for the growth and reduction of the Cr(VI) by *Halomonas sp* was reported, the role of pH, temperature and initial Cr(VI) concentration on Cr(VI) reduction was reported. The CFE produced by the bacteria was tested for its potential to reduce Cr(VI). Various electron donors were tested to improve the reduction capacity of the CFE.

4.2. Materials and Methods

4.2.1. Microorganism and culture conditions

Halomonas sp was procured from the Institute of Microbial Technology, Chandigarh, India and cultivated at 37 ºC in a liquid medium at pH 7 with agitation of 150 rpm on a rotary shaker (Daihan LabTech Co Ltd, Model LSI 3016-R). *Halomonas sp* culture was maintained on a modified media (YEPG-NaCl media). The modified YEPG-NaCl media had glucose as electron donor. Subcultures of the parent culture were made on every 15 days of duration and stored at 4 ºC.

4.2.2 Media

The growth media for bacteria contained peptone 5 g L^{-1}, yeast extract 5 g L^{-1}, NaH_2PO_4 and glucose 1g L^{-1}. The medium for the Cr(VI) reduction contained yeast extract 5 g L^{-1}, glucose 1g L^{-1}, K_2HPO_4 0.03 g L^{-1}, KH_2PO_4 0.03 g L^{-1}, $MgSO_4$ 0.01 g L^{-1}, NaCl 20 g L^{-1}. The pH of the media was adjusted to 7±0.1 with 0.1 N HCl and/or 0.1 N NaOH. All the media were autoclaved (Indfos 110 PB) at 120 °C for 15 min. A total of 7 different substrates such as acetate, citrate, lactate, oxalate, succinate, glycerol and tryptone were tried as replacements for glucose in both the growth and the reduction media.

4.2.3. Cr(VI) stock solution

The reduction of Cr(VI) was studied in an aqueous solution. A Cr(VI) stock solution was prepared by dissolving 2.82 g of $K_2Cr_2O_7$ in 1000 mL deionized water, shaking it at 150 rpm for 15 min to get complete dissolution. Cr(VI) spiked medium was prepared by diluting this solution to required concentration in the growth media.

4.2.4. Bioreduction experiments

The factors affecting the growth and bioreduction were investigated in 250 mL conical flasks containing 100 mL of Cr(VI) spiked medium for 48 h. The pH of the solution is an important parameter in wastewater research. Studies were performed to optimize the pH of the Cr(VI) bioreduction process. The studies on optimizing pH were performed at 37 ºC with an initial glucose concentration of 1g L^{-1}. The bioreduction media was prepared with different pH ranging 1 to 8 for various concentration of Cr(VI). The solution pH was measured using a pH meter (Sartorius AG 37070 Goettingen, Germany). At pH 1, 2, 3 formation of Cr(III) occurred naturally due to the acidic environment. The Cr(III) thus formed was oxidized using potassium permanganate to convert Cr(III) to Cr(VI) [7]. Experiments were performed to study optimal concentration of carbon source, glucose on the growth and reduction of Cr(VI) by *Halomomnas sp.* In

order to estimate the concentration of glucose required for the growth and reduction of Cr(VI) by *Halomomnas sp* the other two parameters, pH and temperature were fixed at pH 7 and 37 ºC respectively. The bioreduction media with an initial Cr(VI) concentration of 40 mg L^{-1} was prepared by varying the concentration of glucose between 250 mg L^{-1} to 2000 mg L^{-1}. Samples were taken at specific time intervals (3 h) and centrifuged at 12000 X g, the biomass concentration was measured as OD_{600}. The concentration of Cr(VI) was measured spectrophometrically using diphenyl carbazide (DPC) at 540 nm using an UV spectrophotometer (Perkin Elmer, Model Lambda 35). Interference of growth was eliminated using calibration curves obtained with the same medium. Total Cr concentration was measured by Atomic absorption spectrophotometer (Varian AA240 FS).

The influence of Cr(VI) on reduction was studied by varying Cr(VI) concentration from 10 mg L^{-1} to 40 mg L^{-1}. Glucose at an initial concentration of 1 g L^{-1} was used as sole carbon source. pH was maintained at 7. The flasks containing the bioreduction media with various concentration of Cr(VI) were inoculated with a loopful of overnight grown cultures of *Halomonas sp*. The flasks were incubated in incubator shaker (Daihan LabTech Co Ltd, Model LSI 3016 –R) at 37 ºC and 180 rpm. Samples were withdrawn aseptically at regular time intervals of 3 h and centrifuged at 15000 X g for 15 min and the supernatant was used for metal analysis. A part of the supernatant was used for the analysis of total Cr, and another part of the supernatant was used for analysis of Cr(VI). Bioreduction was reported as the change in the concentration of Cr(VI) to Cr(III) at regular time intervals.

The effect of yeast extract on the growth and bioreduction was studied by varying the yeast extract concentration in the bioreduction media. The concentration of the yeast extract was varied between 1 g L^{-1} to 10 g L^{-1}. The experiments on optimization of yeast extract was conducted by keeping the other physical parameters constant, viz. the pH was maintained at 7, temperature at 37 °C and glucose at an initial concentration of 1 g L^{-1}. An initial Cr(VI) concentration of 40 mg L^{-1} was used.

The time required for the reduction of Cr(VI) to Cr(III) was performed at pH 7 and 37 °C. The bioreduction was prepared with 1 g L^{-1} of glucose as carbon source and 40 mg L^{-1} of initial Cr(VI) was used. The experiments were conducted for prolonged time period. The experiments were continued till an equilibrium concentration of Cr(III) was attained.

Various electron donors were tested for enhancing the bioreduction of Cr(VI). The effectiveness of each of the donor was tested by using a bioreduction media with an initial Cr(VI) concentration of 40 mg L^{-1}, pH 7 and 37 ºC. The bioreduction experiments were carried out for a period of 48 h. Samples were withdrawn at regular intervals and centrifuged at 12000X g. The supernatant was used for Cr(VI) and biomass analysis.

The bioreduction of Cr(VI) by CFE was performed. The effect of temperature, pH, electron donors and inhibitors was studied. pH of the CFE was optimized by varying the pH between (1-7) at a constant temperature of 37 °C. The optimum temperature for the CFE on reduction of Cr(VI) was studied using a 40 mg L^{-1} of Cr(VI) solution at pH 6.5. Various electron donors were screened for their role in enhancement of Cr(VI) reduction by CFE. Studies using various electron donors were carried out at pH 6.5 and 37 °C at an initial Cr(VI) concentration of 40 mg L^{-1} and 6 h.

4.2.5. Preparation of CFE

Cell free extract was prepared by making modifications to the method used by Bopp and Elrich (1988) [8]. Cultures grown overnight were centrifuged and stored by suspending it in phosphate buffer of pH 7. The suspended cultures were then disrupted by ultrasonication (Sonics, VCX 500, 20 kHz). Power was supplied in pulses at 50 W each. A portion of sonicate was then centrifuged at 12000 X g for 10 min. The supernatant (S_1) was decanted and checked for the viable cells. Another portion of sonicate was centrifuged at 24000 X g for 20 min at 4°C and the supernatant (S_2) was used as soluble fraction. The sediment obtained was used as membrane fractions (MF).

4.3. Results and Discussion

4.3.1 Studies on free cells of *Halomonas sp*

In this section the free cells of *Halomonas sp* was investigated for their ability to reduce Cr(VI). Effect of media components (glucose and complex nutrients) on the reduction of Cr(VI) by free cells of *Halomonas sp* was reported. The potential eight different electron donors on the reduction of Cr(VI) was also reported. The effect of different treatment methods to study the role of metabolism of *Halomonas sp* on the reduction of Cr(VI) was also reported.

4.3.1.1 Effect of glucose on growth and reduction of Cr(VI)

Cr(VI) at an initial concentration of 40 mg L^{-1} was reduced to Cr(III) under aerobic condition. The cell density was found to increase with the increase in the glucose concentration ranging up to 1000 mg L^{-1} (Fig. 4.2). It was observed that there was a drop in the cell density for glucose concentrations exceeding 1000 mg L^{-1}. It can be attributed to the fact that glucose at concentration greater than 1000 mg L^{-1} affected the growth of the *Halomonas sp* whereas the concentration of Cr(VI) decreased with increase glucose concentration ranging up to 1000 mg L^{-1}.

The increase in concentration of Cr(VI) for glucose concentration exceeding 1000 mg L^{-1} was due to the substrate inhibition of glucose. The results indicated that the cell density had an important role in the Cr(VI) reduction. It was observed that the growth of the *Halomonas sp* had a significant effect on the reduction of the Cr(VI).

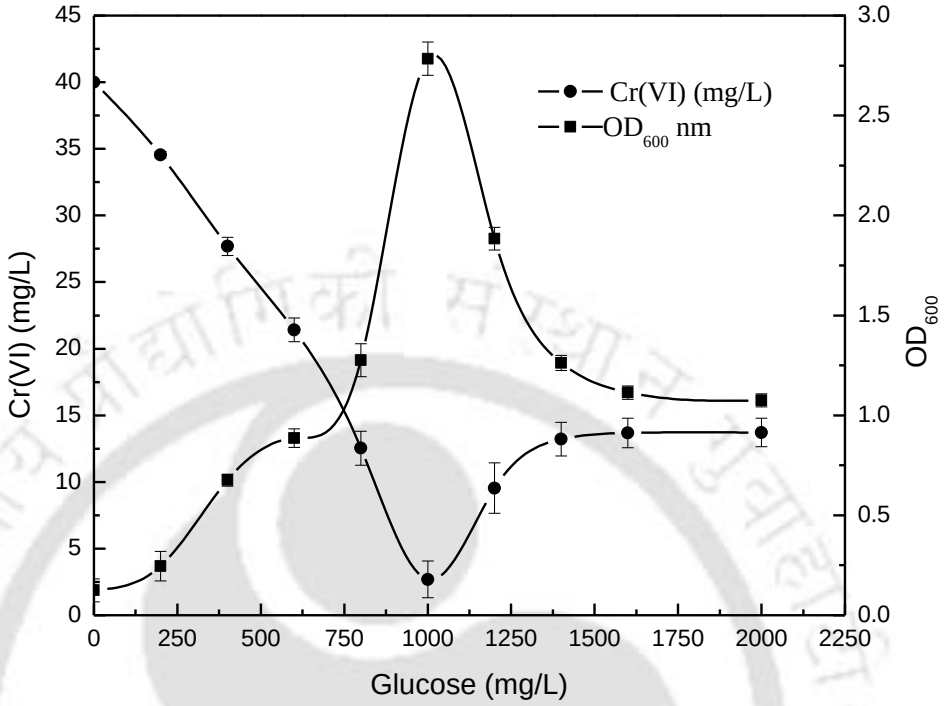

Fig. 4.2. Growth and reduction of Cr(VI) by *Halomonas sp.* at pH 7 and temperature 37 ºC, initial Cr(VI) concentration 40 mg L^{-1}

4.3.1.2 Selection of electron donors for the reduction of Cr(VI)

Microbial reduction of Cr(VI) is an electron dependant process, eight different carbon sources were studied as electron donors namely acetate, citrate, lactate, oxalate, succinate, glycerol, tryptone for the reduction of Cr(VI). It can be observed from Table 4.1, that glucose was found to be an effective electron donor as it yielded a maximum specific reduction rate of 0.74 mg g^{-1} h^{-1}. The maximum reduction was obtained with glucose owing to the fact that, glucose was readily oxidized by *Halomonas sp*. Moreover the culture was acclimatized in glucose media for a prolonged time with an initial Cr(VI) concentration of 40 mg L^{-1} at 37 °C and pH 7. Except for succinate all the other electron donors increased the specific Cr(VI) reduction rate. The reduction rate obtained with

succinate and control are 0.17 and 0.14 mg g^{-1} h^{-1}. The results indicated that electron donors had a significant role on the Cr(VI) reduction rate.

Table 4.1. Effect of different organic substrates on Cr(VI) reduction by *Halomonas sp*. at pH 7 and temperature 37ºC, Initial Cr(VI) concentration 40 mg L^{-1}

Organic substrate	Specific Cr(VI) reduction rate (mg/g/h)
Acetate	0.39
Citrate	0.42
Lactate	0.37
Oxalate	0.42
Succinate	0.17
Glucose	0.74
Glycerol	0.35
Tryptone	0.47
No substrate	0.14

4.3.1.3 Effect of yeast extract on the reduction of Cr(VI)

The growth media for the *Halomanas sp* contained high concentration of yeast extract. Studies were conducted to determine the amount of yeast extract required for the reduction of Cr(VI). Yeast extract supplements the growth of the bacteria. It was observed that 5 g L^{-1} of yeast extract was essential for the reduction of Cr(VI) by *Halomonas sp* (Fig. 4.3). Cr(VI) reduction decreased with yeast extract concentrations exceeding 5 g L^{-1}.

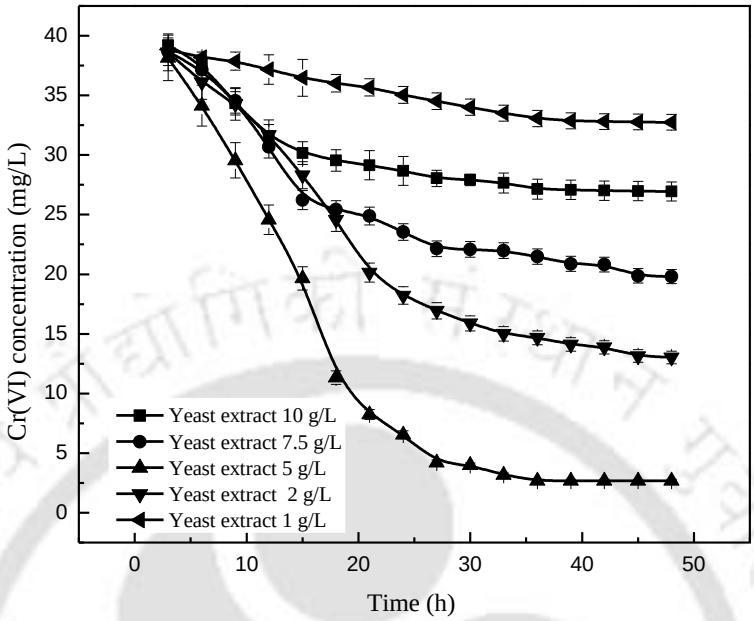

Fig. 4.3. Effect of Yeast extract on reduction of Cr(VI) at pH 7 and temperature 37ºC, initial Cr(VI) concentration 40 mg L^{-1} and glucose concentration 1 g L^{-1}

It was also observed that the reduction of Cr(VI) decreased when the concentration of yeast extract was decreased below 5 g L^{-1}. The decrease in Cr(VI) reduction at 1, 2 g L^{-1} of yeast extract was due to the non availability of the free amino acids which also acts as electron donors for the Cr(VI) reduction. Higher amount of yeast extract (exceeding 5 g L^{-1}) inhibited the uptake of the glucose. Experiments were conducted to study the Cr(VI) reduction with other complex nutrients such as beef extract and liver extracts. The percentage reduction of Cr(VI) obtained was not significant with beef extract (23.1% of Cr(VI) reduction at an initial Cr(VI) concentration of 10 mg L^{-1}) and liver extracts (18. 61% of Cr(VI) reduction at an initial Cr(VI) concentration of 10 mg L^{-1}).

4.3.1.4 Effect of initial Cr(VI) concentration on the reduction of Cr(VI)

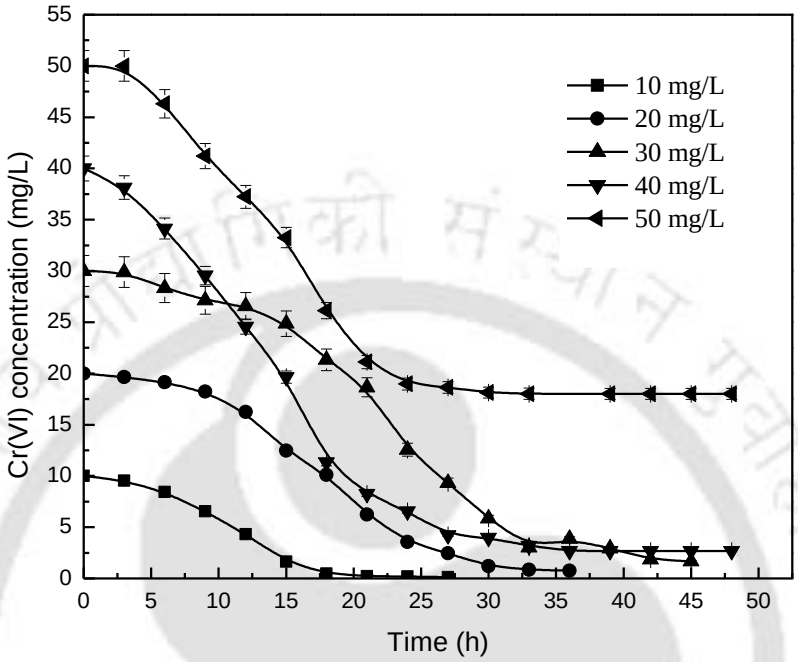

Fig. 4.4. Initial metal ion concentration on Cr(VI) reduction at pH 7 and temperature 37 ºC, initial Cr(VI) concentration 40 mg L^{-1} and glucose concentration 1 g L^{-1}

The effect of initial Cr(VI) ions on the reduction process was evaluated. The concentration of the Cr(VI) was varied between 10 to 50 mg L^{-1} in the reduction media. It was observed that the Cr(VI) concentration decreased from initial concentration of 10, 20, 30 mg L^{-1} to near zero (Fig. 4.4).

The reduction obtained with an initial concentration of 50 mg L^{-1} of Cr(VI) was insignificant (61%) when compared with the other concentrations studied. 10 , 20, 30 and 40 mg L^{-1} of initial concentration of Cr(VI) in the media resulted in 98.6, 96.3, 94.4 and 93.3 % of Cr(VI) reduction respectively. The decrease reduction in the Cr(VI) concentration for an initial concentration beyond 40 mg L^{-1} was due to the toxicity of

the Cr(VI) on *Halomonas sp* at higher concentrations. It was observed that the organism was able to reduce Cr(VI) to near zero level for concentrations ranging up to 40 mg L^{-1} .

4.3.1.5 Effect of pH on the reduction of Cr(VI)

The pH of a solution is an important parameter for any wastewater treatment process. pH of the media affects solubility of Cr(VI) and the growth of the bacteria. Bioreduction of Cr(VI) was studied for a wide range of pH (1-7). The reduction of Cr(VI) was found to be dependent on the pH of the growth media. It was observed that the maximum reduction of 98.6 % was obtained with an initial concentration of 10 mg L^{-1} at pH 7 (Fig. 4.5). The decrease in the reduction on either side of this pH range was due to the fact that the pH affected the growth of the organism. The growth of the bacteria was affected at acidic pH as the organism was adapted to grow initially under pH 7. As the pH of the media was modified the growth was affected which had a significant effect on the metabolism and the Cr(VI) reduction by *Halomonas sp*. At acidic pH (1-4) the cells disintegrated resulting in the loss of their metabolic activity. pH 6 and 7 favored the growth and bioreduction of Cr(VI). At pH 6, 7, the cells were intact and showed resistance to an initial Cr(VI) concentration of 40 mg L^{-1}. The bacterial growth was slightly affected when the pH of the growth media was adjusted to pH 8. The cell growth was initially affected. The 88 % bioreduction obtained with pH 8 was due to fact that there was a drop in the pH of the media due to the depletion of glucose. This drop in pH favored the growth of the bacteria and bioreduction of Cr(VI).

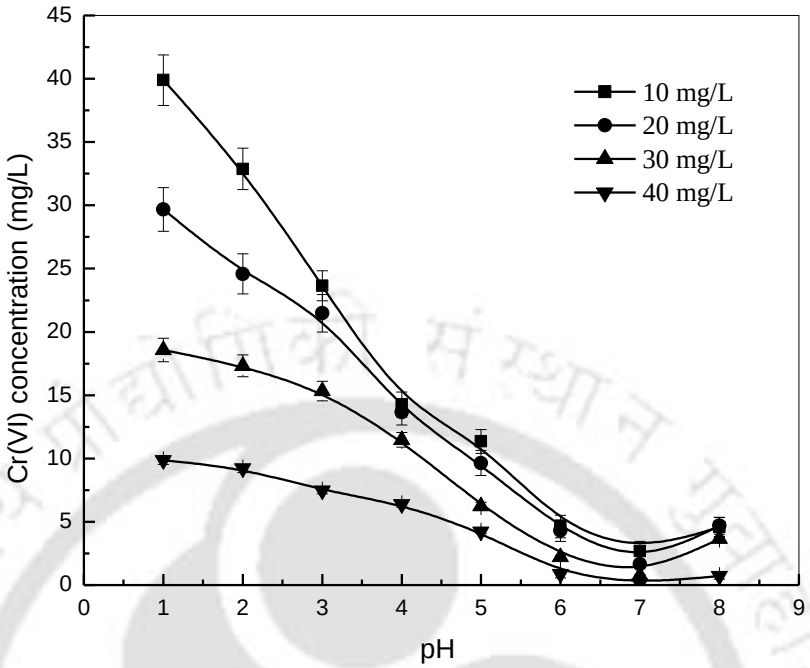

Fig. 4.5. Effect of pH on Cr(VI) reduction at temperature 37ºC, initial Cr(VI) concentration 40 mg L^{-1} and glucose concentration 1 g L^{-1}

4.3.1.6 Effect of time on Cr(VI) reduction

The simultaneous production of Cr(III) and Cr(VI) reduction by *Halomonas sp* was reported (Fig. 4.6). It was observed that the concentration of Cr(VI) dropped from an initial concentration of 40 mg L^{-1} to 2.68 mg L^{-1} during the time period studied. The concentration of Cr(III) increased with time. The maximum amount of Cr(III) produced was found to be 37.32 mg L^{-1}. The maximum reduction of Cr(VI) was obtained at 48 h. Fig. 4.6 clearly indicated that the Cr(VI) reduction was a time dependant process. There was a gradual increase in the concentration of the Cr(III) up to 33 h for an initial Cr(VI) concentration of 40 mg L^{-1} and thereafter it was observed to be constant.

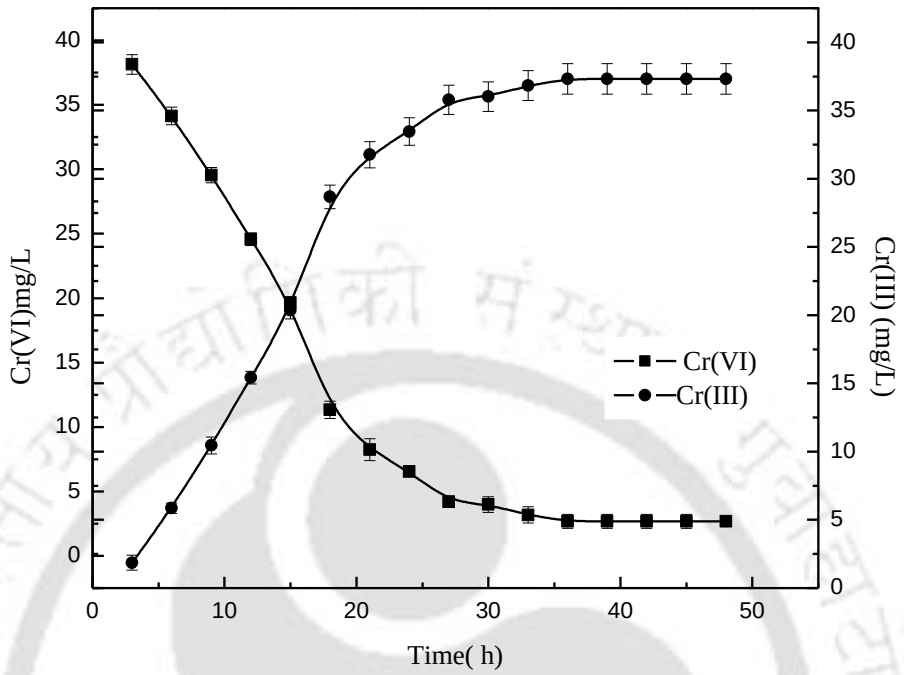

Fig. 4.6. Effect of time on Cr(VI) reduction at pH 7 and temperature 37 ºC, initial Cr(VI) concentration 40 mg L^{-1} and glucose concentration 1 g L^{-1}

4.3.1.7 Effect of different treatment methods on Cr(VI) reduction

In order to study the physiological state of the cells during the bioreduction of Cr(VI) the cells were subjected to different treatments like inactivation by heat, treatment with sodium azide (NaN_3), pentachlorophenol (PCP) and potassium cyanide (KCN). The cell free media was used as control for all these studies. It was observed that the maximum percentage of Cr(VI) reduction was obtained with untreated free cells (Fig 4.7). The free cells were metabolically active throughout the study yielded a maximum reduction of 93.3% Cr(VI) reduction.

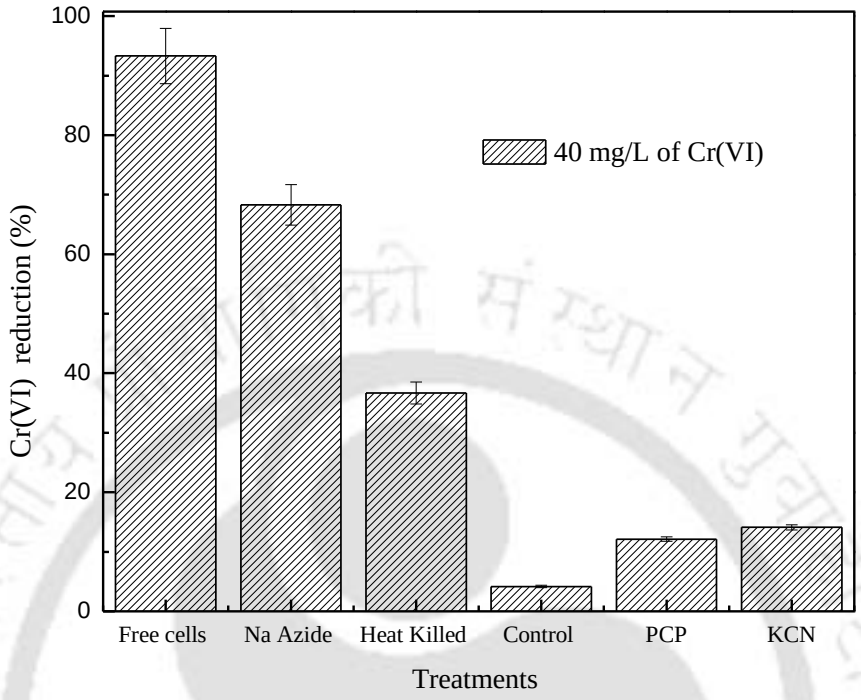

Fig. 4.7. Effect of different treatments on Cr(VI) reduction at pH 7 and temperature 37ºC, initial Cr(VI) concentration 40 mg L^{-1} and glucose concentration 1 g L^{-1}

The heat killed and the NaN_3 treated cells yielded 36.7 and 68.3% of Cr(VI) reduction respectively. PCP and KCN had a drastic effect on the Cr(VI) reduction. PCP and KCN in the growth media resulted in mere 12.14, 14.11 % reduction of Cr(VI). The decrease in the reduction rate was due to the fact that treatment with heat, NaN_3, PCP and KCN affected the metabolism of the organism. NaN_3 is an inhibitor of respiratory system [6]. Addition of NaN_3 affects the respiratory chain and thereby reducing the production of free electrons. The percentage reduction with the heat inactivated, NaN_3, PCP, KCN treated cells were found to decrease significantly as the physiological status of the bacteria was affected. There was no significant reduction observed with the control

media. The results indicated that the metabolic activity of the cells are essential for the reduction of Cr(VI).

4.3.2. Studies on cell free extracts of *Halomonas sp*

Cell free extracts (CFE) are metabolites produced during the Cr(VI) reduction. CFE consists of a large amount of crude protein and are intercellular materials. CFE have a great potential in the reduction of the Cr(VI) at concentrations ranging up to 50 mg L^{-1}. CFE have an advantage over the free cells by reducing 40 mg L^{-1} Cr(VI) in a time interval of 6 h. The bioreduction capacity of the CFE was studied by varying the physical parameters such as temperature, pH and the initial Cr(VI) concentration. The effect of the inhibitors, metal ions and electron donors on Cr(VI) reduction by CFE was reported in this section.

4.3.2.1 Effect of temperature on the reduction of Cr(VI) by CFE

The effective temperature for reduction of Cr(VI) by CFE was investigated by varying the temperature ranging from 10 °C to 60 °C. Maximum reduction of 89.65 % was observed at 37 °C. Fig. 4.8 showed a rapid decrease in the percentage reduction on either side of 37 °C, which can be attributed to the fact that the enzymes active sites were affected by the temperature change. The reduction percentage was lowest at 10 °C and 60 °C. The optimal temperature for the CFE was 37 °C.

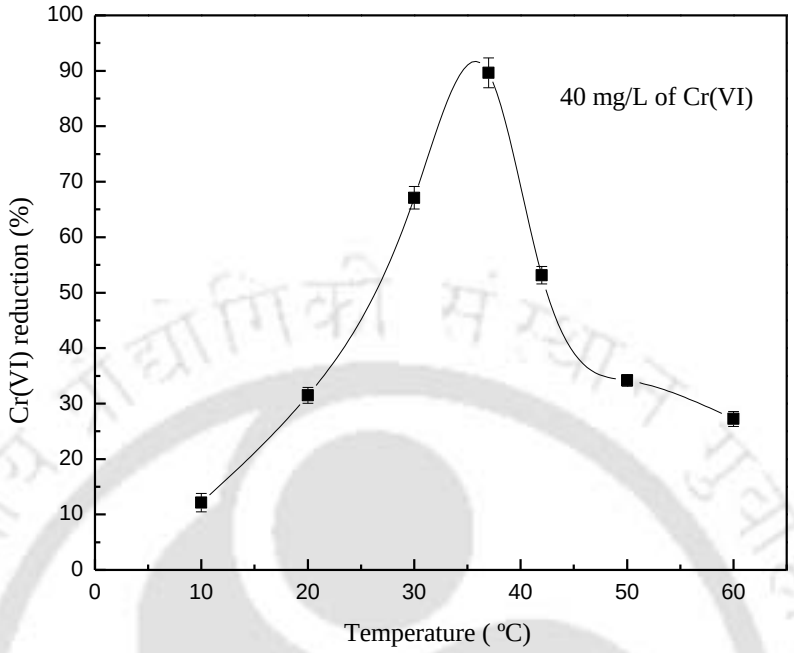

Fig. 4.8. Effect of temperature on Cr(VI) reduction by CFE at pH 6.5, initial Cr(VI) concentration 40 mg L^{-1} and glucose concentration 1 g L^{-1}

4.3.2.2 Effect of pH on the reduction of Cr(VI)

The optimal pH for the reduction of Cr(VI) was studied by varying the pH from 1 to 7. It was observed that the maximum reduction with CFE was obtained with the pH ranging between 6 to 7 (Fig. 4.9). A maximum of 89.65% of reduction was obtained at pH 6.5. The soluble enzymes showed no significant reduction when the pH was below 4. The decrease in the percentage reduction of Cr(VI) was due to loss of activity of the CFE at acidic pH. The change in the pH brings about conformational changes in the structure of the CFE [9]. The active sites responsible for the interaction with the Cr(VI) may be affected due to the conformational changes and percentage reduction of Cr(VI) was drastically affected.

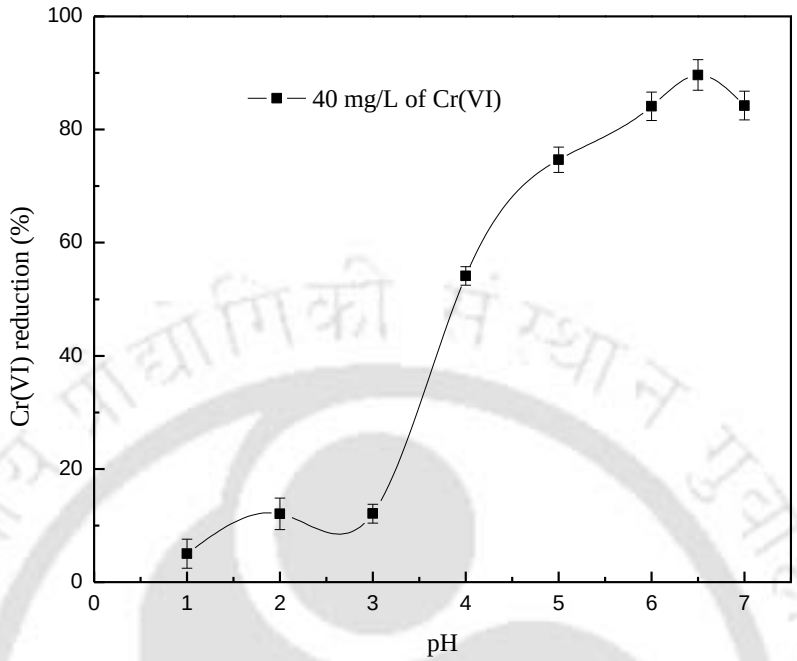

Fig. 4.9. Effect of pH on Cr(VI) reduction by CFE at 37 ºC, initial Cr(VI) concentration 40 mg L^{-1}

4.3.2.3. Effect of electron donors

Studies were conducted with cell free extracts to investigate the role of metabolites in the Cr(VI) reduction process. *Halomonas sp* can utilize a variety of electron donors for the reduction of Cr(VI). By selecting a suitable electron donor it was possible to develop a new microbial method for the reduction of Cr(VI). A maximum of nine different electron donors were tested for the ability to increase the reduction capacity of CFE. It was observed that NADH and NADPH were the suitable electron donors for the reduction of Cr(VI) with CFE of the *Halomonas sp* NADH and NADPH yielded 89.65% and 79.62 % of reduction respectively (Fig. 4.10). The other electrons such as citrate, lactose, glucose, fructose, acetate and succinate yielded 51.65, 52.25, 59.17, 58.82, 56.15 and 49.65% reduction of Cr(VI) respectively.

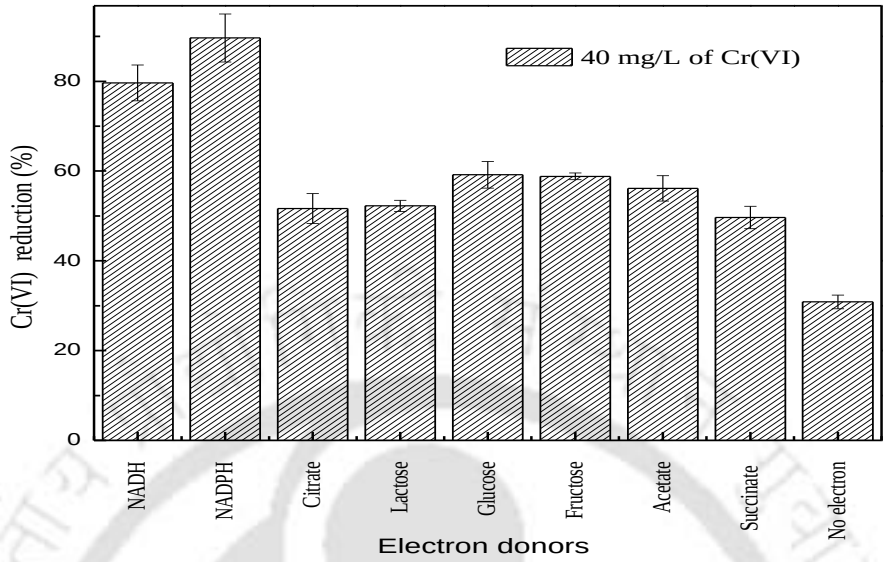

Fig. 4.10. Effect of different electron donors on Cr(VI) reduction by CFE at pH 6.5 and temperature 37 ºC, initial Cr(VI) concentration 40 mg L^{-1}

Hence NADH and NADPH were used as electron donors for the bioreduction studies using sonicate, supernatant of the sonicates [S_1(12000Xg), S_2 (24000Xg)] and membrane fractions (MF). Electron free CFE was used as control. It was observed that the presence of the NADPH in the CFE increased the percentage reduction of Cr(VI) by the sonicate, S_1, S_2 (Fig. 4.11). The sonicate reduced 40 mg L^{-1} to 8.14 mg L^{-1} in the presence of NADH as the electron donor. The uncentrifuged sonicate reduced 40 mg L^{-1} of Cr(VI) to 4.16 mg L^{-1} in the presence of NADPH (Fig. 4.11 a). It was found that the reduction of Cr(VI) obtained with S_1, S_2 are almost similar. S_1 reduced Cr(VI) from an initial concentration of 40 mg $L^{-1}$to 8.08 mg L^{-1} in the presence of NADH as the electron donor. NADPH increased the reduction as S_1 reported a reduction of Cr(VI) from 40 mg L^{-1} to 4.02 mg L^{-1}(Fig. 4.11 b). S_2 reduced Cr(VI) from an initial concentration of 40 mg L^{-1} to 2.81 mg L^{-1} in the presence of NADPH as electron donor, whereas in the presence of NADH as the electron donor S_2 reduced 40 mg L^{-1} to 6.14 mg L^{-1}(Fig. 4.11 c). It was observed that there is a gradual decrease in the Cr(VI) concentration for both the electron donors studied up to 4 h. After 4 h the reduction of Cr(VI) by CFE in the slowed and

reached a equilibrium value at 6h. The results indicated that the partially purified CFE yielded increased reduction. The reduction percentage obtained with the MF was negligible (Fig. 4.11 d).

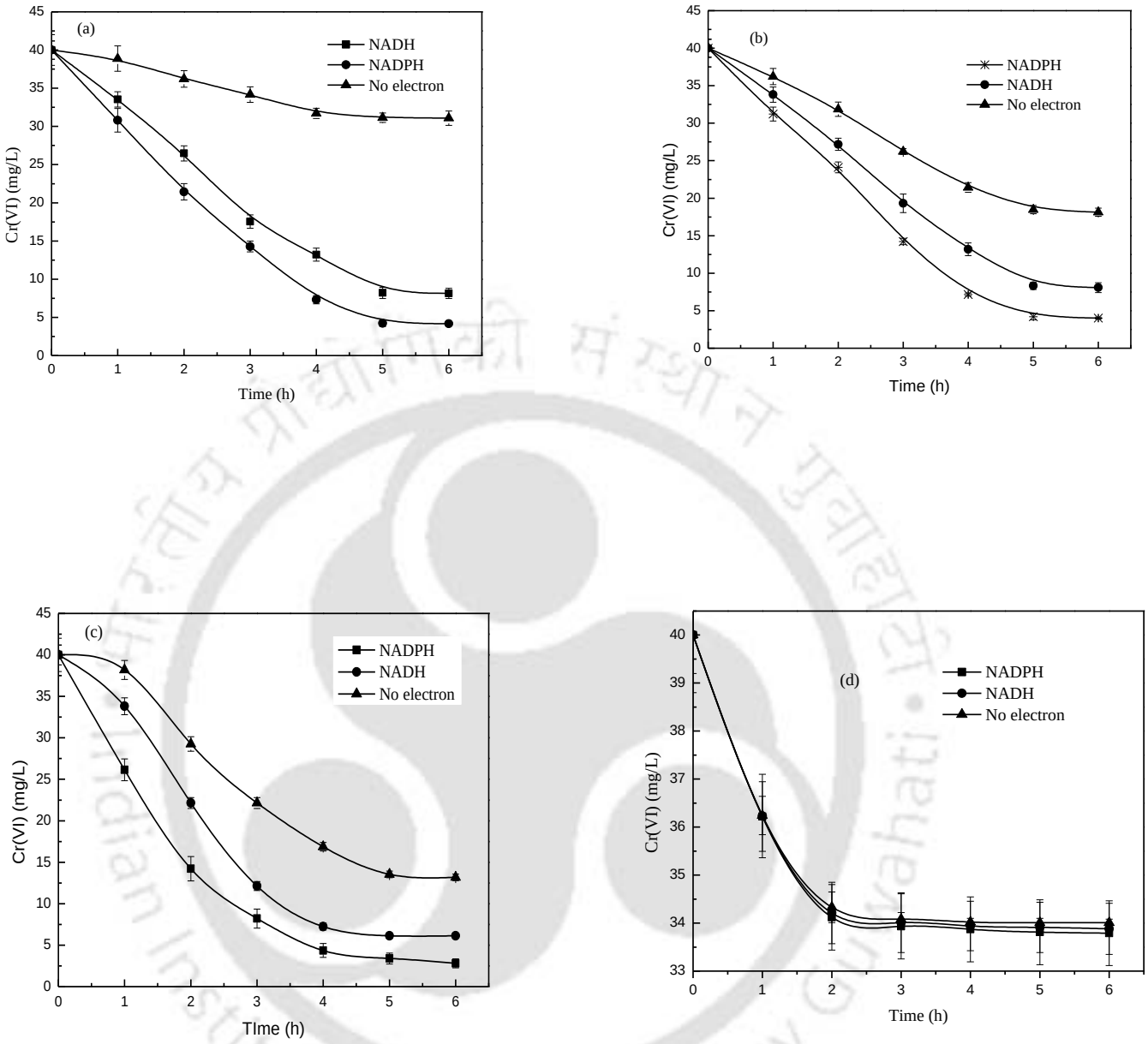

Fig. 4.11. Cr(VI) reduction by: (a) Sonicate in the presence of NADH and NADPH; (b) S_1(12000Xg) in the presence of NADH and NADPH (c) S_2 (24000Xg) in the presence of NADH and NADPH (d) Membrane fractions in the presence of NADH and NADPH

4.3.2.4. Effect of metal ions on CFE

The inhibitory effect of various metal ions on the CFE during the Cr(VI) reduction was studied. It was observed that the percentage reduction of Cr(VI) by CFE was drastically affected by the presence of other metal ions (Fig. 4.12). Cu had a lesser inhibitory effect as it yielded 63.14 % of reduction. Mg and Na resulted in 45.61 and 43.14 % respectively. Cd was the most toxic heavy metal as it yielded in 12.16 % of reduction. The results indicate that heavy metals cannot be used as electron donors for the CFE as they result in decreasing the activity of the soluble enzymes.

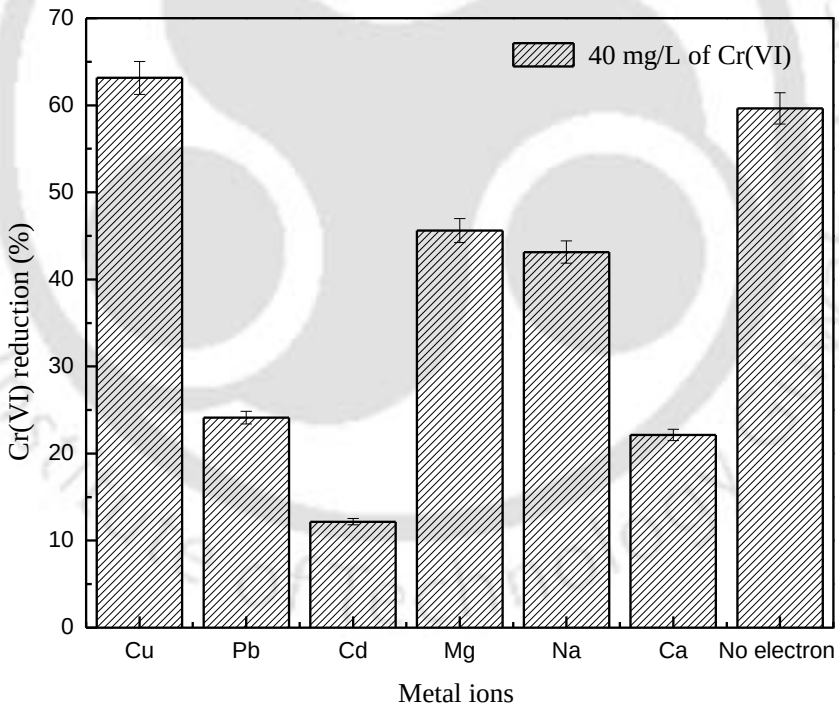

Fig. 4.12. Effect of metal ions on Cr(VI) reduction by CFE at pH 6.5 and temperature 37 ^{o}C, initial Cr(VI) concentration 40 mg L^{-1}

4.3.2.5 Effect of inhibitors on the CFE

The catalytic activity of the CFE was investigated by treatment with metabolic inhibitors like KCN, azide, pentachlorophenol and EDTA. Untreated CFE was used as the control. The metabolic inhibitors compete for the active sites in the CFE. A drastic drop in the percentage reduction of Cr(VI) was observed when metabolic inhibitors were used with CFE. CFE in the presence of EDTA reported lowest percentage reduction (8.22%). Azide, KCN, PCP reported 26.11, 8.66, 9.14 % reduction respectively (Fig. 4.13).

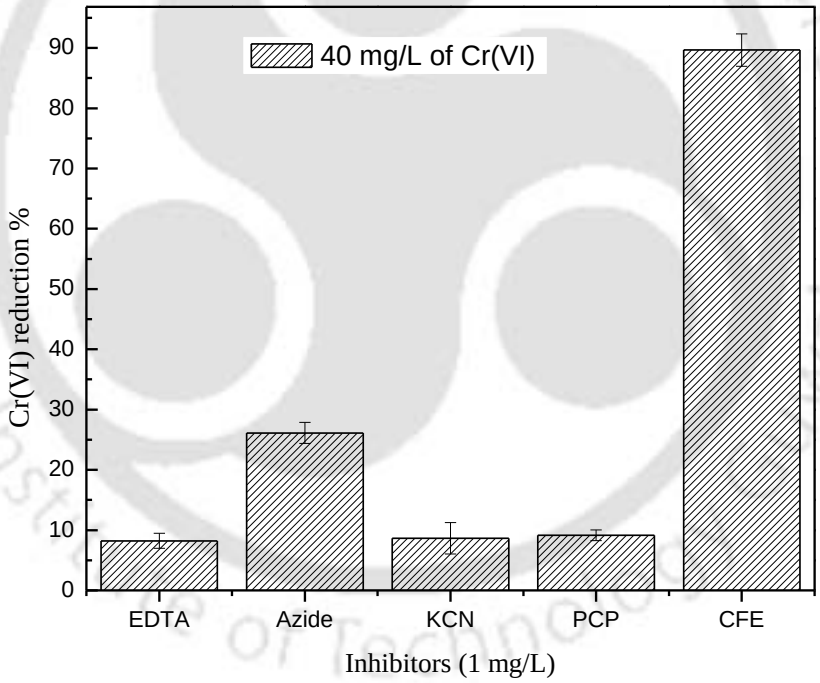

Fig. 4.13. Effect of inhibitors on Cr(VI) reduction by CFE at pH 6.5 and temperature 37 ºC, Initial Cr(VI) concentration 40 mg L^{-1}

The reason behind the drop in reduction was due to competitive inhibition between the inhibitors and the metals for active sites which results in drop of percentage reduction. The results clearly indicated that the reduction of the Cr(VI) by CFE was drastically affected by the presence of inhibitors. Reduction of Cr(VI) was by solely by soluble enzymes and no other factors were involved.

4.4. Conclusion

Investigation on Cr(VI) reduction was performed using *Halomonas sp*. A modified media (YEPG-NaCl) for the growth and reduction of the Cr(VI) was designed. Initial Cr(VI) concentration of 10 , 20 and 30 mg L^{-1} of initial concentration of Cr(VI) in the media resulted in 98.6, 96.3, 94.4 % of Cr(VI) reduction respectively. The reduction capacity of the culture was found to be 93.3% for an initial concentration of 40 mg L^{-1}. It was observed that yeast extract at 5 g L^{-1} was essential for reduction process. NaCl at 20 g L^{-1} did not inhibit the growth of the halophillic bacteria. pH 7 was found to be optimal for the reduction of Cr(VI) with the free cells of *Halomonas sp*. Sodium azide, KCN, PCP and heat treatment decreased the percentage reduction of Cr(VI). The CFE was effective in reducing 40 mg L^{-1} of Cr(VI) and yielded 89.65% in 6 h. NADPH, NADH were the suitable electron donors for the CFE during bioreduction of Cr(VI). Addition of other metals and biochemical inhibitors affected the activity of CFE and resulted in decreased reduction of Cr(VI). pH 6.5 was optimal for the CFE at 37°C. The results obtained from the present study signifies the potential of *Halomonas sp* in the treatment of lower concentration of Cr(VI) in water and wastewater. The cell free extracts were capable of reducing Cr(VI) in less than 6 h of time. The free cells of *Halomonas sp* and the CFE obtained from the *Halomonas sp* was able to reduce Cr(VI) at an initial concentration of 40 mg L^{-1}. It was evident that the free cells of *Halomonas sp* and its CFE can be used for the reduction of lower concentration Cr(VI) in an eco-friendly manner.

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Chapter V

Bioreduction of Cr(VI) by Immobilized Cells of *Halomonas sp*

5.1. Introduction

Immobilization of whole cells provides a wide range of advantage, easy to regenerate, immobilized cells are more stable and have the ability to withstand higher Cr(VI) load. It was observed from chapter (III) that immobilization increases stability of the organism.

The choice of the immobilization matrix is an important factor in the application of whole cell immobilization. The polymer matrix determines the mechanical and chemical resistance of the microbial cells towards Cr(VI) [1]. Alginates, agar, poly acrylamide are the commonly used matrices for the immobilization of the microbial cells [2, 3]. Biopolymers are non toxic, selective, efficient and inexpensive [4]. Among the different immobilization methods employed, entrapment of the microbial cells in a polymer matrix is the common method. The aim of the present study was to investigate the Cr(VI) bioreduction capacity of the immobilized *Halomonas sp*. Various parameters such as the stability of the matrix, the biomass loading rate, pH and reusability of the different matrices were studied.

5.2. Materials and Methods

5.2.1 Microorganism and culture conditions

Halomonas sp was obtained from the Institute of Microbial Technology, Chandigarh, India and cultivated at 37 ºC in a liquid medium at pH 6 with agitation of 180 rpm on an incubator shaker (Daihan LabTech Co Ltd, Model LSI 3016-R). The

culture was maintained on YEPG- agar media. Subcultures were made on every 15 days of duration and stored at 4 ºC.

5.2.2 Media

The growth media for bacteria consisted of peptone 5 g L^{-1}, yeast extract 5 g L^{-1}, NaH_2PO_4. The medium for the Cr(VI) reduction consisted of yeast extract 5 g L^{-1}, glucose 1g L^{-1}, K_2HPO_4 0.03 g L^{-1}, KH_2PO_4 0.03 g L^{-1}, $MgSO_4$ 0.01 g L^{-1} and NaCl 0.01 g L^{-1}. The pH of the media was adjusted to 7±0.1 with 0.1 N HCl and/or 0.1 N NaOH. The media was autoclaved at 120 °C for 15 min.

5.2.3 Preparation of stock solution

Bioreduction of Cr(VI) was studied in an aqueous solution using immobilized cells of *Halomonas sp*. The Cr(VI) stock solution was prepared by dissolving 2.82 g of $K_2Cr_2O_7$ in 1000 mL of deionized water. The bioreduction media was prepared by diluting the appropriate quantity of Cr(VI) stock solution in the growth media.

5.2.4 Immobilization methods

Halomonas sp was entrapped in various matrices such as Ca alginate, polyacryl amide and agar. The details of preparation of these immobilized cells are already given in section 3.2.3 of Chapter (III).

5.2.5 Bioreduction studies

The immobilized beads containing the *Halomonas sp* were first transferred to the growth media of 100 mL in a 250 mL flask for activation and incubated in an orbital shaker (Daihan LabTech Co Ltd, Model LSI 3016-R) at 37 ºC for 52 h. The growth was periodically monitored using a microscope (Zeiss Axiostar, Germany). The metabolically active cultures of the immobilized *Halomonas sp* was transferred from growth media to

the bioreduction media containing varying concentration of Cr(VI) ranging from 10 to 40 mg L^{-1} of Cr(VI). For all the experiments carried out with immobilized *Halomonas sp*, bacteria free matrices were used as control to study the effect of the matrices on bioreduction. The effect of media pH, temperature and initial Cr concentration on the bioreduction were also studied. The reusability of the matrices was also studied.

5.2.6 Analytical procedure

After the bioreduction, the supernatants were collected by centrifugation (Remi, R-24) and analyzed for total chromium concentration using Atomic Absorption Spectrophotometer (Varian, AA140). The Cr(VI) concentration was measured spectrophotmetrically (Perkin Elmer, Model Lambda 35) at 540 nm by complexation with 1,5 diphenyl carbazide.

5.3 Results and Discussion

The substrate utilization of the bacteria during the bioreduction process, effect of temperature and pH on the bioreduction of Cr with immobilized *Halomonas sp* was studied and the results are presented in this section. Various matrices for immobilization like Ca alginate, acryl amide and agar were investigated. The best suitable matrix with high mechanical stability and chemical resistance was optimized. The reusability of the beads was also reported.

5.3.1 Utilization of glucose during bioreduction of Cr(VI)

Glucose was tested as the sole carbon source for the growth and reduction of Cr(VI) by *Halomonas sp*. 1 g L^{-1} of glucose was added to the bioreduction media and concentration of glucose was analyzed periodically using dinitro salilic acid (DNS) method. The glucose utilization profile of the *Halomonas sp* immobilized in Ca alginate was presented in Fig. 5.1. It was observed that the glucose was completely utilized for all

the initial concentration of Cr(VI) studied. This was a clear indication that the bacteria were metabolically active throughout the study. Glucose supplemented the energy required for the metabolic activity. The decrease in glucose consumption rate for concentration exceeding 30 mg L^{-1} was due to the inhibitory effect of the Cr(VI) present in the media. The control cultures completely utilized the glucose in less than 24 h of time, the presence of Cr(VI) affected the metabolic activity thereby increasing the time required for the metabolic activity of the organism.

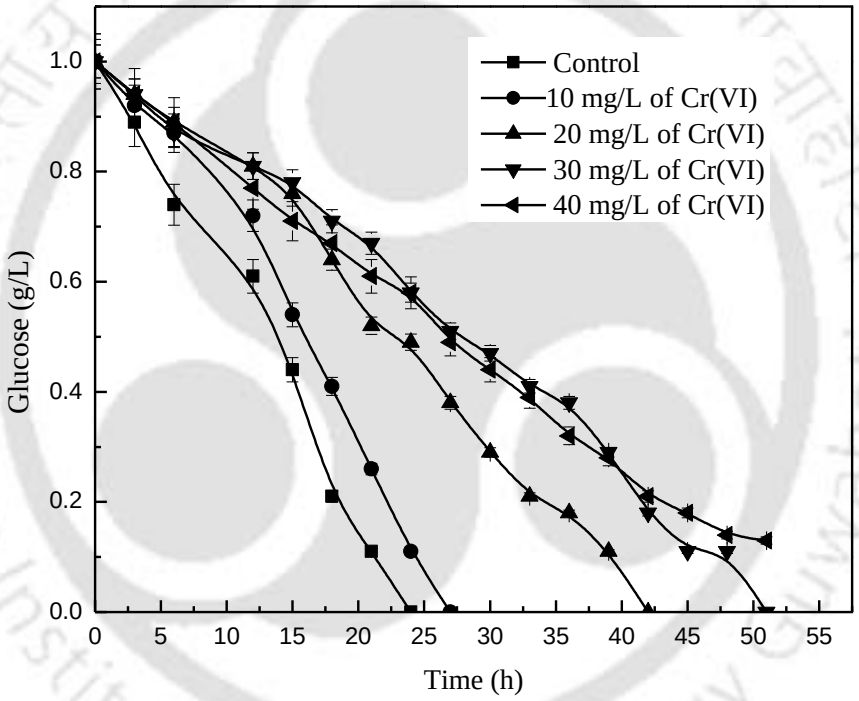

Fig. 5.1. Glucose consumption profile of immobilized *Halomonas sp*

5.3.2 Effect of pH on bioreduction

The solubility and the ionization state of the Cr(VI) was influenced by the pH of the bioreduction media. The bioreduction was studied at various pH ranging from 2 to 7.

Maximum bioreduction was obtained with pH 6. A maximum of 96.66, 98.99, 91.34, 74.16, 21.66, 18.21, 2 % of bioreduction were reported for pH 7, 6, 5, 4, 3, 2, 1 respectively with an initial Cr(VI) concentration of 10 mg L^{-1} (Fig. 5.2). The increase in bioreduction from pH 4 indicates the physico-chemical interaction between the metal ions and the bacteria. As the pH was increased further it favored the growth of the organism and thereby the bioreduction capacity increased. It was found that pH 6 was the optimum pH for the bioreduction using immobilized beads.

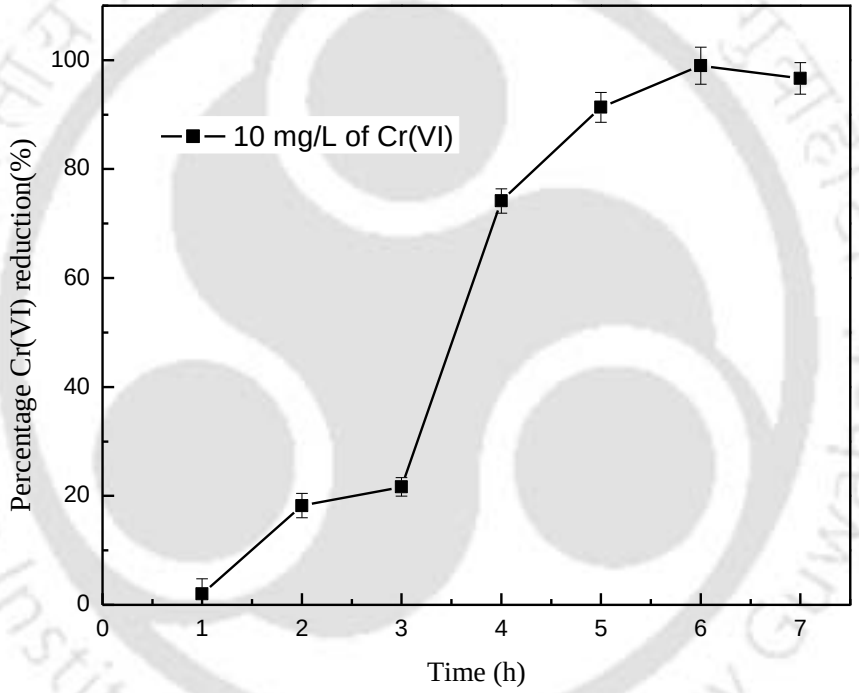

Fig. 5.2. Effect of pH on Cr(VI) bioreduction

5.3.3 Cr(VI) reduction by alginate immobilized cell

The Cr(VI) bioreduction by alginate immobilized cells of *Halomonas sp* was reported in Fig 5.3. It was observed that the efficiency of the immobilized cells in the

bioreduction Cr(VI) decreased with the increase in the initial Cr(VI) concentration. It was also observed from the figure that the time taken for the bioreduction also increased with the increase in the initial Cr(VI) concentration. This can be attributed to the fact that the presence of higher concentration of Cr(VI) increased the lag phase of the bacteria. Alginate immobilized cells were able to reduce Cr(VI) completely for an initial Cr(VI) concentration of 10 mg L^{-1} 28 h (Fig. 5.3 a). It was observed that the Cr(VI) in the range of 20 and 30 mg L^{-1} were reduced to 2.02 and 3.16 mg L^{-1} respectively within 48 h (Fig. 5.3 c and Fig. 5.3 d). Ca alginate immobilized *Halomonas sp* reported a minimum of 45.30% bioreduction for an initial Cr(VI) concentration of 40 mg L^{-1} (Fig. 5.3 d). As discussed earlier the increase in the Cr(VI) affected the metabolic activity of the organism thereby reducing the bioreduction percentage of Cr(VI). The decrease in the bioreduction capacity can also be attributed to the fact, that the mechanical stability of the beads was affected by the presence of the borate and nitrate.

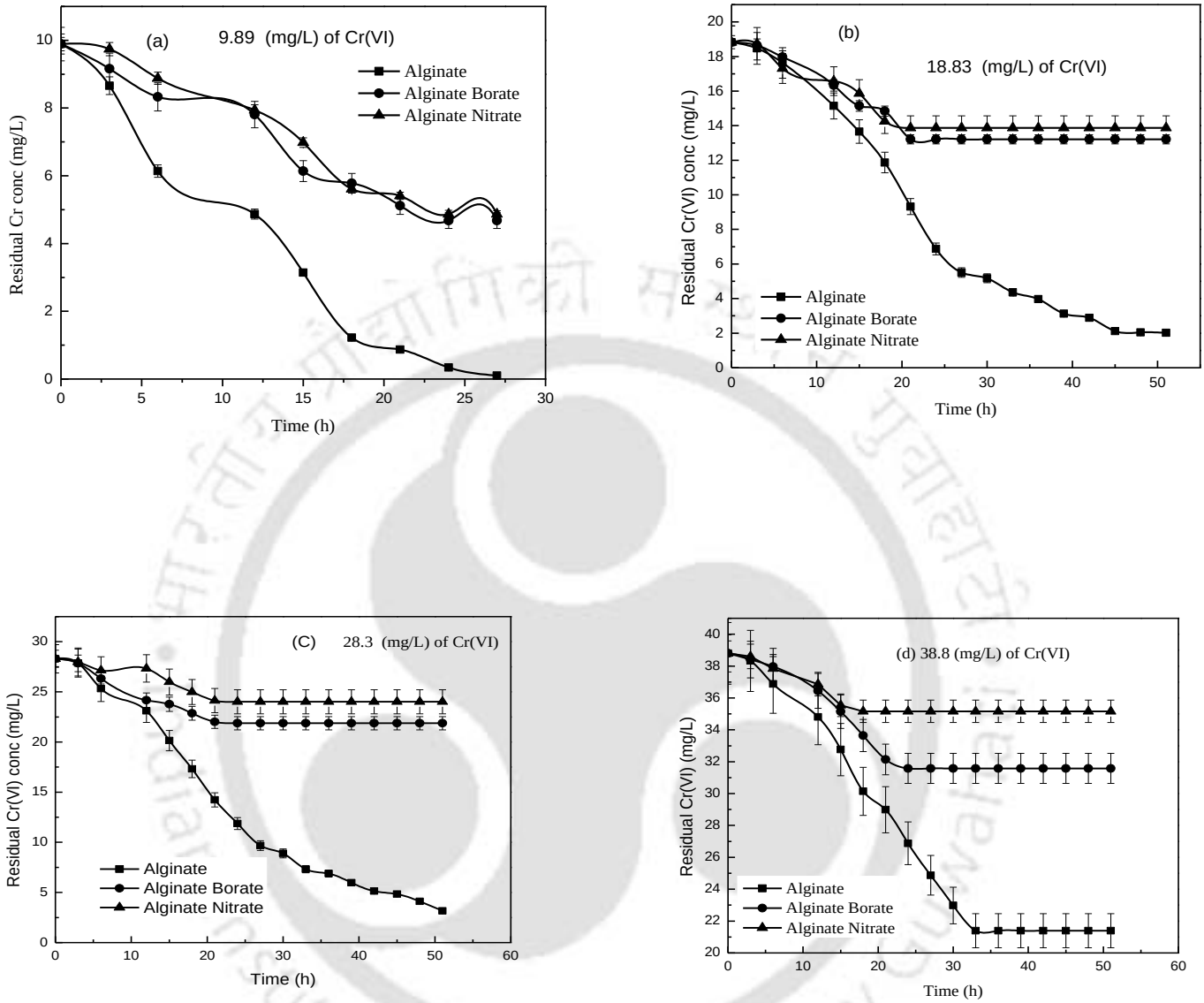

Fig. 5.3. Cr(VI) reduction by *Halomonas* immobilized in Alginate beads : (a) Initial Cr(VI) concentration 10 mg/L; (b) Initial Cr(VI) concentration 20 mg/L; (C) Initial Cr(VI) concentration 30 mg/L; Initial Cr(VI) concentration 40 mg/L.

5.3.4 Effect of cell concentration

The cell concentration in the immobilization matrix was varied from 0.05% (w/v) to 2.5 % (w/v). Fig. 5.4 clearly indicated the increase in the bioreduction capacity of the *Halomonas sp* immobilized in alginate beads. The increase in bioreduction with increase in cell concentration was due to the fact that the bioreduction was dependant on the growth and metabolic activity of the organism. The number of viable colonies increased with increasing inoculums dosage. A maximum of 98.9 % of bioreduction was obtained with an initial Cr concentration of 10 mg L^{-1} for a cell concentration of 0.2 % (w/v). The bioreduction obtained reached a constant value for inoculums dosage exceeding 0.2 % (w/v).

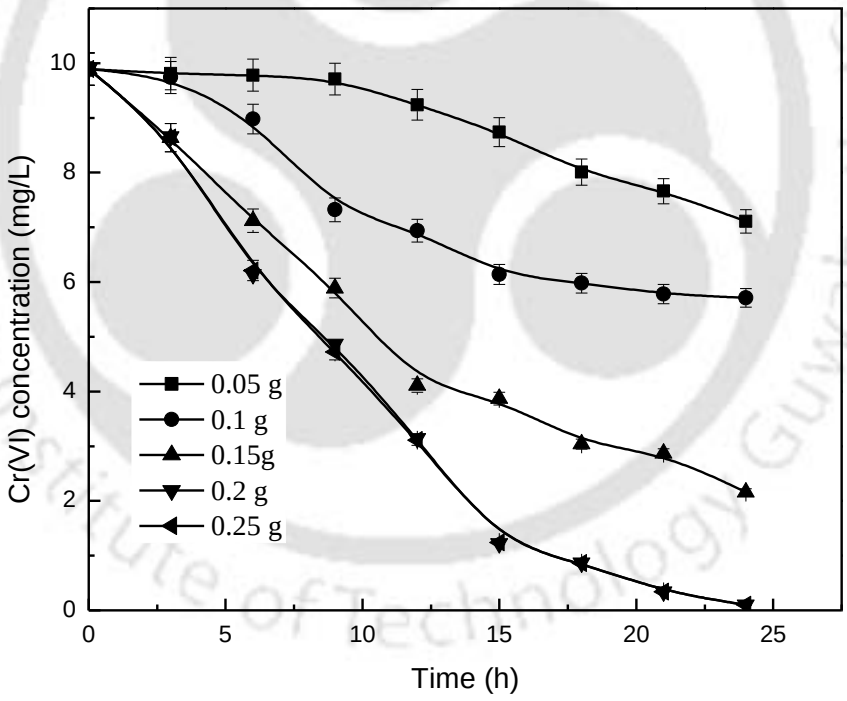

Fig. 5.4. Effect of cell concentration on bioreduction of Cr(VI)

5.3.5 Screening of different matrices

A maximum of 98.9% reduction of Cr(VI) was reported with alginate matrix for an initial Cr(VI) concentration of 10 mg L^{-1}. The acryl amide and agar matrices yielded a Cr(VI) reduction of 94.14 and 91.2 % of Cr(VI) bioreduction respectively. The acryl amide matrix was found to disintegrate as the study was continued for a period of 52 h. The agar matrix was found to be soluble in the media causing difficulty in analyzing the Cr(VI) concentration. Table 5.1 showed the percentage reduction of Cr(VI) obtained for different matrices studied with different initial concentrations of Cr(VI) ranging from 10 mg L^{-1} to 40 mg L^{-1}. Addition of borate and nitrate decreased the bioreduction capacity with the alginate. The mechanical stability of the beads was an important parameter in the selection of the matrix. It was observed that the alginate beads disintegrated when nitrate and borate was added to the matrix. Similar results were reported by various earlier researchers [2, 6, 7].

Table 5.1. Screening of various matrices

Immobilization matrix	Initial (0 h)	Residual (24 h)	Reduction (%)	Bead integrity
Calcium alginate	40	21.4	45.30	Retained
Agarose	40	31.6	17.70	Disintegrated
Agar	40	33.2	13.54	Disintegrated
Calcium alginate	30	3.16	88.83	Retained
Agarose	30	8.14	71.24	Disintegrated
Agar	30	8.84	68.76	Disintegrated
Calcium alginate	20	2.04	89.17	Retained

Agarose	20	2.87	84.76	Disintegrated
Agar	20	3.14	83.32	Disintegrated
Calcium alginate	10	0.1	98.99	Retained
Agarose	10	0.58	94.14	Disintegrated
Agar	10	0.87	91.20	Disintegrated
Alginate borate	10	4.68	53.11	Disintegrated
Alginate nitrate	10	4.87	51.20	Disintegrated

5.3.6 Cr(VI) reduction in the presence of other metals

Cr(VI) reduction of the alginate immobilized *Halomonas sp* was studied in the presence of the other metals. Cd was more toxic to the organism as it drastically affected the bioreduction of Cr(VI). It was observed that only 12.59% of bioreduction was obtained for an initial Cr(VI) concentration of 10 mg L^{-1} in the presence of Cd. The presence of Mn, Pb and Cu also affected the bioreduction capacity of the immobilized *Halomonas sp* as depicted in Fig. 5.5.

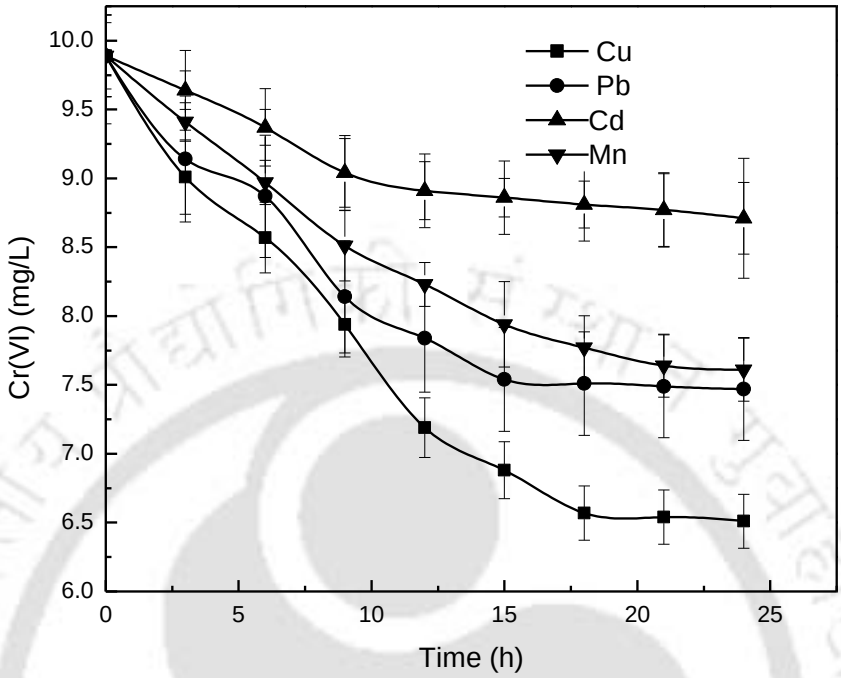

Fig. 5.5. Effect of other metals on bioreduction of Cr(VI) by *Halomonas sp*

5.3.7 Effect of initial Cr(VI) on bioreduction

Effect of the initial Cr(VI) on the bioreduction was reported in Fig. 5.6 . It was observed that the bioreduction time prolonged as the initial Cr(VI) concentration was increased from 10 mg L^{-1} to 40 mg L^{-1}(Fig 5.6). Increase in Cr(VI) concentration decreased the percentage reduction. A maximum of 98.9% was reported for an initial Cr(VI) concentration of 10 mg L^{-1}. A minimum of 45.30 % of Cr(VI) was reported for an initial Cr(VI) concentration of 40 mg L^{-1}, It can be attributed to the fact that the Cr(VI) at concentration exceeding 20 mg L^{-1} had an inhibitory effect on the growth.

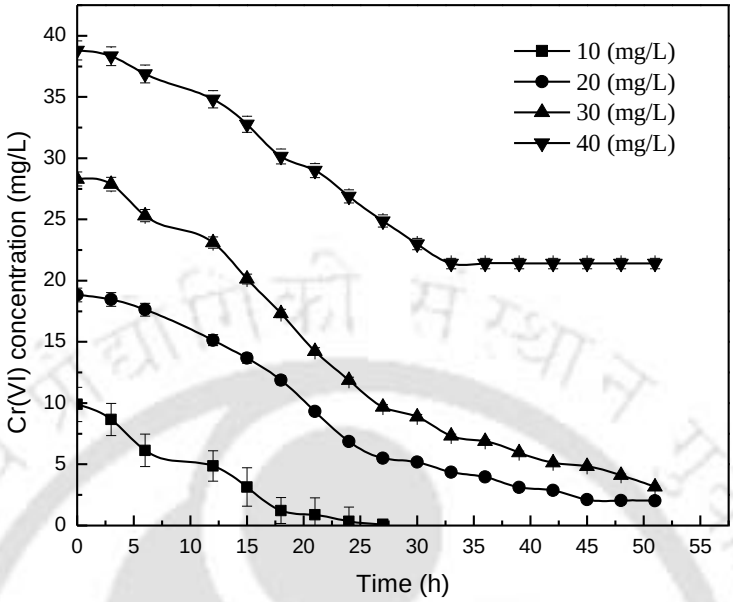

Fig. 5.6. Effect of initial Cr(VI) on bioreduction

5.3.8 Bioreduction profile

The bioreduction of Cr(VI) was studied for 27 h. It was observed that the bioreduction of the Cr increased with time. A maximum of bioreduction 98.9% of Cr(VI) was reported at 27 h for an initial concentration of 10 mg L^{-1} with *Halomonas sp* entrapped in Ca alginate (Fig. 5.7). The Cr(VI) reduction profile clearly indicated the bioreduction capacity of the Ca alginate immobilized cells of *Halomonas sp.* It was observed that the concentration of the Cr(III) increased with time. The simultaneous reduction of Cr(VI) and production of Cr(III) is a clear indication of the metabolic activity of the live and active immobilized cells. Fig. 5.7 Clearly showed that the increase in the Cr(III) concentration was proportionate to the decrease in the Cr(VI) concentration.

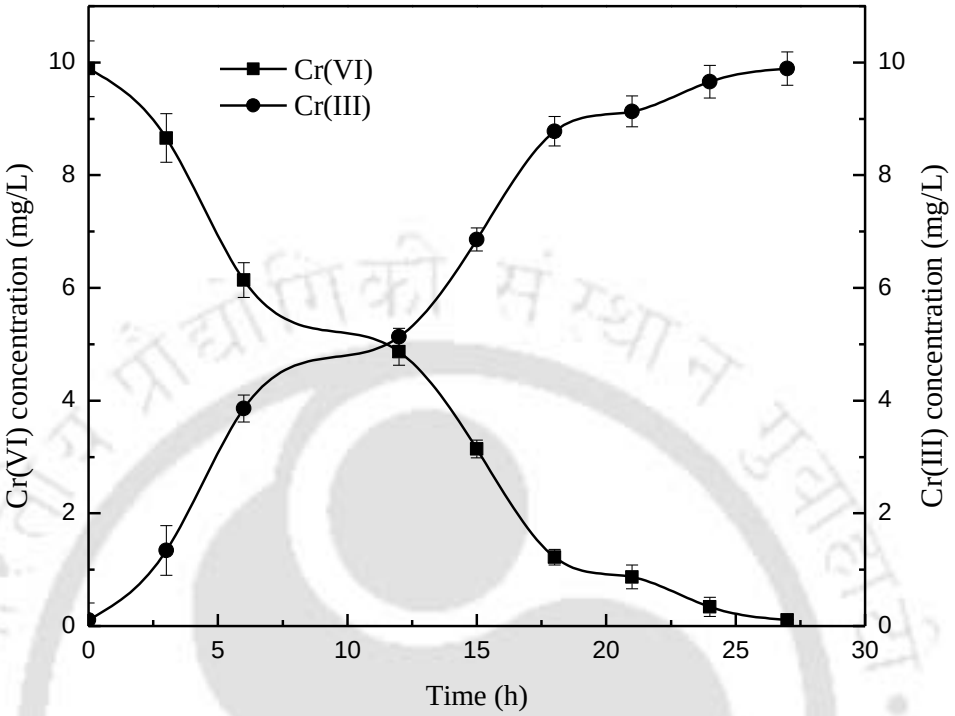

Fig. 5.7. Cr(VI) bioreduction profile

5.3.9 Reusability

Ca alginate immobilized cells can be reused for 5 times. The percentage reduction with the number of reuses was found to decrease. It was observed that a maximum of 98.99 % of bioreduction was obtained with an initial concentration of 10 mg L^{-1}. The percentage reduction dropped to 88.64 in the second use (Fig. 5.8). The mechanical strength of the beads were completely affected with the reuse. The number of viable colonies also decreased with the number of uses.

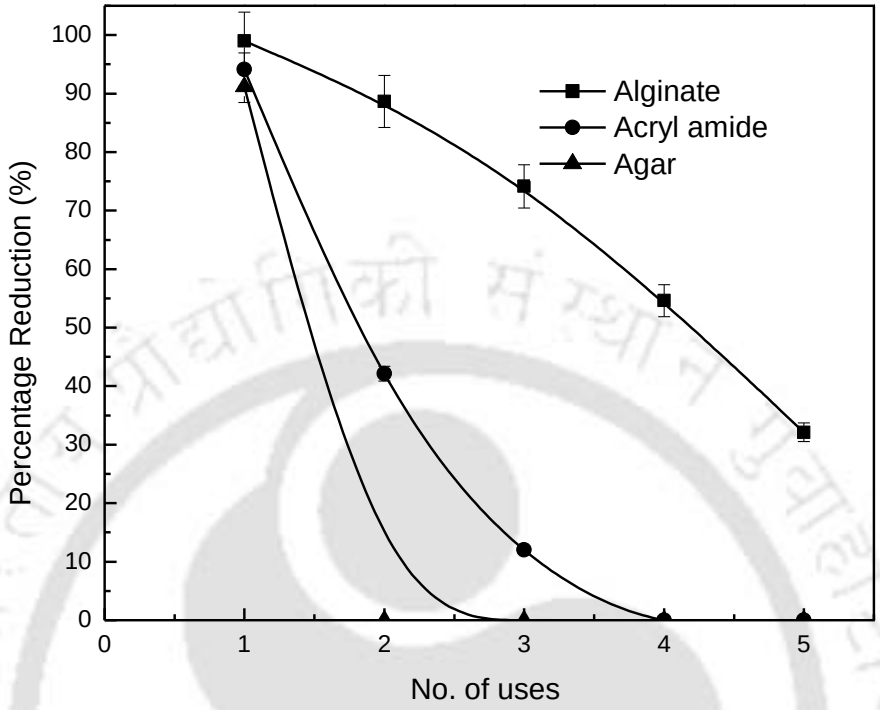

Fig. 5.8. Reusability of beads on Cr(VI) bioreduction

5.4. Conclusion

Bioreduction of Cr(VI) using the immobilized *Halomonas sp* was dependant on the pH, initial Cr(VI) concentration and metabolic activity of the organism. A maximum of 98.9 % of bioreduction was reported for 10 mg L^{-1} of initial Cr(VI) concentration. Results indicated that the stability of the beads was necessary for significant reduction of Cr(VI). The optimum reduction was observed at pH 6. Ca alginate was found to be the best suitable matrix for the immobilization of the bacteria. Cd was found to be more toxic for the bioreduction of Cr(VI) by *Halomonas sp*. Ca alginate immobilized cells of *Halomonas sp* can be effectively used for the treatment of water bodies containing lower concentration Cr(VI), which cannot be treated by the chemical methods. The immobilization provides advantage as the separation of the cells is easier and the cells can be reused.

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Chapter VI

Isolation, Identification and Characterization of Cr(VI) Reducing *Bacillus cereus* from Chromium Contaminated Soil

6.1. Introduction

Bacteria found in chromium contaminated sites have been reported to reduce Cr(VI) to less toxic Cr(III) [1-3]. In general bacteria protect themselves from the toxic components by transforming the toxic components through reduction, oxidation and precipitation [2]. Bacteria can reduce relatively low concentrations of Cr(VI) which cannot be treated by the conventional methods. The chemical method used currently for the reduction of Cr(VI) requires lowering the pH of waste stream to 2 or 3 [3]. In the other hand biological reduction of Cr(VI) can be achieved at near neutral pH [4]. The microbes in the chromium contaminated sites produce soluble reductase as a result of cometabolism [5]. Enzymatic reduction of Cr(VI) to Cr(III) is one of the defense mechanisms of microorganisms to protect themselves from the toxicity of Cr(VI). The enzymes produced by bacteria mediate the transfer of electrons from NADH to chromate [6]. Earlier studies on the reduction of Cr(VI) have reported NADH and NADPH as the suitable electron donors [7,8]. Chromium bioreduction capacity of the bacteria was dependant on the availability of the substrate. Glucose was reported as the suitable substrate for the growth as well as bioreduction of Cr(VI) by bacteria [8-12].

The objectives of the present work was to isolate Cr(VI) reducing strains from chromium contaminated soil and to determine the optimum condition for the reduction of Cr(VI). The role of electron donors, cell density and initial Cr(VI) concentration on Cr(VI) reduction was also evaluated. The Cr(VI) reduction of cell free extracts of the

new isolate was also studied. The role of electron donors on the reduction of Cr(VI) was also investigated.

6.2. Materials and Methods

6.2.1 Growth media

The general growth media for bacteria consisted of peptone 5 g L^{-1}, yeast extract 5 g L^{-1}, NaH_2PO_4 5 g L^{-1}. The bioreduction media consisted of peptone 5 g L^{-1}, yeast extract 5 g L^{-1}, glucose 1g L^{-1}, $MgSO_4$ 0.01 g L^{-1}, NH_4Cl 0.03 g L^{-1}, K_2HPO_4 0.03 g L^{-1}, and KH_2PO_4 0.03 g L^{-1}. The pH of the media was adjusted to 7±0.1 with 0.1 N HCl and/or 0.1 N NaOH. All the media were autoclaved at 120 °C for 15 min. Seven different substrates such as acetate, citrate, lactate, oxalate, succinate, glycerol and tryptone were used as replacements for glucose in bioreduction media.

6.2.2 Isolation and culture conditions

Bacterial strain was isolated from soil sample collected from Vellore, TamilNadu, India where tannery wastes are discharged. 1 g of the soil was added to 100 mL of growth media and was incubated for 48 h at 37 ºC at 180 rpm in a rotary shaker. After 36 h when significant growth was obtained, one loopful of the grown culture was transferred to media spiked with 10 mg L^{-1} of Cr(VI) and incubated till significant growth was observed. This process was continued up to a final concentration of 60 mg L^{-1} of Cr(VI). When significant growth was observed in the presence of 60 mg L^{-1} of Cr(VI), serial dilution of the culture was done and culture was streaked on agar plates and incubated at 37 ºC for 48 h. The culture was stored at 4 ºC for further use.

6.2.3 Characterization and identification of strain

The isolated culture was sent to DRL Tezpur, India for biochemical characterization and 16S rDNA sequence determination. The sequence results were submitted to Gene bank data base to sort out the similarity match for nucleotides using an online BLAST tool (http://www.ncbi.nlm.nih.gov). The thermodynamic properties of the isolated culture were calculated using an online tool BIOTOOL (http://www.unc.edu/~cail/biotool/oligo/index.html) [13].

6.2.4 Cr(VI) stock solution

Bioreduction of Cr(VI) was studied in an aqueous medium. Cr(VI) stock solution of 1000 mg L^{-1} was prepared by dissolving 2.82 g of $K_2Cr_2O_7$ in 1000 mL deionized water. The Cr(VI) spiked media was prepared by diluting the stock solution in growth media to obtain the desired concentrations.

6.2.5 Bioreduction experiments

The bioreduction experiments were carried out in 250 mL conical flasks containing 100 mL of Cr(VI) spiked medium for 48 h. The experiments on optimizing the pH of the bioreduction process were performed at 37 ºC with an initial glucose concentration of 1g L^{-1}. The media for the bioreduction process was prepared for a wide range of pH ranging from 3 to 8. The solution pH was measured using a pH meter (Sartorius AG 37070 Goettingen, Germany). The optimum concentration of glucose required for the growth and bioreduction of Cr(VI) was studied by varying the glucose concentration in the media from 0 to 2000 mg L^{-1} whereas the pH and temperature were kept constant at 6 and 37 ºC respectively. Samples were withdrawn at regular intervals (3 h) and centrifuged at 12000 X g and the corresponding biomass concentration was reported as OD_{600}. The concentration of Cr(VI) was measured using an UV spectrophotometer (Spectrascan UV 2700, ThermoFisher, Germany). The standard method using diphenyl carbazide (DPC) at 540 nm was followed to determine the

concentration of Cr(VI). Interference of growth was eliminated using calibration curves. The concentration of total chromium was measured using an Atomic absorption spectrophotometer (Varian AA240 FS).

The role of initial Cr(VI) concentration on bioreduction of Cr(VI) was estimated by varying concentration of Cr(VI) from 10 mg L^{-1} to 70 mg L^{-1}. Glucose (1 g L^{-1}) was used as the sole carbon source, pH and the temperature were maintained constant at 6 and 37 ºC respectively. The flasks with the bioreduction media containing various concentration of Cr(VI) were inoculated with one loop of overnight grown isolated culture. The flasks were incubated in an incubator shaker (Daihan LabTech Co Ltd, Model LSI 3016–R) at 37 ºC and 180 rpm. Samples were withdrawn under sterile conditions at regular time intervals of 3 h and centrifuged at 12000 X g for 15 min and the supernatant was used for metal analysis.

The amount of yeast extract required for the bioreduction was studied by varying the yeast extract concentration between1 g L^{-1} to 10 g L^{-1} in the bioreduction media. Gluocse, pH and temperature were maintained at 1 g L^{-1}, 6, and 37 °C respectively.

The time required for the reduction of Cr(VI) to Cr(III) was performed at pH 6 and 37 °C. The bioreduction media was prepared with 1 g L^{-1} of glucose as carbon source and 60 mg L^{-1} of initial Cr(VI) was used. The experiments were conducted till an equilibrium concentration of Cr(III) was attained.

The role of various electron donors on their ability to enhance the Cr(VI) bioreduction capacity of the free cells of the isolated culture was studied using a media spiked with 60 mg L^{-1} of Cr(VI). The pH was maintained at 6 and the temperature at 37 ºC. The experiments were conducted for a time period of 48 h. Samples were withdrawn at regular intervals and centrifuged at 12000X g. The supernatant was used for determination of Cr(VI), Cr(III) and biomass.

6.2.6 Preparation of CFE

Cell free extract was prepared by using a modified procedure proposed by Bopp and Elrich (1988) [6]. Completely grown cultures were harvested by centrifugation at 12000 X g and stored by suspending it in phosphate buffer of pH 6. The suspended cultures were then disrupted by ultrasonication (Sonics, VCX 500, 20 kHz). Power for the sonication process was supplied in pulses at 50 W each. A part of sonicate was then centrifuged at 12000 X g for 10 min. The supernatant (S_{12}) was decanted and checked for the viable cells. Another part of sonicate was centrifuged at 24000 X g for 20 min at 4 °C and the supernatant (S_{24}) was used as soluble fraction. The sediment obtained during the centrifugation was used as membrane fractions (MF).

6.3. Results and Discussion

6.3.1 Isolation and identification

The morphology of the isolated strain was studied using SEM images and was found to be rod shaped (Fig. 6.1 a) and gram positive (Fig. 6.1 b). The complete details of the biochemical and physiological characteristics of the strain are given in Table 6.1. The strain was characterized as catalase and oxidase positive.

Partial 16S rDNA sequencing results obtained from DRL Tezpur, India, showed that *Bacillus cereus* had 756 base pairs. Gene analysis by online BLAST tool showed that the isolated culture was similar to *Bacillus sp*. The morphological and biochemical characteristics showed that the isolated culture was more similar to *Bacillus cereus*. The biolog analysis by using the Biogen III plates also revealed that the isoated culture was more similar to *Bacillus cereus*. The thermodynamic property obtained by online BIOTOOL showed that the culture has 53% of GC content with ΔG= 1251.2 kcal mol^{-1}, enthalpy ΔH= 6648.1 kcal mol^{-1}, ΔS = 17383.9 cal K mol^{-1}.

Table 6.1. Physiological and biochemical characteristics of the isolated *Bacillus cereus* (+: positive; -: negative; w: weak)

Tests	Results	Tests	Results	Tests	Results
Morphological tests					
Margin	Entire	Pigment	-	Cell shape	Rods
Elevation	Raised	Opacity	Opaque	Size (µm)	2-4
Surface	Flat	Gram's reaction	+	Motility	+
Physiological tests					
Tests	Results	Tests	Results	Tests	Results
Growth at Temp °C		Growth on NaCl (%)		Growth at pH	
4	-				
10	-	2	+	4	+(w)
15	-	4	+	5	+
25	+(w)	6	+	6	+
30	+	8	+(w)	7	+
37	+	10	-	8	+(w)
42	+(w)			9	-
45	-			10	-
55	-			11	-
65	-				

Biochemical tests

Tests	Results	Tests	Results	Tests	Results
Indole test	-	Esculin hydrolysis	+	Arginine dihydrolase	+
Methyl reductase test	+	Gelatin hydrolysis	+	Lysine decarboxylase	-
Voges proskauer test	-	Nitrate reduction	-	Ornithine decarboxylase	-
Growth on MacConkey agar	ND	Catalase test	+	Phenylalanine deamination	ND
Citrate utilization		Oxidase test	+	Tween 80 hydrolysis	-
H2S production		Caesin hydrolysis	+	Tween 40 hydrolysis	+(w)
Gas production		Urea hydrolysis	+	Tween 20 hydrolysis	+

Acid production from carbohydrates

Tests	Results
Dextrose	+
Mannitol	+
Xylose	+

Use of sugar as sole carbon source

Tests	Results
Fumarate	-
Glycerol	+
Lactose	-

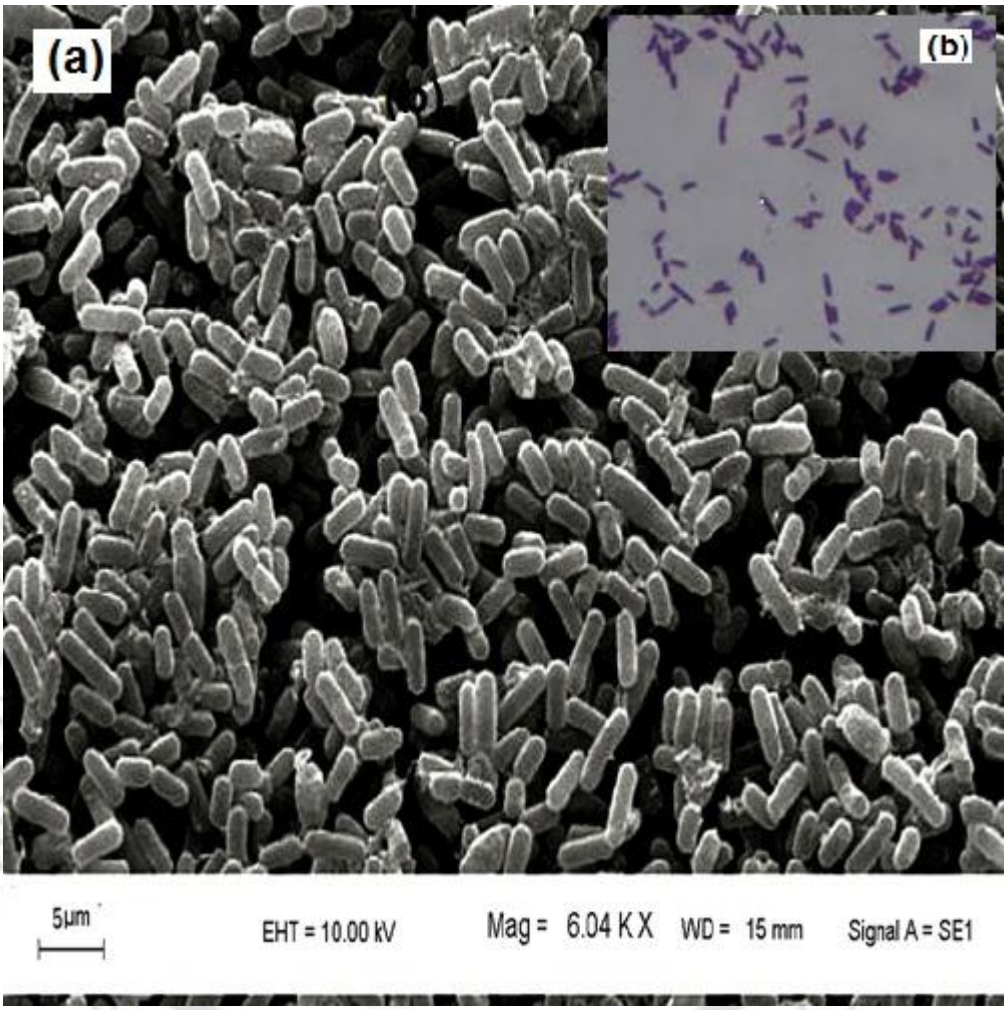

Fig. 6.1. (a) SEM image of *Bacillus cereus* isolated from soil contaminated with Cr(VI); (b) gram staining picture of *Bacillus cereus* isolated from soil contaminated with Cr(VI)

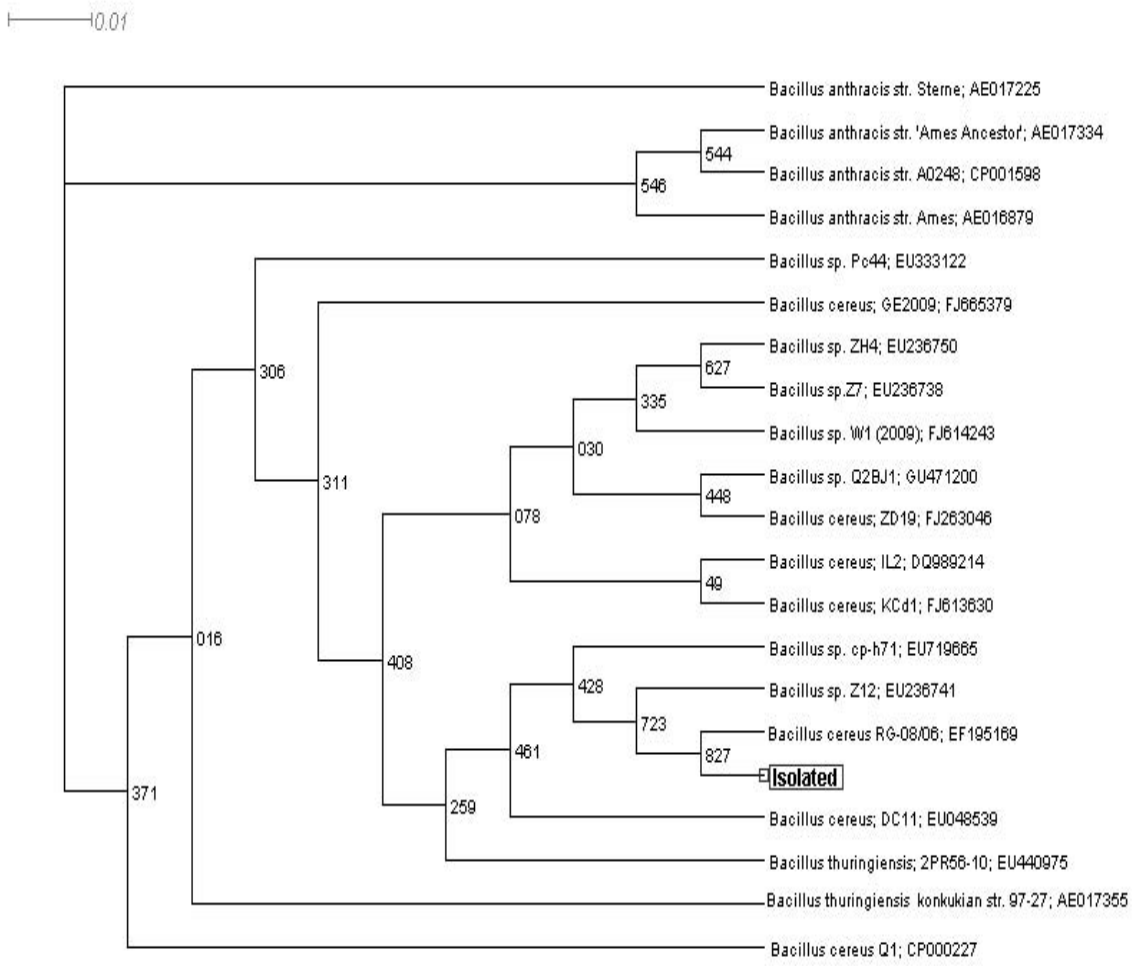

Fig. 6.2. Phylogenetic tree constructed by Neighbor joining method using BIONJ tool. Bootstrap values are noted on the branch and the scale bar (=0.01) represents nucleotide substitution per 1000 nucleotide.

The phylogenetic tree was derived from the 16s rDNA sequence. Neighbor joining method was followed for the construction of the tree using online tool BIONJ (http://www.phylogeny.fr/version2_cgi/advanced.cgi). The tree was constructed with 1000 iterations, the bootstrap values are noted on the branch (Fig. 6.2). It was observed that the isolated culture showed maximum similarity to *Bacillus cereus* (827 times out of 1000 iterations). The similarity search was performed using an online tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi#60454) and the result indicated that the isolated culture showed more similarity to *Bacillus cereus* RG-08/06; EF195169, 4 gaps and 0 mismatches were found in the total similarity search.

6.3.2 Studies on free cells of isolated *Bacillus cereus*

The Cr(VI) bioreduction ability of the free cells of the isolated culture was reported in this section. The role of glucose and other media components on the reduction of Cr(VI) was reported. The efficiency of eight different electron donors in enhancing the bioreduction capacity of the free cells of the isolated culture was evaluated. The role of bacterial metabolism on the reduction of Cr(VI) was also studied by subjecting the cells to different treatments.

6.3.2.1 Selection of electron donors for the isolated culture

Cr(VI) reduction is an electron dependant process [8], different carbon sources such as acetate, citrate, lactate, oxalate etc. were tested as electron donors to enhance the reduction of Cr(VI). Glucose was found to be most effective among the eight different electron donors studied. Glucose yielded a maximum specific reduction rate of 0.83 mg $g^{-1}h^{-1}$ (Table 6.2). Pal and Paul (2004) [14], Poopal and Laxman (2009) [15], Focardi et al. (2012) [16] also have reported glucose as the suitable carbon source for Cr(VI) by bacteria and actinomcycetes. The maximum reduction obtained with glucose as electron donor was due to the fact that glucose can easily be oxidized by the bacteria. Another reason may be due to the fact that, the culture was acclimatized in glucose media for an

extended period during its adaptation under Cr(VI) loading. Other electrons donors such as acetate, citrate, lactate, oxalate, succinate, glycerol, tryptone yielded 0.46, 0.31, 0.41, 0.21, 0.65, 0.58 mg g^{-1} h^{-1} of specific Cr(VI) reduction respectively for an initial Cr(VI) concentration of 60 mg L^{-1}. The control culture without any electron donor yielded a specific reduction of 0.17 mg g^{-1} h^{-1}.

Table 6.2. Effect of different organic substrates on reduction of Cr(VI) by *Bacillus Sp.* at pH 7 and temperature 37ºC, initial Cr(VI) concentration 60 mg L^{-1}

Organic substrate	Specific Cr(VI) reduction rate (mg/g/h)
Acetate	0.32
Citrate	0.46
Lactate	0.31
Oxalate	0.41
Succinate	0.21
Glucose	0.83
Glycerol	0.65
Tryptone	0.58
No substrate	0.17

6.3.2.2 Effect of glucose on growth and reduction of Cr(VI)

The cell density of the isolated culture increased gradually from 0.025 to 1.384 for an increase in glucose concentration from 0 to 1 g L^{-1}. The cell density was found to decrease for glucose concentration beyond 1 g L^{-1}. Similar result of drop in cell density with increase in glucose concentration was reported by Murugavelh and Mohanty (2012) [9] for *Halomonas sp.* The decrease in concentration of the cell density for glucose concentration exceeding 1 g L^{-1} is a clear indication of the substrate inhibition of glucose. The Cr(VI) concentration decreased from an initial of 60 mg L^{-1} to 1.89 mg $L^{-1}$for an

initial glucose concentration of 1 g L^{-1} (Fig. 6.3). Pal and Paul 2004 [14] also have reported that *B. sphaericus* reduced 20 mg L^{-1} of Cr(VI) to 8.9 mg L^{-1} with glucose as sole carbon source at 1 g L^{-1} within 24 h. The concentration of Cr(VI) was found to increase when the glucose concentration exceeded 1 g L^{-1}. The increase in the concentration of Cr(VI) when the glucose concentration was increased from 1 g L^{-1} was due to the fact that cell growth was inhibited due to substrate inhibition. The results indicated the role of the cell growth on Cr(VI) reduction.

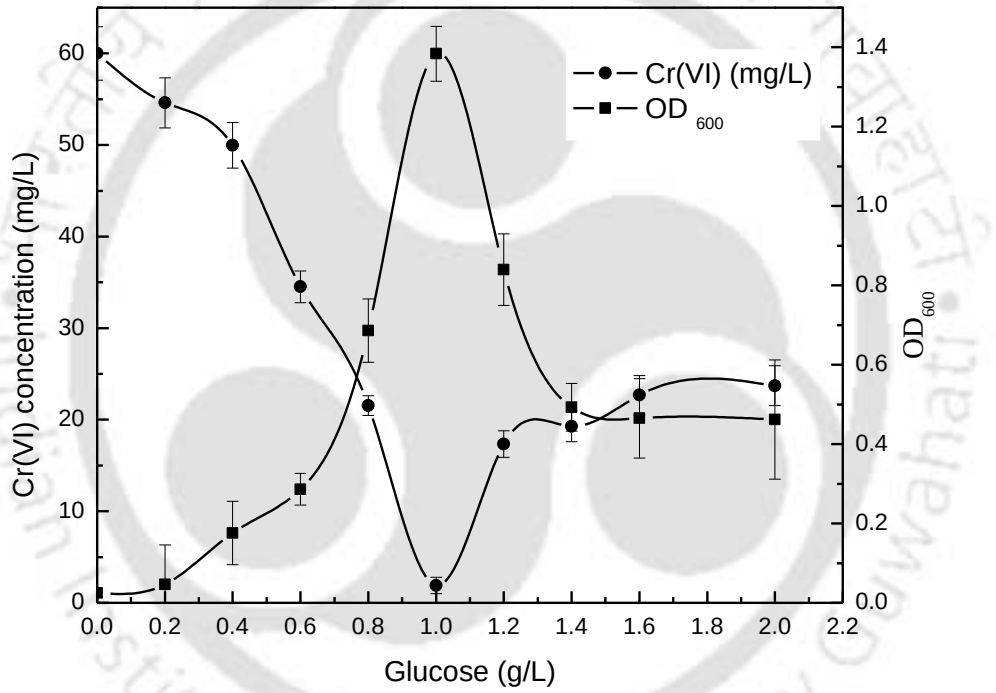

Fig. 6.3. Growth and Reduction of Cr(VI) by *Bacillus cereus* isolated from soil at pH 6 and temperature 37 ºC, initial Cr(VI) concentration 60 mg L^{-1}

6.3.2.3 Effect of yeast extract on the reduction of Cr(VI)

Yeast extract supplements the growth of the bacteria [9]. The growth media contained higher concentrations of yeast extract. Experiments were performed to estimate the yeast extract concentration required for the maximum reduction of Cr(VI). Growth media was prepared with various concentrations of yeast extract ranging from 1 g L^{-1} to 10 g L^{-1}. 5 g L^{-1} of yeast extract in the growth media resulted maximum reduction of 96.7 % of Cr(VI) at an initial concentration of 60 mg L^{-1}. Philip et al. 1998 [7] have reported that yeast extract at 5 g L^{-1} was essential for Cr(VI) reduction by *B. coagulans* isolated from contaminated soil. The other concentration of yeast extract resulted in 19.56, 40.30, 74.84, 24.3 % of Cr(VI) reduction respectively for 10, 7.5, 2 and 1 g L^{-1} of yeast extract (Fig. 6.4).

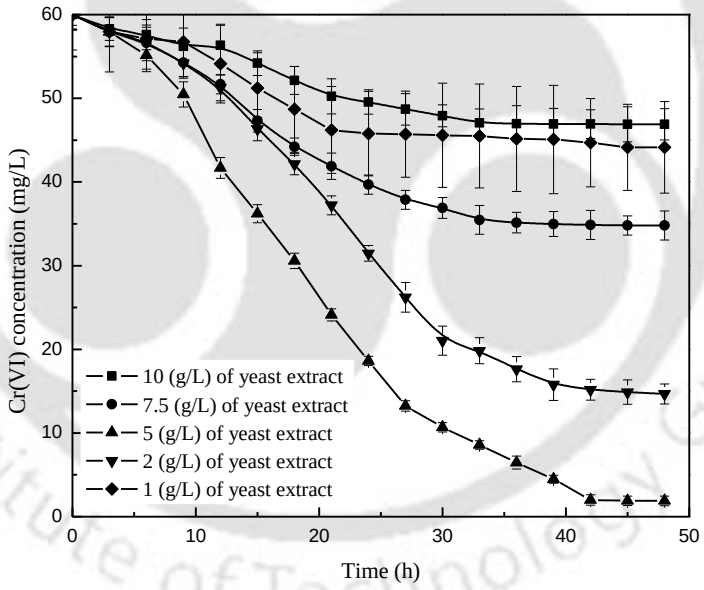

Fig. 6.4. Effect of Yeast extract on reduction of Cr(VI) at pH 6 and temperature 37ºC, initial Cr(VI) concentration 60 mg L^{-1} and glucose concentration 1 g L^{-1}

The decrease in the Cr(VI) reduction with increase in yeast extract concentrations beyond 5 g L^{-1} was due to the fact that the higher amount of yeast extract in the growth media affected the oxygen uptake of the culture. Similar trend of decrease in the reduction of Cr(VI) was observed for growth media containing yeast extract concentration below 5 g L^{-1}. This was due to the scarcity in the availability of the free amino acids which were the electron donors for the reduction of Cr(VI).

Cr(VI) reduction with other complex nutrients like beef extract and liver extracts was also tested. The percentage reduction of Cr(VI) obtained with beef extract (5 g L^{-1}) was 27.81% for an initial Cr(VI) concentration of 60 mg L^{-1} and liver extracts (5 g L^{-1}) reported 21.34% of Cr(VI) reduction for an initial Cr(VI) concentration of 60 mg L^{-1}. The results obtained with the use of liver extract and beef extract showed that yeast extract was the best suitable complex nutrient for Cr(VI) bioreduction process.

6.3.2.4 Effect of initial Cr(VI) concentration on the reduction of Cr(VI)

The role of initial Cr(VI) concentration on the bioreduction process was studied. The growth media was prepared with different concentrations of the Cr(VI) ranging from 10 to 70 mg L^{-1}. Near complete reduction of Cr(VI) was obtained for an initial Cr(VI) concentrations of 10, 20, 30, 40 mg L^{-1}. Focardi et al. (2012) [16] has reported that *Halomonas sp* reduced 92.32% Cr(VI) at an initial Cr(VI) concentration of 3.6 mg L^{-1}, when the Cr(VI) concentration was increased to 54 mg L^{-1} the Cr(VI) reduction dropped to 33.26%. Focardi et al. (2012) [16] have reported that the drop in Cr(VI) reduction with increase in Cr(VI) concentration was due to the cell inhibition by Cr(VI).

An initial Cr(VI) concentration of 60 mg L^{-1} decreased to a final Cr(VI) concentration of 1.89 mg L^{-1}. Cr(VI) reduction obtained with an initial Cr(VI) concentration of 70 mg L^{-1} was less significant when compared with the other concentrations studied. An initial Cr(VI) concentration of 70 mg L^{-1} of Cr(VI) was reduced to 18.21 mg L^{-1}. It was observed that the Cr(VI) reduction decreased when the concentration of the Cr(VI) was increased to 70 mg L^{-1} (Fig. 6.5). The reason for the

decrease in the reduction was due to the toxicity of the Cr(VI) on the cells of the isolated culture at an initial concentration of 70 mg L^{-1}. The other concentration were able to reduce Cr(VI) easily owing to the fact that the culture was acclimatized up to a concentration of 60 mg L^{-1}.

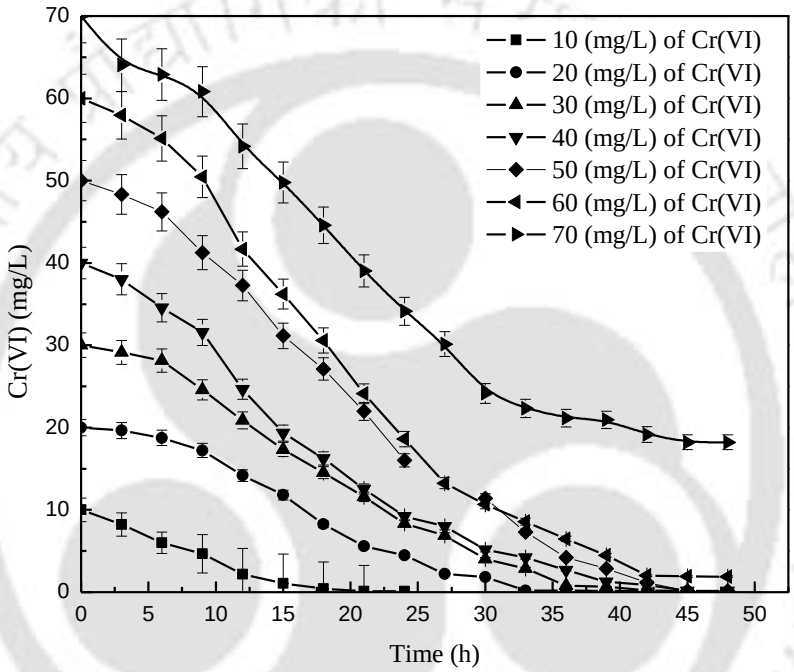

Fig. 6.5. Initial metal ion concentration on Cr(VI) reduction at pH 6 and temperature 37 ºC, initial Cr(VI) concentration 60 mg L^{-1} and glucose concentration 1 g L^{-1}

6.3.2.5 Effect of pH on the reduction of Cr(VI)

pH of a solution has very significant effect on bioreduction experiments. pH of the media affects solubility of Cr(VI) and the bacterial growth is also affected by change in the pH of the media. The bioreduction capacity of the isolated culture was studied under a wide range of pH 3-8. It was observed that the reduction was dependant on the pH of the media. Near 100% reduction was obtained with initial concentrations of 10, 20,

30, 40, 50 mg L^{-1} at pH 6 (Fig. 6.6). Philip et al. (1998) [7], Murugavelh and Mohanty (2012) [8], Focardi et al. (2012) [18] have reported *B. coagulans and Halomonas sp* reduced Cr(VI) under an optimum pH of 6 to 7. An initial Cr(VI) concentration of 60 mg L^{-1} resulted in 96.7 % reduction of Cr(VI). The other pH studied at 3, 4, 5, 7 resulted in 6.29, 7.13, 85.9, 11.6% reduction of Cr(VI) respectively for an initial Cr(VI) concentration of 60 mg L^{-1}. The drop in the percentage reduction of Cr(VI) for these pH values was due to the decrease in the cell growth. It was observed that pH 5 and 6 favored the bioreduction of Cr(VI). It was due to the fact the cell growth was favored at pH 5 and 6. At pH 5 and 6 the bacterial cells remained intact and were able to grow and withstand the toxic effect of the Cr(VI). The bacterial growth was drastically affected when the pH of the growth media was adjusted to pH 8 which decreased the reduction capacity of the *Bacillus cereus*.

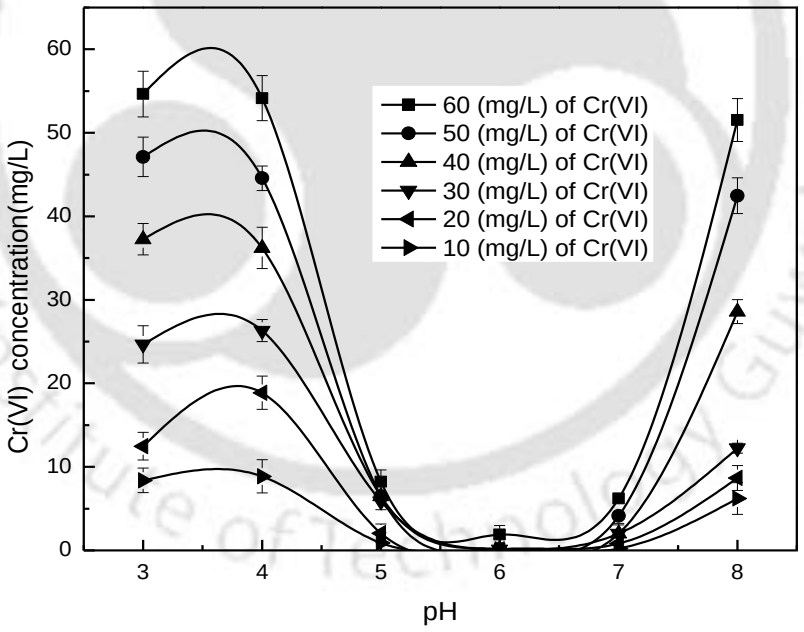

Fig. 6.6. Effect of pH on Cr(VI) reduction at temperature 37 ºC, initial Cr(VI) concentration 60 mg L^{-1} and glucose concentration 1 g L^{-1}

6.3.2.6 Effect of time on Cr(VI) reduction

The reduction of Cr(VI) and simultaneous production of Cr(III) by the isolated culture was presented in Fig. 6.7. The concentration of Cr(VI) gradually decreased from 60 mg L^{-1} to 1.89 mg L^{-1} within 48 h. Similar trend of drop in Cr(VI) concentration with time was reported by Quilntana et al. (2001) [17] for *T. ferroxidans*. It was observed that the decrease in the concentration of Cr(VI) was accompanied by proportionate increase in the Cr(III) concentration. The concentration of Cr(III) increased from an initial concentration of near zero to 58.11 mg L^{-1} within 48 h. The results indicated that Cr(VI) reduction by the isolated cultures is a time dependant process. The concentration of the Cr(VI) decreased gradually up to 39 h and was observed to be constant from 42 h. A maximum of 58.11 mg L^{-1} of Cr(III) was produced.

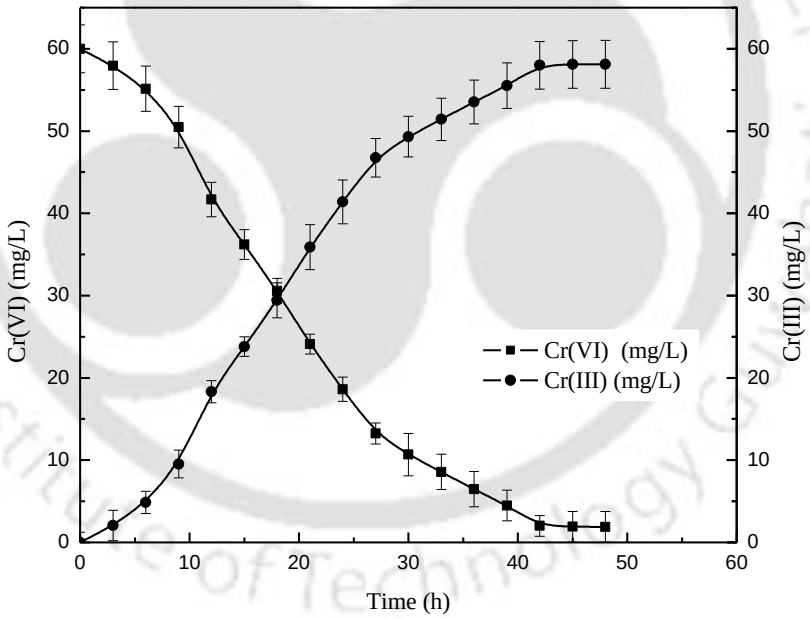

Fig. 6.7. Effect of time on Cr(VI) reduction at pH 6 and temperature 37 ºC, initial Cr(VI) concentration 60 mg L^{-1} and glucose concentration 1 g L^{-1}

6.3.2.7 Effect of different treatment methods on Cr(VI) reduction

The physiological state of the cells play an important role in the bioreduction of Cr(VI). In order to estimate the role of physiological state of the cells during the bioreduction process, the cells were treated with heat, sodium azide, pentachlorophenol and potassium cyanide. Untreated free cells were used as the control. The untreated free cells reported a maximum reduction of 96.7% reduction for an initial Cr(VI) concentration of 60 mg L^{-1}. The heat killed cells reported 35.14% for an initial Cr(VI) concentration of 60 mg L^{-1}. The cells treated with NaN_3 reported 71.1% of Cr(VI) reduction. The cells treated with PCP and KCN reported 10.18% and 10.14 % of Cr(VI) reduction respectively (Fig. 6.8).

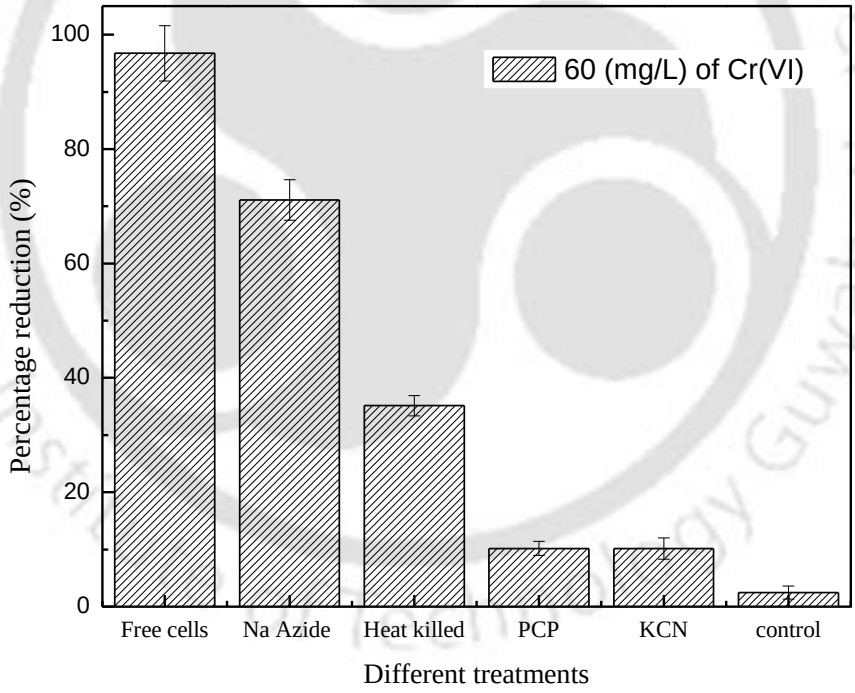

Fig. 6.8. Effect of different treatments on Cr(VI) reduction at pH 6 and temperature 37 ºC, initial Cr(VI) concentration 60 mg L^{-1} and glucose concentration 1 g L^{-1}

Murugavelh and Mohanty (2012) [8] reported that metabolic activity of the cells of *Halomonas sp* was essential for Cr(VI) reduction. Barrera et al. (2008) [18] also have reported that cell density plays an important role in *H. tawa* during Cr(VI) reduction. The maximum percentage reduction obtained with the free cells was due to the fact that the cells were metabolically active throughout the study which favored the bioreduction process. The reduction obtained with the heat killed cells was due to the interaction of denatured organic components of cells with the Cr(VI). It can be observed that the reduction decreased for cells treated with heat, PCP, KCN and NaN_3. It was due to the fact that the metabolism of the bacterial cells was affected by various treatments. The results confirmed that the reduction of Cr(VI) is a metabolism dependant process.

6.4 Studies on cell free extracts of isolated *Bacillus cereus*

Cell free extracts (CFE) are the metabolites formed during the bioreduction process and it contains huge amount of crude protein and other intercellular components. The CFE of the isolated culture was tested for its ability to reduce Cr(VI). The pH of the CFE was varied between 1-7 keeping the temperature constant at 37 °C. Similarly the optimum temperature for the CFE on reduction of Cr(VI) was studied using 60 mg L^{-1} of Cr(VI) solution at pH 6. Studies using various electron donors were carried out at pH 6 and 37 °C for an initial Cr(VI) concentration of 60 mg L^{-1}. All experiments using CFE were performed for 6 h. CFE has the ability to reduce 60 mg L^{-1} of Cr(VI) within 6 h. The bioreduction capacity and role of the physical parameters on CFE are reported in this section. The role of inhibitors, electron donors on the reduction of Cr(VI) by CFE was also reported.

6.4.1 Effect of temperature on the reduction of Cr(VI) by CFE

The optimum temperature required for the reduction of Cr(VI) using CFE was determined by varying the temperature between 10 °C to 60 °C. The CFE reported a maximum reduction of 96.54 % of Cr(VI) for an initial Cr(VI) concentration of 60 mg L^{-1}

at 37 °C. Elangovan et al. (2010) [19] also reported that optimum temperature for CFE of *Arthobacter rhombi* in Cr(VI) reduction to be 37 °C.

It was observed that 78.11% of Cr(VI) reduction was obtained at 30 °C. Other temperature ranges studied did not result in significant reduction of Cr(VI). 10, 20, 42, 50 and 60 °C reported 18.14, 42.47, 28.1, 9.67 and 2.14 % reduction of Cr(VI) respectively (Fig. 6.9). The drop in the percentage reduction on both sides of 37 °C was due to the fact that the active sites on the soluble enzyme were inactivated by the change in the temperature. The optimum temperature for the reduction of Cr(VI) with CFE was thus found to be 37 °C.

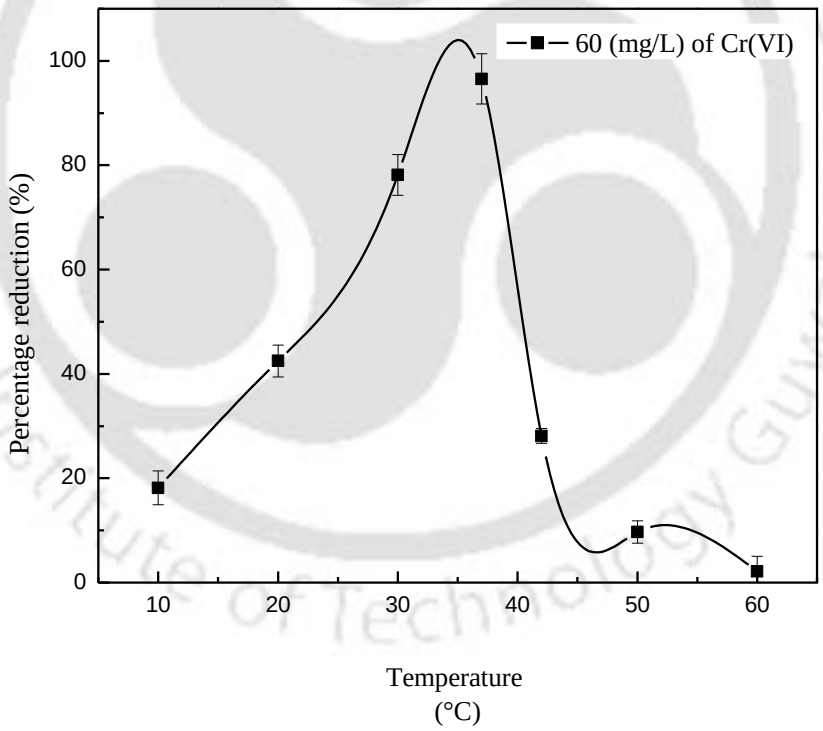

Fig. 6.9. Effect of temperature on Cr(VI) reduction by CFE at pH 6.5**,** initial Cr(VI) concentration 60 mg L^{-1} and glucose concentration 1 g L^{-1}

6.4.2 Effect of pH on the reduction of Cr(VI)

In order to determine the optimum pH for the reduction of Cr(VI) with CFE, studies were conducted by varying the pH of the solution between 1-7. A maximum of 96.57% of Cr(VI) reduction was obtained with pH 6.5 for an initial Cr(VI) concentration of 37 °C. Poopal and Laxman (2009) [15], Sau et al. (2010) [20], Focardi (2012) [16], Murugvelh and Mohanty (2012) [8] have reported that the optimum pH for CFE obtained from *S. griseus, B. firmus, Halomonas sp.* was 6.5. The percentage reduction obtained with other pH values studied are 2.87, 16.57, 26.47, 45.68, 72.41, 94.38, 88.26 respectively for pH 1, 2, 3,4, 5, 6, 7 (Fig. 6.10). The percentage reduction obtained with pH ranging from 1-4 was very insignificant. The decrease in the percentage reduction at a pH (1-4) was due to fact that the CFE's active sites were inactivated in acidic medium. The percentage reduction obtained with pH 7 and 8 are slightly lesser (94.38%, 88.26%) when compared to reduction obtained with pH 6.5 (96.57%) and 6 (94.38%). The change in the conformation of the enzymes inactivates the CFE and thereby reduces the percentage reduction of Cr(VI).

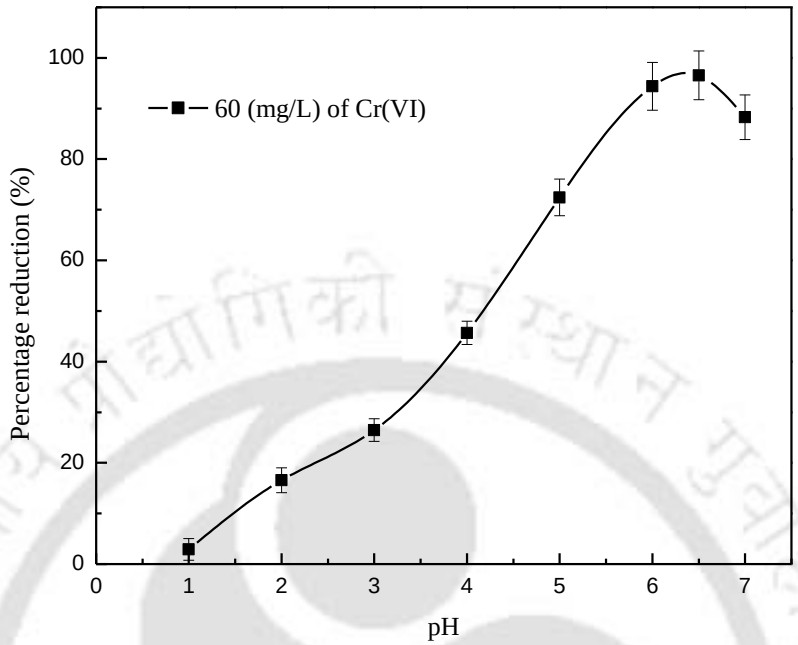

Fig. 6.10. Effect of pH on Cr(VI) reduction by CFE at 37 ºC, Initial Cr(VI) concentration 60 mg L^{-1}

6.4.3 Effect of electron donors

CFE can utilize a wide range of electron donors during the reduction of Cr(VI). Selecting a suitable electron donor can increase the rate of Cr(VI) reduction. The reduction capacity of the CFE was tested with nine different electron donors. It was found that CFE with NADH and NADPH as electron donors yielded a maximum of 91.52 and 96.54 % reduction of Cr(VI) for an initial Cr(VI) concentration of 60 mg L^{-1}. Desai et al. 2008, Poopal and Laxman (2009) [15], Murugavelh and Mohanty (2012) [8], have reported that NADPH increased the percentage reduction of Cr(VI). The other electrons such as citrate, lactose, glucose, fructose, acetate and succinate yielded 57.28, 49.61, 67.58, 67.51, 52.11 and 43.54 % reduction of Cr(VI) respectively (Fig. 6.11).

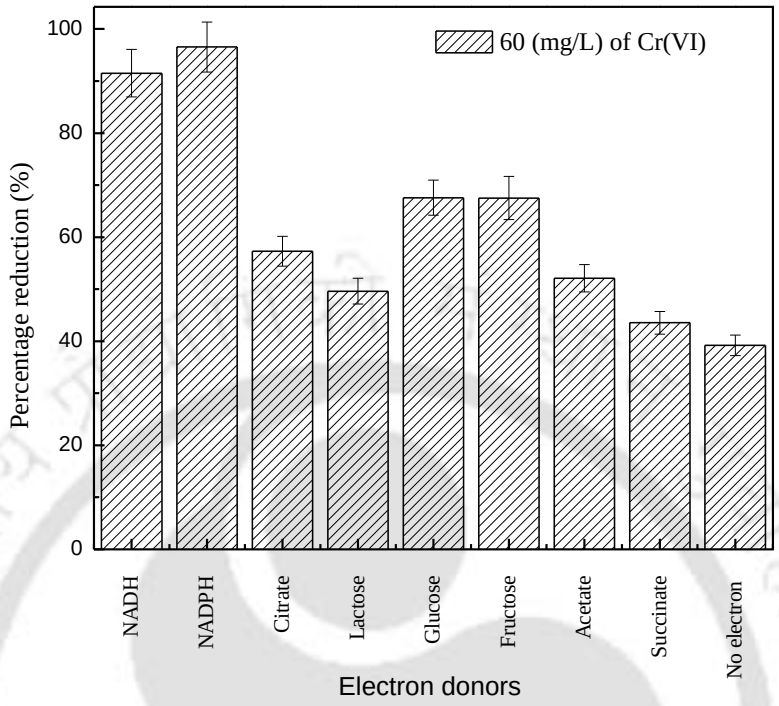

Fig. 6.11. Effect of different electron donors on Cr(VI) reduction by CFE at pH 6.5 and temperature 37ºC, initial Cr(VI) concentration 60 mg L^{-1}

Based on the screening of electron donors for CFE, NADH and NADPH were used as electron donors for further studies. The sonicate, supernatants of the sonicate [S_{12} (12000Xg), S_{24}(24000xg)] were studied for their capacity to reduce Cr(VI). CFE without the addition of any electron donor was used as the control for all the experiments. It was found that the presence of electron donor in the CFE increased the percentage reduction of Cr(VI). The uncentrifuged sonicate reduced 60 mg L^{-1} to 7.14 mg L^{-1} in the presence of NADH (Fig. 6.12 a). NADPH increased the reduction percentage as it was found that sonicate reduced 60 mg L^{-1} Cr(VI) to 5.28 mg L^{-1} in the presence of NADPH. The sonicate S_{12} also showed a similar trend in the presence of NADPH. It was reported that S_{12} reduced 60 mg L^{-1} of Cr(VI) to 4.22 mg L^{-1} in the presence of NADPH as electron

donor. The Sonicate S_{12} reduced 60 mg L^{-1} of Cr(VI) to 6.28 mg L^{-1} Cr(VI) in the presence of NADH within 6 h (Fig. 6.12 b). It was clear that NADPH enhances the reduction capacity of the CFE. The electron donor free S_{12} reduced 60 mg L^{-1} of Cr(VI) to 35.45 mg L^{-1} of Cr(VI) within 6 h. Fig. 6.12 c shows that the partially purified sonicate S_{24} reduced 60 mg L^{-1} to 1.38 mg $L^{-1}$of Cr(VI) in the presence of NADPH as electron donor within 6 h. Partially purified sonicate S_{24} in the presence of NADH reduced 60 mg L^{-1} to 6.14 mg L^{-1} within 6 h (Fig 6.12c). The sonicates S_{12} and S_{24} showed almost similar reduction capacity in the presence of NADH as the electron donor. It was observed that the reduction obtained with CFE was rapid for 5 h and then slowed down (Fig. 6.12 c). Partially purified CFE in the presence of NADPH reported a maximum of 96.7% reduction of Cr(VI). The membrane fraction (MF) did not report any significant reduction (Fig. 6.12 d).

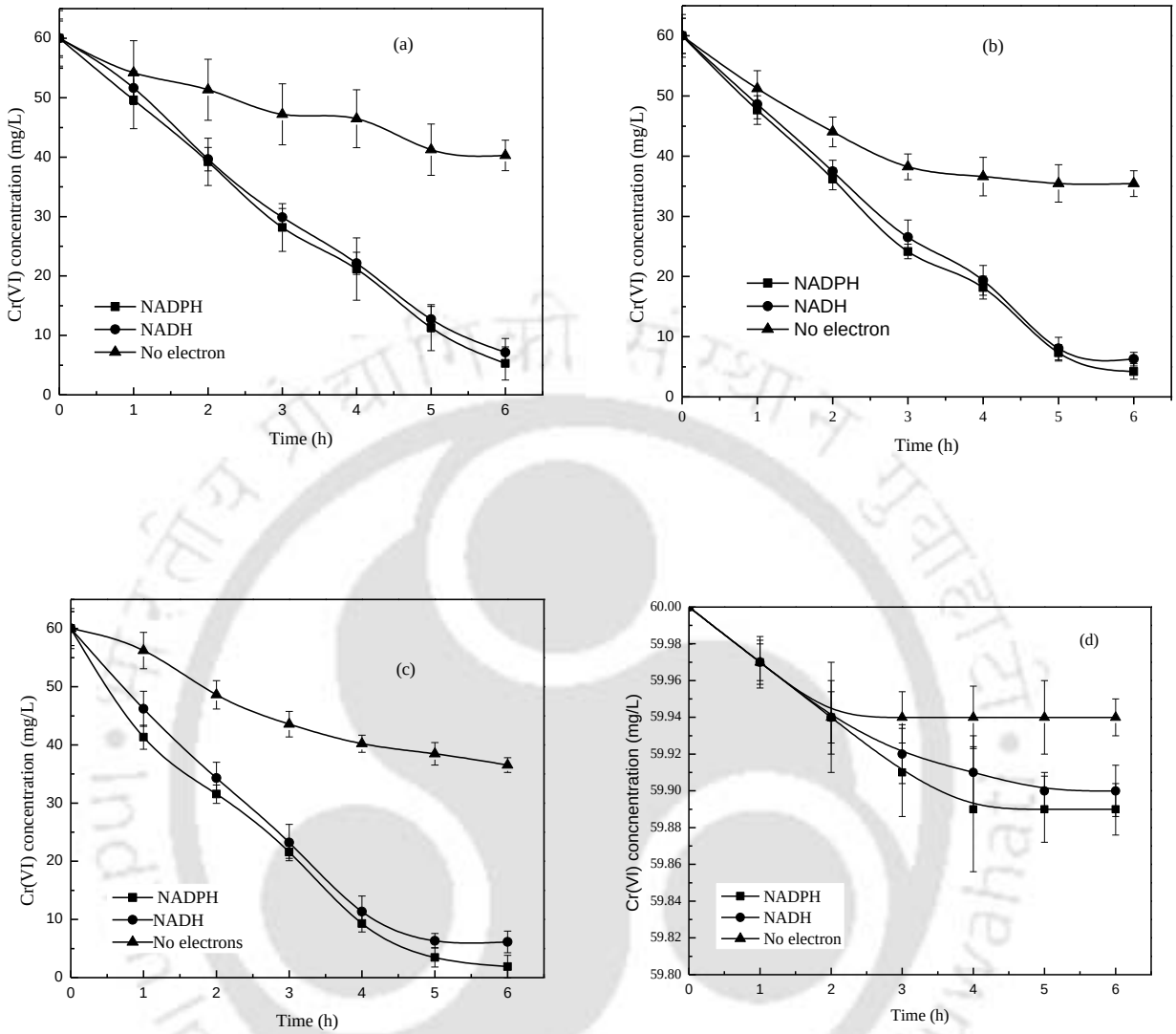

Fig. 6.12. Cr(VI) reduction by: (a) Sonicate in the presence of NADH and NADPH; (b) S_1(12000Xg) in the presence of NADH and NADPH; (c) S_2 (24000Xg) in the presence of NADH and NADPH; (d) Membrane fractions in the presence of NADH and NADPH

6.4.4 Effect of metal ions on CFE

Different metal ions were tested for their role during the Cr(VI) reduction using CFE. The presence of other metal ions drastically reduced the percentage reduction of Cr(VI). Among the different metal ions studied Cu was found to have lesser inhibitory effect on Cr(VI) reduction using CFE as it yielded 66.14% reduction of Cr(VI).

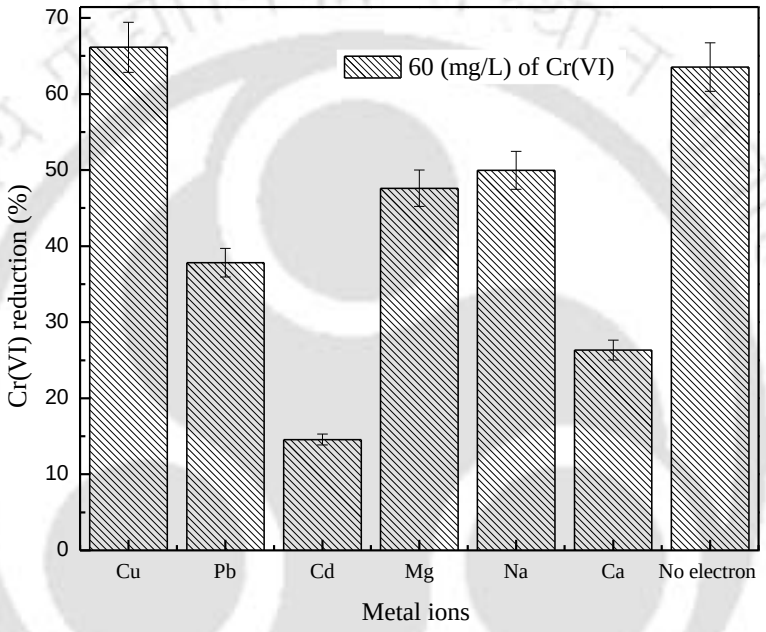

Fig. 6.13. Effect of metal ions on Cr(VI) reduction by CFE at pH 6.5 and temperature 37ºC, initial Cr(VI) concentration 60 mg L^{-1}

It was observed that Mg and Na reported 47.61%, and 49.98% reduction of Cr(VI). Cd was found to have more inhibition on Cr(VI) reduction by CFE. Presence of Cd in the CFE reduced its reduction capacity to mere 14.57 %. Ca also had similar inhibitory effect as the maximum reduction reported by the CFE in the presence of Ca was 26.32% (Fig. 6.13). Sarangi and Krishnan (2008) [21] have reported that the presence of other metal ion decreased the percentage reduction of Cr(VI) by CFE . It can

be inferred that other metal ions cannot be used as an electron donor for the CFE. Other heavy metal has resulted in decreasing the enzyme activity.

6.4.5 Effect of inhibitors on the Cr(VI) reduction using CFE

The CFE was treated with metabolic inhibitors to investigate its enzymatic activity. CFE without any treatment was used as control. Metabolic inhibitors work by competing for the active sites in the CFE. It was observed that the percentage reduction was found to drop drastically in the presence of metabolic inhibitors.

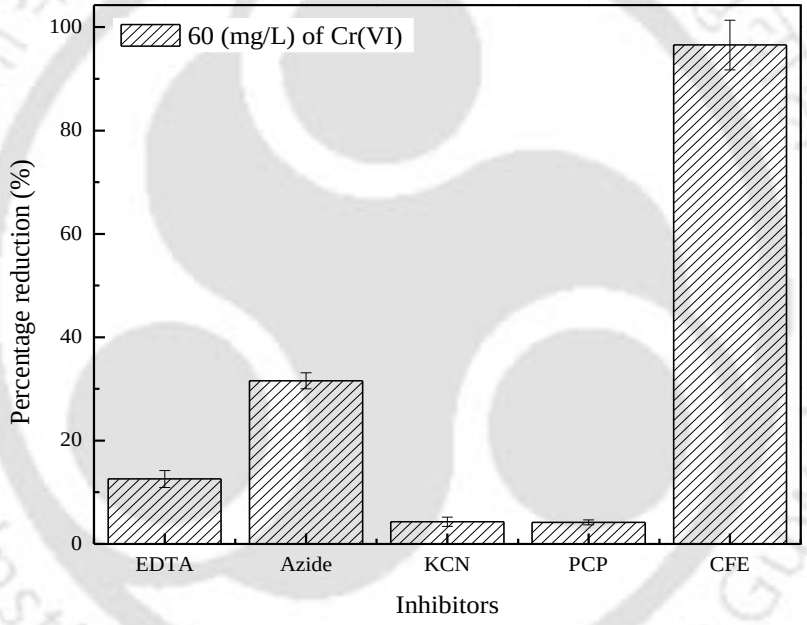

Fig. 6.14. Effect of inhibitors on Cr(VI) reduction by CFE at pH 6.5 and temperature 37ºC, initial Cr(VI) concentration 60 mg L^{-1}

Among the treatment with different metabolic inhibitors, the Cr(VI) reduction decreased drastically. It was found that CFE with PCP and KCN has reported 4.32% and 4.17 % reduction of Cr(VI) respectively (Fig. 6.14). The effect of metabolic inhibitors on

CFE of *Halomonas sp* was reported in our previous study (Murugavelh and Mohanty, 2012) [8]. The results obtained with the CFE of *Bacillis cereus* and *Halomonas sp* in the presence of metabolic inhibitor are similar. The metabolic inhibitors reduce the percentage reduction of CFE by competing for the active sites through competitive inhibition mechanism. CFE in the presence of EDTA and Azide reported 12.57 and 31.57% reduction of Cr(VI) respectively.

6.5. Conclusion

Investigation on Cr(VI) reduction using isolated strain of *Bacillus cereus* was reported. A modified YEPG media was used for this study. Near complete reduction of Cr(VI) was obtained for concentration of Cr(VI) ranging from 10 mg L^{-1} to 50 mg L^{-1}. Glucose at 1 g L^{-1} was essential for the reduction of Cr(VI). Maximum reduction of 96.7% was reported for 60 mg L^{-1} of Cr(VI) at pH 6. Heat treatment, treatment with sodium azide, PCP and KCN reduced the percentage reduction of Cr(VI). CFE reduced 60 mg L^{-1} of Cr(VI) to 1.88 mg L^{-1} within 6 h. NADPH was the suitable electron donor for Cr(VI) reduction with CFE. pH 6.5 was the optimum pH for the CFE for Cr(VI) reduction. The free cells and the CFE of the *Bacillus cereus* strain isolated was capable of completely reducing Cr(VI) up to an initial concentration of 60 mg L^{-1}. Thus, the free cells and CFE of isolated *Bacillus cereus* can be used for the treatment of Cr(VI) containing wastewater in an environment friendly method. The free cells and CFE of the *Bacillus cereus* can be used for the treatment of Cr(VI) containing wastewater under neutral pH. Cr(VI) containing wastewater can be treated in less than 6 h.

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Chapter VII

Performance of *Halomonas Sp* to Reduce Cr(VI) in Batch and Continuous Fixed Film Reactor

7.1. Introduction

The bacterial reduction of Cr(VI) is a potential alternate to the conventional methods [1]. Many researchers have reported the bacterial reduction of Cr(VI). Shen and Wang (1994) [2] studied reduction of Cr(VI) using *E. coli*. Wang and Shen [3] used *Bacillus sp* and *Pseudomonas fluorescens* LB 300 for reduction for an initial Cr(VI) concentration of 27 mg L^{-1}. Philip et al., [4] reported *Bacillus coagulans* as a potential organism for the reduction of Cr(VI). In particular *Halomonas sp* was reported for its potential in rapid reduction of Cr(VI) for an initial concentration range upto 40 mg L^{-1} [5]. Previous study showed that yeast extract and glucose are the essential carbon source for the growth and reduction of the Cr(VI) by *Halomonas sp*. Most of the chromium reduction studies reported was conducted under batch conditions [2, 6]. In batch operations microbial cells are guaranteed to receive the nutrients for the growth [7]. In continuous mode the intermittent seeding of the reactor is difficult and the chance of loss of limiting substrate is there which can lead to loss of metabolic activity [1]. In the recent years the application of continuous reactors for the reduction of Cr(VI) is gaining importance. Chirwa and Wang [8] were the first to study the application of fixed film reactor in the Cr(VI) bioreduction process. Shen and Wang [9] studied chromium reduction in a two stage reactor.

The present work reports the bioreduction capacity of *Halomonas sp* in batch and continuous mode under limited carbon source. The biokinetic parameters are also evaluated.

7.2. Materials and Methods

7.2.1 Bacterial culture

The *Halomonas sp* was purchased from Institute of Microbial Technology, Chandigarh, India and grown at 37 ºC in a liquid medium at pH 7. The subcultures of the *Halomonas sp* was prepared and stored at 4 ºC for future use.

7.2.2 Media

The growth media for the *Halomonas sp* was prepared by dissolving 5 g of yeast extract, 5 g of peptone and 1 g of glucose in 1 L of deionized water. The feed to the fixed film reactor was prepared by dissolving, 0.03 g of K_2HPO_4, 0.03 g of KH_2PO_4, 0.01 g of $MgSO_4$, 5 g of yeast extract, 5 g of peptone and 1 g of glucose in 1 L of deionized water. The media was sterilized by autoclaving (Indfos, India) at 120 °C for 15 min.

7.2.3 Cr(VI) stock solution

The stock solution of Cr(VI) was prepared by dissolving 2.82 g of $K_2Cr_2O_7$ in 1 L of deionized water. The feed to the reactor containing various concentrations of Cr(VI) was prepared by diluting the stock solution in the growth media. All media was adjusted to pH 7.0 using 0.1 N HCl.

7.2.4. Analytical procedure

Cr(VI) concentration was analyzed at 540 nm using UV spectrophotometer (SpectraScan, ThermoFisher, Germany) by using the DPC method [10]. Total chromium was measured using atomic absorption spectrophotometer (Varian AA240 FS).

COD was determined using HACH COD digester (Model DRB 200 USA) following the standard methods [14]. The Dissolved oxygen concentration was measured using a DO meter (VSI-14 ATC, India).

Glucose was measured following the standard dinitro salicylic method [11]. Viable cells are measured by plating small volume of the effluent in PYG agar. The plates were incubated for 24 h and the colonies were counted. The morphology of the culture was monitored to check for the contamination. Total suspended cells were measured by plating diluted sample (10^{1}) on PYG agar. The total attached cells were calculated by centrifuging the glass beads (with drawn under sterile condition after the reactor attained steady state) in centrifuged in 9 mL of 0.85% NaCl for 10 mins and the sediments of the centrifugation was reported as attached cell growth, the supernatant was plated on PYG agar and incubated for 24 h. Colonies were counted after incubation, microscopic examination of the culture was performed.

7.2.5 Reactor setup

The fixed film reactor was fabricated to operate under completely mixed and aerated conditions. The schematic diagram of the reactor set up was shown in Fig. 7.1. The fixed film reactor was 42 cm long with an internal diameter of 35 cm. The reactor was made from pyrex glass. The reactor bed was packed with 19241 glass beads of diameter 3 mm to provide an external surface area of 1631.6 cm^2 for the growth of the *Halomonas sp*. The reactor was operated at 30 °C (room temperature). The feed to the reactor was provided in up flow mode. Aeration to the fixed film reactor was provided using an aquarium pump. An aeration chamber of 25 cm length and internal diameter of

2.5 cm was used for collecting the effluent from the fixed film reactor. The aeration chamber was aerated by another aquarium pump. The microbial volume in the fixed film reactor was maintained by recycling the effluent at a ratio of 100:1. Previously calibrated peristaltic pump (ENPD- 100 Express) was used to feed the reactor with Cr(VI) spiked media. The pumps were calibrated to maintain a HRT of 24, 12, 6 h respectively for batch studies and 24 h for the continuous study. The media from the feed tank was plated in PYG agar to check for the contamination.

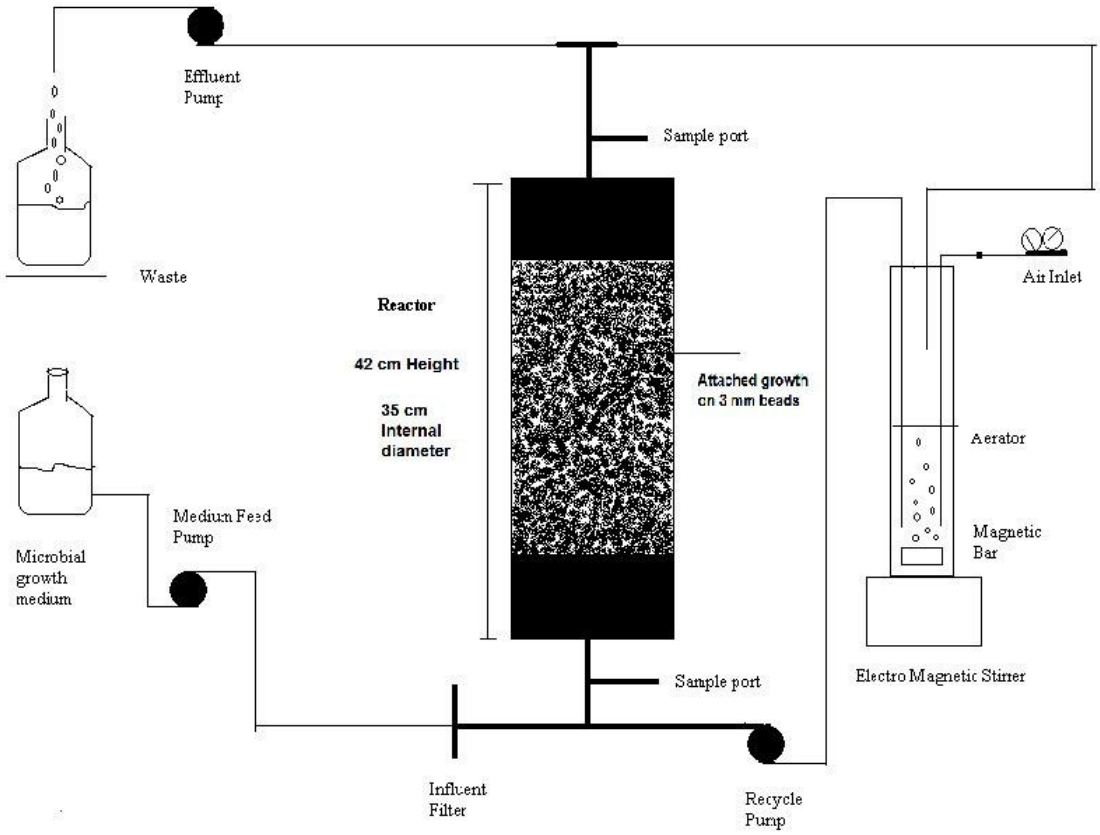

Fig. 7.1. Schematic diagram of fixed film reactor

7.2.6. Reactor start up

The reactor column, media tanks, glass beads, silicone tubes were sterilized by autoclaving for 120 °C for 15 min. The fixed bed reactor was assembled in a laminar flow hood (Aeromech, India). The reactor was fed with influent media containing 10 mg L^{-1} of Cr(VI). The reactor was operated under HRT of 24 h without inoculum. The effluent from the reactor was analysed for Cr(VI) and total Cr. 1 mL of the effluent was plated in PYG agar to check for contamination. No growth was visible, which indicated the reactor set up was sterile. The influent and effluent Cr(VI) was found to be same. This indicated that there was no abiotic Cr(VI) reduction.

For batch studies the reactor was inoculated with 10 mL of overnight grown culture of *Halomonas sp*. After inoculation the reactor was operated at an HRT of 24 h. Once visible growth was obtained in the glass beads, the reactor was fed with Cr(VI) at an lowest initial concentration of 10 mg L^{-1}. The HRT was maintained at 12 and 6 h to study the Cr(VI) reduction.

The continuous study the reactor was inoculated with 10 mL of pregrown culture of *Halomonas sp*. and operated under 24 h HRT. Once significant growth was visualized on the glass beads, the reactor was fed with 10 mg L^{-1} of Cr(VI) and the concentration of Cr(VI) was steadily increased.

7.3. Results and Discussion

The reactor was operated under batch and continuous condition for a wide range of influent Cr(VI) concentration (10 – 100 mg L^{-1}). The role of glucose as sole carbon source on growth of the *Halomonas sp* was studied and reported in this section. The COD reduction by *Halomonas sp* was also reported. For batch studies the HRT was studied

between 24, 12 and 6 h. The biological activity in the fixed film reactor was also reported.

7.3.1 Batch studies

Batch studies was performed to estimate the COD and Cr(VI) bioreduction by *Halomonas sp*. The inhibitory effect of the influent Cr(VI) on the growth of the *Halomonas sp* was reported in Fig. 8.2. A maximum biomass concentration of 457 h^{-1} was reported for control culture. It was observed that the initial lag for the control culture was less than 3 h. The lag period for cells grown in media spiked with an initial Cr(VI) concentration of 40 mg L^{-1} was slightly above 3 h. An initial Cr(VI) concentration of 50 mg L^{-1} in the growth media reported a lag period of 12 h. Other concentration of Cr(VI) studied 60, 70 and 80 mg L^{-1} had a greater inhibitory effect on the growth of the *Halomonas sp*. A prolonged lag period of 18 h was reported for an initial Cr(VI) concentration beyond 50 mg L^{-1}. An initial Cr(VI) concentration of 90 and 100 mg L^{-1} completely inhibited the growth of the bacteria as the biomass obtained was negligible (Fig. 7.2).

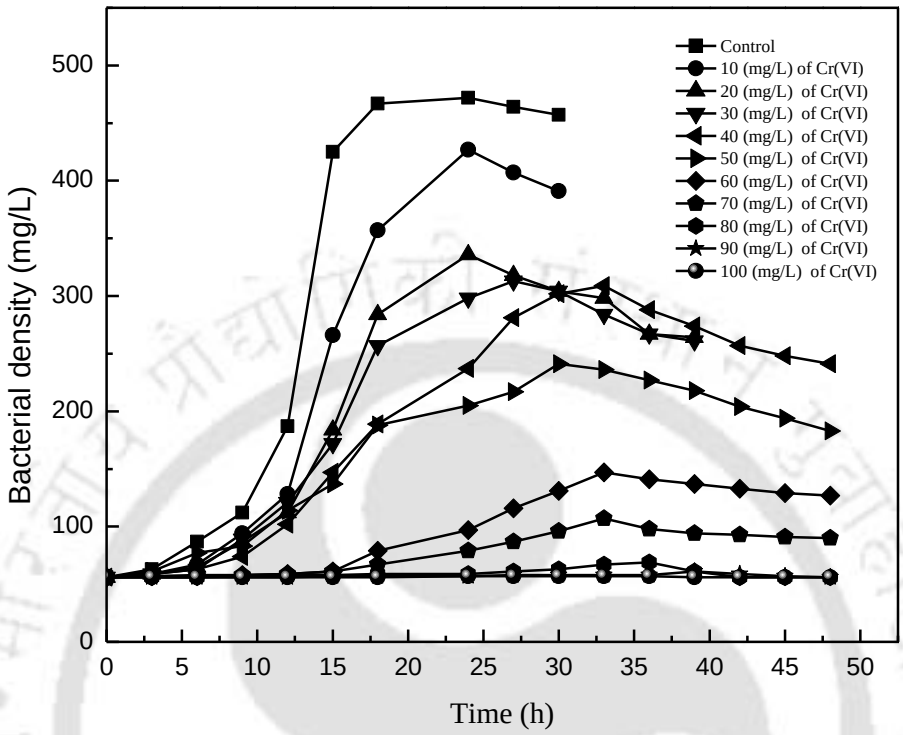

Fig. 7.2. Growth of *Halomoans sp* in the presence of different concentrations of Cr(VI)

The results showed that the increase in influent Cr(VI) concentration inhibited the growth of the bacteria. COD was reported as the substrate concentration and calculated to be 3000 mg L^{-1} for the total media composition. It was observed that the *Halomonas sp* was able to reduce the COD significantly (Fig 7.3). The control culture reported 84.1 % of COD removal. COD at an initial concentration of 3000 mg L^{-1} was reduced to a final concentration of 524 and 654 respectively in the presence of 10 and 20 mg L^{-1} of Cr(VI). Increase in the initial Cr(VI) concentration decreased the COD reduction. It was observed from Fig. 7.3 that, 30, 40 and 50 mg L^{-1} of Cr(VI) in the media resulted in final COD of 721, 791 and 921 mg L^{-1} from an initial COD of 3000 mg L^{-1}. The COD reduction was

insignificant when the concentration of Cr(VI) in the media was increased beyond 50 mg L^{-1}. The decrease in the removal of COD in the presence of the higher concentration of Cr(VI) was possibly due to the inhibitory effect of the Cr(VI). Substrate utilization is a metabolic process. Cr(VI) is a toxic substance, presence of the Cr(VI) affects the growth of the bacteria as the biomass concentration decreased with increase in Cr(VI) concentration, this in turn affected the substrate utilization ability of the *Halomonas sp*.

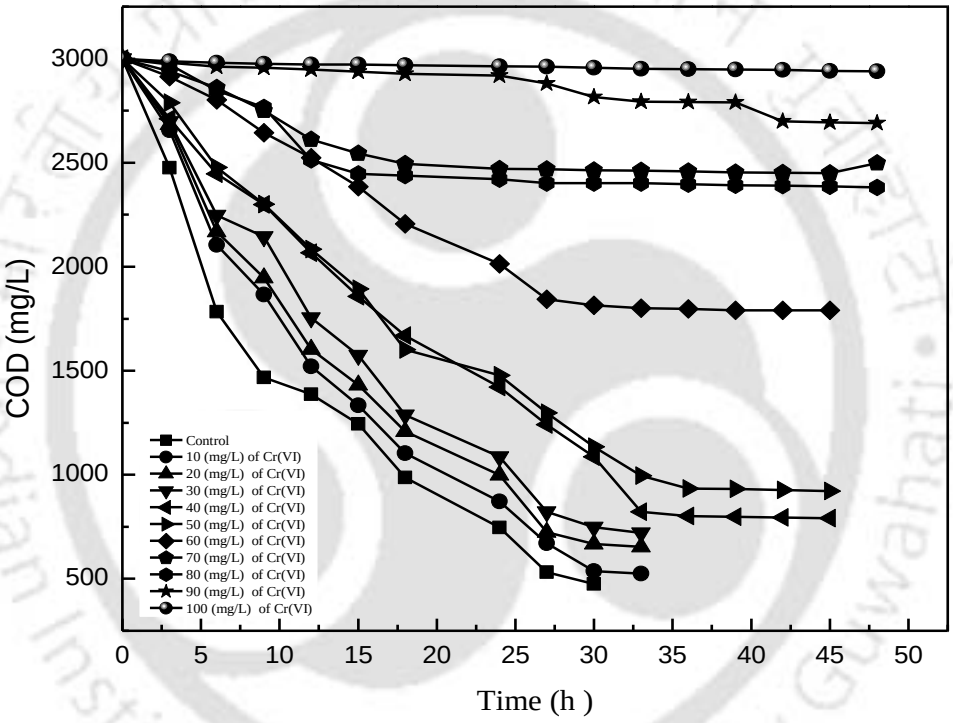

Fig. 7.3. COD removal in batch studies

The bioreduction of Cr(VI) was studied in eleven phases. The influent Cr(VI) concentration to the reactor was varied from 10 to 100 mg L^{-1}. It was observed that the HRT did not had any significant effect on the reduction for initial Cr(VI) concentration

ranging up to 30 mg L^{-1} (Table 7.1). The reduction of Cr(VI) reported with 12 h and 6 h for an initial Cr(VI) concentration was less. It was due to the fact that the reduction was a metabolism dependant process and the organism needed sufficient time for the growth and there by reduction of Cr(VI). The percentage reduction of Cr(VI) was found to decrease with increase in Cr(VI) concentration beyond 50 mg L^{-1}.

A minimum of 5.68 % reduction of Cr(VI) concentration was reported for an initial Cr(VI) concentration of 100 mg L^{-1}. An initial Cr(VI) concentration of 60, 70 and 80 mg L^{-1} in the influent resulted in 50, 33 and 14 % bioreduction of Cr(VI) respectively. A maximum of 99.94 % of Cr(VI) reduction was reported for an initial Cr(VI) concentration of 10 mg L^{-1}. It was observed that when the Cr(VI) concentration in the reactor was decreased from 100 mg L^{-1} to 10 mg $L^{-1}$the Cr(VI) reduction recovered drastically. The results suggest that higher metabolic activity has significant effect on the reduction of Cr(VI). The metabolic activity of the cells are drastically affected by the presence of Cr(VI) in the media. A maximum of 4.3X 10^{14} colonies were reported for Cr(VI) loading rate of 240 mg L^{-1} day^{-1}. The number of attached colonies was also found to be maximum (2013 mg) for Cr(VI) loading rate of 240 mg L^{-1} day^{-1} (Table 7.2). As the Cr(VI) was increased further the number of colonies decreased drastically. Only 21 cells were observed when the Cr(VI) loading rate was 1920 mg L^{-1} day^{-1}. The number of viable colonies obtained with a Cr(VI) loading rate of 2160 mg L^{-1} day^{-1} was insignificant as only 3 viable colonies were visible (Table 7.2). The attached and suspended biomass reported with different Cr(VI) loading rate of 1920, 2160 mg L^{-1} day^{-1} was 41, 60 and 6, 4 mg respectively. No viable cells were observed with further increase in the Cr(VI) loading.

Table 7.1. Performance of the fixed film bioreactor operated in batch mode

Phase	Days	Batch time (h)	Influent Cr(VI) concentration (mg L^{-1})	Effluent Cr(VI) concentration (mg L^{-1})	Effluent Cr(III) concentration (mg L^{-1})	DO (mg L^{-1})	Reduction (%)
I	1 to 3	24	10	0.06	9.94	3.71	99.4
	3 to 5	12	10	0.09	9.91	2.74	99.1
	5 to 7	6	10	0.121	9.879	2.72	98.79
II	1 to 3	24	20	0.14	19.86	2.8	99.3
	3 to 5	12	20	1.29	18.71	2.5	93.55
	5 to 7	6	20	2.32	17.68	2.1	88.4
III	1 to 3	24	30	1.46	28.54	2.7	95.13
	3 to 5	12	30	2.52	27.48	2.5	91.6
	5 to 7	6	30	4.71	25.29	2.1	84.3
IV	1 to 3	24	40	2.91	37.09	2.9	92.72
	3 to 5	12	40	4.26	35.74	2.7	89.35
	5 to 7	6	40	8.44	31.56	2.8	78.9
V	1 to 3	24	50	9.14	40.86	2.1	81.72
	3 to 5	12	50	14.16	35.84	2.1	71.68
	5 to 7	6	50	21.82	28.18	2.1	56.36
VI	1 to 3	24	60	29.48	30.52	3.3	50.86
	3 to 5	12	60	37.41	22.59	3.1	37.65
	5 to 7	6	60	46.89	13.11	3.5	21.85
VII	1 to 3	24	70	46.38	23.62	3.7	33.74
	3 to 5	12	70	59.14	10.86	3.5	15.51
	5 to 7	6	70	63.88	6.12	3.7	8.74

	1 to 3	24	80	68.66	11.34	3.7	14.17
	3 to 5	12	80	74.32	5.68	3.4	7.1
VIII	5 to 7	6	80	76.89	3.11	3.4	3.88
	1 to 3	24	90	80.88	9.12	3.1	10.13
	3 to 5	12	90	83.14	6.86	3.4	7.62
IX	5 to 7	6	90	83.88	6.12	3.7	6.8
	1 to 3	24	100	94.32	5.68	2.7	5.68
	3 to 5	12	100	94.31	5.69	2.7	5.69
X	5 to 7	6	100	94.32	5.68	2.7	5.68
	1 to 3	24	10	0.06	9.94	2.7	99.4
	3 to 5	12	10	0.08	9.92	2.7	99.2
XI	5 to 7	6	10	0.08	9.92	2.7	99.2

Table 7.2. Biological activity in the bioreactor operated in batch mode

Biomass distribution	Total suspended cells (mg)	Attached cells (mg)	Viable Cell count
I	238	2013	$4.3X\ 10^{14}$
II	234	1028	$6.1\ X\ 10^{11}$
III	166	1016	$3.4x\ 10^{9}$
IV	134	941	$2X\ 10^{6}$
V	96	719	$1X10^{4}$
VI	96	708	$1X10^{2}$
VII	94	296	$2X10^{1}$
VIII	41	60	21
IX	6	4	3
X	6	Not detectable amount	Not detectable amount
XI	263	Not detectable amount	Not detectable amount

7.3.2 Continuous operation

The reactor was operated with HRT of 24 h. An initial influent Cr(VI) concentration of 10 mg L^{-1} was fed to the reactor. The percentage reduction obtained with an initial Cr(VI) concentration of 10 and 20 mg L^{-1} was near 100%. When the concentration of Cr(VI) in the influent was increased to 30 and 40 mg L^{-1} the effluent Cr(VI) concentration was found to be 2.94 and 3.49 mg L^{-1} respectively. When the concentration of Cr(VI) was increased beyond 40 mg L^{-1} the percentage reduction was drastically affected. The Cr(VI) concentration in the effluent was found to increase for

an influent Cr(VI) concentration beyond 40 mg L^{-1}. A maximum of 96.12 mg L^{-1} of effluent Cr(VI) concentration was reported for an initial Cr(VI) concentration of 100 mg L^{-1} (Fig. 7.4). The drop in percentage reduction with increased influent Cr(VI) was due to the loss of biological activity of the cells. Increase in the Cr(VI) concentration affected the growth of the *Halomonas sp* which resulted in the decreased percentage reduction of Cr(VI).

The COD removal was also dependant on the influent Cr(VI) concentration. A maximum of 94.7 % of COD removal was attained when the initial Cr(VI) was 10 mg L^{-1}. The COD removal percentage decreased with increase in Cr(VI) concentration. The COD removal attained on day 3, day 4 and day 5 was found to be 78, 75, 73 % respectively (Fig. 7.5). The percentage reduction of COD reduction attained on day 9 and 10 are found to be 10.32 and 2 % respectively. The influent Cr(VI) concentration to the reactor on day 9 and day 10 of the study was in the range of 60 to 100 mg L^{-1}. The decrease in the COD removal after day 6 of the study was due to the fact that the higher influent Cr(VI) concentration affected the growth of *Halomonas sp*. The organism was unable to sustain under such a high chromium load and that affected the substrate utilization (COD) of the *Halomonas sp.*

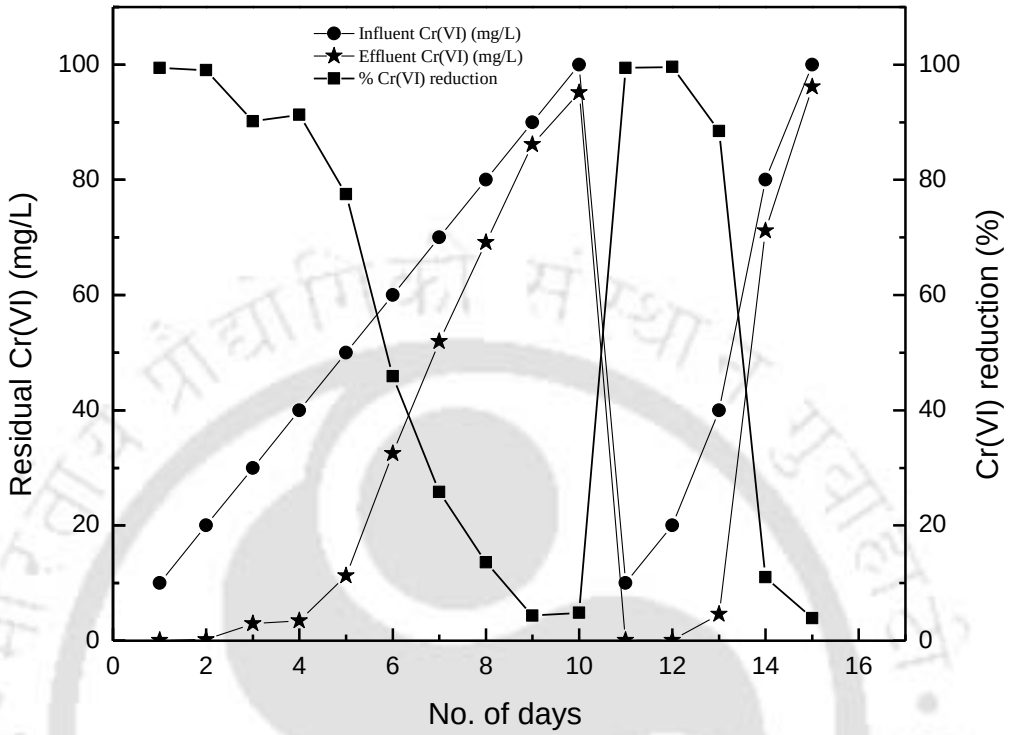

Fig. 7.4. Cr(VI) reduction in continuous reactor

Cr(VI) reduction was studied in fifteen phases. The HRT for the phase I to X was maintained at 24 h. The HRT for the phase XI to XV was maintained at 12 h. The DO was found to be 3.1 ± 0.4. Complete reduction of Cr(VI) was obtained for 10 and 20 mg L^{-1} of Cr(VI). The percentage reduction obtained was 91.275 and 90.2 for phase III and IV (Table 7.3). The percentage reduction was found to decrease drastically from phase VI as the influent Cr(VI) concentration was above 60 mg L^{-1}. The effluent Cr(VI) concentration matched with the influent Cr(VI) during the IX^{th} and X^{th} phase of the study. No significant Cr(VI) reduction was observed during phase IX and X . It was due to the loss of biological activity in the reactor. It can be observed from the Table 7.4 that the cell count was near 0 for Phase IX and X. It was observed that the biological activity

regained when the Cr(VI) loading rate was reduced from 2400 mg L^{-1} day^{-1} for 240 mg L^{-1} day^{-1}.

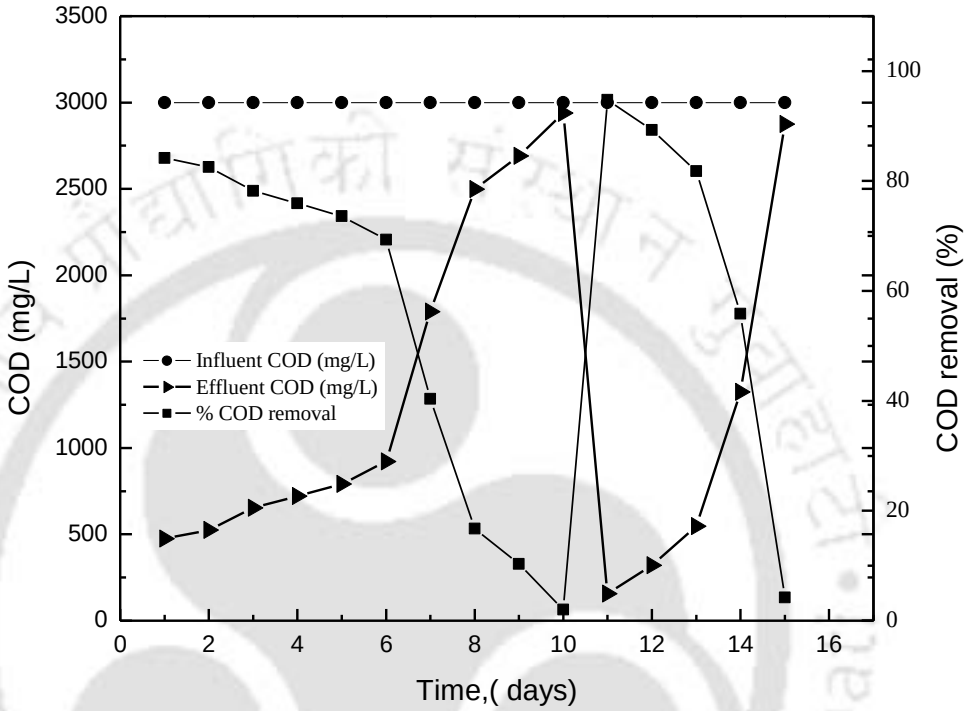

Fig. 7.5. COD removal in continuous reactor

Table 7.3. Performance of the fixed film bioreactor operated in continuous mode

Phase	Days	Batch time (h)	Influent Cr(VI) concentration (mg L^{-1})	Effluent Cr(VI) concentration (mg L^{-1})	Effluent Cr(III) concentration (mg L^{-1})	DO (mg L^{-1})	Reduction (%)
I	0-1	24	10	0.067	9.933	2.7	99.33
II	1 - 2	24	20	0.089	19.91	2.7	99.55
III	2 - 3	24	30	2.94	27.06	2.7	90.2
IV	3 - 4	24	40	3.49	36.51	3.1	91.27
V	4 - 5	24	50	11.26	38.74	3.1	77.48
VI	5-6	24	60	32.46	27.54	3.1	45.9
VII	6-7	24	70	51.92	18.08	2.5	25.82
VIII	7-8	24	80	69.14	10.86	2.5	13.57
IX	8-9	24	90	86.11	3.89	2.5	4.32
X	9-10	24	100	95.16	4.84	2.1	4.84
XI	10-12	12	10	0.06	9.94	2.1	99.4
XII	12-14	12	20	0.08	19.92	2.1	99.6
XIII	14-16	12	40	4.61	35.39	3.3	88.47
XIV	16-20	12	90	81.18	8.82	3.3	9.8
XV	20-24	12	100	96.12	3.88	3.1	3.88

Table 7.4. Biological activity in the bioreactor operated in continuous mode

Biomass distribution	Total suspended cells (mg)	Attached	Cell count
I	269	2542	6.2×10^{16}
II	249	2568	3.8×10^{14}
III	212	1949	3.4×10^{8}
IV	208	1962	1.6×10^{2}
V	184	708	1×10^{2}
VI	93	321	241
VII	84	247	210
VIII	33	84	21
IX	8	16	3
X	8	3	3
XI	247	2439	2×10^{12}
XII	238	2439	3×10^{9}
XIII	169	1841	1×10^{2}
XIV	84	116	21
XV	12	12	2

Glucose was the sole carbon used for the growth and reduction of Cr(VI) by *Halomonas sp*. Complete reduction of Cr(VI) was obtained when the glucose concentration was in the range of 1000 mg L^{-1} (Fig. 7.6). When the glucose concentration was increased the cumulative Cr(VI) concentration reduced was found to increase. It was evident that the glucose concentration is not a limiting factor in the reduction of Cr(VI) in the bioreactor.

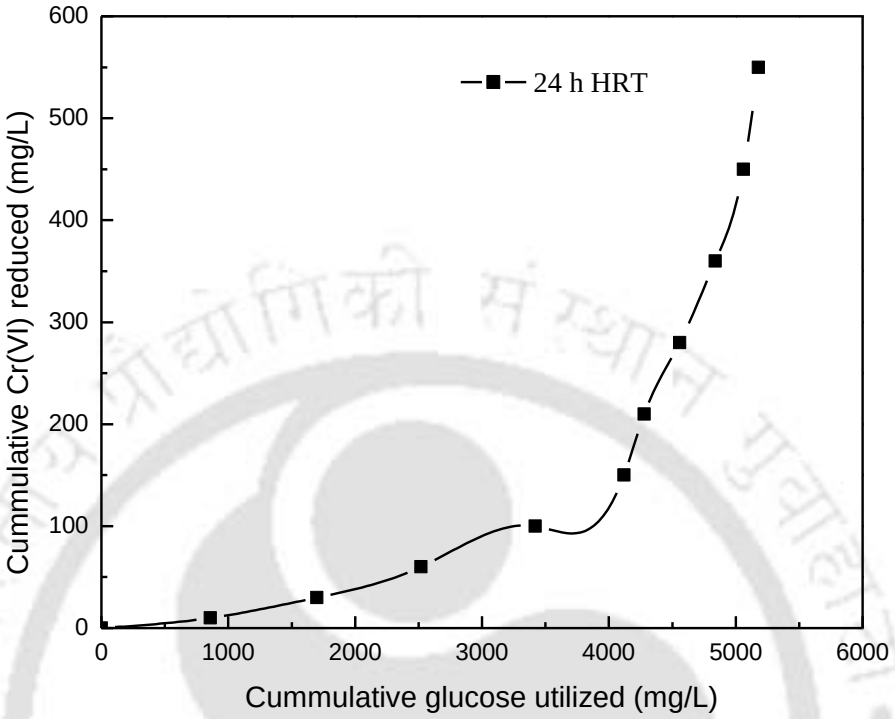

Fig. 7.6. Relationship between cumulative Cr(VI) reduced and cumulative glucose utilized

7.3.3 Determination of biokinetic parameter

The maximum specific growth rate, half saturation constant was calculated using Monod's equation. The inhibition constant K_I was determined by using the equation (2) [11].

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

$$\mu = \frac{\mu_{max} S}{K_S + S}\left(\frac{K_I}{K_I + Cr(VI)}\right) \qquad (2)$$

where μ, μ_{max} are the specific growth rate (h^{-1}) and maximum specific growth rate (h^{-1}), K_s is the half saturation constant of COD (mg L^{-1}), S is the COD (mg L^{-1}), Cr(VI) is the hexavalent concentration (mg L^{-1}), K_I is the inhibition constant (mg L^{-1}). The yield coefficient was calculated from the ratio of the specific growth rate to the substrate utilization rate. The kinetic parameters obtained are tabulated (Table 7.5). The maximum specific growth rate obtained was 7.16 mg L^{-1}. The half saturation constant for COD was 475 mg L^{-1}. The yield coefficient was found to be 0.209. The inhibition constant was found to be 4.07 mg L^{-1}. K_I specifies the concentration of chromium in which the growth rate of the microbe was found to decrease.

Table 7.5. Biokinetic parameters

Biokinetic parameters	
μ_{max}	7.16
K_s	475
K_I	3
Y_T	0.20
k_d	0.005

7.4. Conclusion

Halomonas sp showed an optimal growth in the batch and continuous reactor. Near complete reduction of Cr(VI) was reported for an initial Cr(VI) concentration of 10 and 20 mg L^{-1}. Glucose did not limit the bioreduction process. Influent Cr(VI) concentration is the limiting factor in the batch and continuous reactor. The continuous reactor was operated for 24 days. The DO concentration was found to be in the range of

3.1±0.4 mg L^{-1}. The COD reduction obtained was 84.1 %. The biokinetic parameter was evaluated, the maximum specific growth rate reported was 7.16 mg L^{-1}, and the K_I reported was 4.07 mg L^{-1}. The increase in the influent Cr(VI) concentration increased the lag period of the *Halomonas sp*. The results obtained showed that the fixed film reactor has a great potential in the reduction of Cr(VI).

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Chapter VIII

Conclusion and Future Scope

This chapter summarizes the most important results of the present investigation. The prospect for continuing further study by implementing advancements to the current study is also discussed.

8.1. Conclusion

In the first part of the present study, bioreduction of Cr(VI) was performed with *Phanerochaete chrysosporium*, a white rot fungus. The fungus was procured from Institute of Microbial Technology, Chandigarh, India. The organism was maintained on Malt extract agar media. It was observed that the bioreduction of Cr(VI) was dependant on the physical parameters viz., pH and initial Cr(VI) concentration. A maximum of 98.29% of Cr(VI) reduction was obtained for an initial Cr(VI) concentration of 10 mg L^{-1} at pH 6. The bioreduction capacity of the immobilized cells of the *P. chrysosporium* was also carried out. Higher cell concentrations (2 X 10^8) was essential for significant reduction of Cr(VI). The immobilized cells *P. chrysosporium* reported a maximum of 98.3% of Cr(VI) reduction at pH 5 and 25 ºC. Ca alginate was optimized to be the best suitable matrix for immobilization studies using *P. chrysosporium*. The mechanism of Cr(VI) by the white rot fungus was elucidated. The detoxification of Cr(VI) was found to be a two step process. The initial step in detoxification of Cr(VI) involves the charge based interaction of Cr(VI) with the functional groups on the cell wall. The SEM showed the surface binding of Cr ions on to *P. chrysosporium* cell wall. FTIR studies confirmed that the amide and amine groups of the chitin and the polysaccharides were the functional groups involved in the surface binding of the Cr with the cell wall. The second step

involves the intracellular accumulation of Cr(VI). The TEM coupled with the EDX showed that the Cr ions were found in the cell wall as well as in the cytoplasm.

In the next stage *Halomonas sp,* a halophilic bacteria was used for the bioreduction of Cr(VI) as the organism has ability to grow under alkaline condition. The culture was purchased from Institute of Microbial Technology, Chandigarh, India. The growth media was optimized. A modified media containing (YEPG-NaCl) for the growth and reduction of the Cr(VI) was designed. The reduction capacity of the culture was found to be 93.3% for an initial concentration of 40 mg L^{-1}. Yeast extract was essential for the growth and bioreduction of Cr(VI). pH 7 was found to be optimal for the reduction of Cr(VI) with the free cells of *Halomonas sp*. The CFE was effective in reducing 40 mg L^{-1} of Cr(VI) and yielded 89.65% within 6 h. The optimum pH for bioreduction using CFE was 6.5 at 37 °C. NADPH, NADH were the suitable electron donors for the CFE during bioreduction of Cr(VI). Presence of metabolic inhibitors (NaN_3, KCN, PCP) and heat treatment decreased bioreduction capacity of the bacteria. The bioreduction of Cr(VI) using the immobilized cells of the *Halomonas sp* was also reported. A maximum of 98.9 % of bioreduction was reported for 10 mg L^{-1} of initial Cr(VI) concentration. The stability of the beads are necessary for the bioreduction using immobilized cells of *Halomonas sp*. The immobilization provides advantage as the separation of the cells is easier and the cells can be reused. Na alginate was best suitable matrix.

Investigation on Cr(VI) reduction was conducted using a novel bacteria isolated from soil samples contaminated with tannery waste. The 16s rDNA sequence placed the isolated culture close to *Bacillus cereus*. The BIOGEN plate III also confirmed that the isolated culture closely matched *Bacillus cereus*. A phylogenetic tree was constructed with 1000 iterations showed maximum similarity to *Bacillus cereus* (827 times out of 1000 iterations). The similarity search conducted using BLAST indicated that the isolated culture showed more similarity to *Bacillus cereus* RG-08/06; EF195169 with 4 gaps and 0 mismatches. Near complete reduction of Cr(VI) was obtained for concentration of Cr(VI) ranging from 10 mg L^{-1} to 50 mg L^{-1}. CFE reduced 60 mg L^{-1} of Cr(VI) to 1.88

mg L^{-1} within 6 h. NADPH was the suitable electron donor for Cr(VI) reduction with CFE. The optimum pH for the free cells and CFE of the *Bacillus cereus* was found to be 6 and 6.5 respectively.

Finally the bioreduction studies were performed in a batch and continuous reactor using *Halomonas sp*. The reactor was made from pyrex glass. The reactor bed was packed with 19241 glass beads of diameter 3 mm to provide an external surface area of 1631.6 cm^2 for the growth of the *Halomonas sp*. The DO concentration in the continuous reactor was found to be 3.1±0.4 mg L^{-1}. A maximum of 84% of COD reduction was obtained. Near complete reduction of Cr(VI) was obtained for an initial Cr(VI) concentration of 10 – to 20 mg L^{-1} at a HRT of 24 h.

In a bottom line, the whole study showed the bioreduction of Cr(VI) with fungi, halophilic bacteria and an isolated culture. Initially *P. chrysosporium*, a less exploited white rot fungi was tested for Cr(VI) reduction in its live form where it could reduce 10 mg L^{-1} Cr(VI) completely. As the bioreduction with the fungi was a slow process, *Halomonas sp, a* halophilic bacteria capable of growing in saline condition was investigated. *Halomonas sp.* reduced Cr(VI) from 10-50 mg L^{-1} completely. Immobilization of the *P. chrysosporium* as well as *Halomonas sp* was carried out to provide more stability and ease of separation of cells during the bioreduction process. However it was found that in both the cases immobilization does not give good results. The main reason for this was the degeneration of immobilized beads during bioreduction process. *Bacillus cereus* isolated from contaminated soil was applied for Cr(VI) reduction as the bacteria was naturally adapted to the toxicity of the Cr(VI) in the environment. This isolated culture also reduced Cr(VI) from 10-50 mg L^{-1} completely. The cell free extracts of the *Halomonas sp* and the *Bacillus cereus* were more efficient as they could reduce Cr(VI) up to 60 mg L^{-1} and the reduction of Cr(VI) was faster than free cells. Packed bed reactor study was also conducted with an aim to provide better aeration for bacteria. The potential of *Halomonas sp* in continuous study was carried out in a fixed film reactor. *Halomonas sp* in the fixed film reactor thus reduced Cr(VI) and COD simultaneously. From all these study it was observed that microbial reduction of Cr(VI)

can be applied for the treatment of wastewater containing Cr(VI) in the range of 10-60 mg L^{-1} in an more economical and environmental friendly way.

8.2. Future scope

This work was focused on reduction of Cr(VI) to Cr(III). By doing so the toxicity of wastewater comes down to permissible limits, however Cr(III) remains in the wastewater. More work should be done to remove Cr(III) from wastewater after bioreduction u various methods. From the findings of the present work, it was inferred that there is enough scope for carrying future work on bioreduction of Cr(VI).

- The bioreduction capacity of the bacteria and fungi can be increased by methods like protoplast fusion, gene mutation.
- The role different cell organelles on the bioreduction of Cr(VI) can be investigated, there by the complete mechanism of the bioreduction can be understood.
- The purification of the cell free extracts can be performed in order to improve the bioreduction capacity of the CFE.
- Immobilization of CFE can be performed; there by the CFE can be easily recovered and reused.
- The exopolysaccharides produced by the microbes can be tested for Cr(VI) reduction.

Research Output

In Peer-reviewed Journals

S. Murugavelh, K. Mohanty, Bioreduction of Hexavalent Chromium by Live and Active *Phanerochaete chrysosporium*: Kinetics and Modeling, **CLEAN: Soil, Air, Water**, 40, 2012, 746-751.

S. Murugavelh, K. Mohanty, Bioreduction of hexavalent chromium by free cells and cell free
extracts of *Halomonas sp*., **Chemical Engineering Journal**, 203, 2012, 415-422.

S. Murugavelh, K. Mohanty, Bioreduction of Cr(VI) using live and immobilized Phanerochaete chrysosporium, **Desalination and Water Treatment** (Article in Press)

S. Murugavelh, K. Mohanty, Mechanism of Cr(VI) bioaccumulation by *Phanerochaete Chrysosporium*, **Environmental Engineering and Management Journal** (Article in Press)

S. Murugavelh, K. Mohanty, Bioreduction of chromate by immobilized cells of *Halomonas* sp., **International Journal of Energy and Environment** (Article in Press)

Submitted and under review

S. Murugavelh, K. Mohanty, Isolation, identification and characterization of Cr(VI) reducing *Bacillus cereus* from chromium contaminated soil.

S. Murugavelh, K. Mohanty, Performance of *Halomonas sp* to reduce hexavalent chromium in batch and continuous fixed film reactor.

Conference Presentations

S. Murugavelh, K. Mohanty, Cr(VI) reduction by *Bacillus cereus* isolated from chromium contaminated soil, TEZCON-2012, Tezpur, 6-8 November, 2012.

S. Murugavelh, K. Mohanty, Cr(VI) reduction by indigenous bacteria isolated from Cr rich wastewater, CHEMCON-2011, Bangalore, 27th -29th December, 2011.

S. Murugavelh, K. Mohanty, Biological Reduction Cr(VI) by Halomonas Sp. International Congress of Environmental Research (ICER 2011), Surat, 15th -17th December, 2011.

S. Murugavelh, K. Mohanty, Mechanism of Cr(VI) Bioaccumulation by Phanerochaete Chryosporium, International Conference on Recent Advances in Chemical Engineering and Technology (RACET 2011), Cochin, 10-12 March, 2011.

S. Murugavelh, K. Mohanty, Cr(VI) Bioaccumulation Using Live and Immobilized Phaenerohaete Chrysosporium, CHEMCON 2010, 27-29 December 2010, Annamalai Nagar, India.

S. Murugavelh, K. Mohanty, Bioreduction of hexavalent chromium by immobilized P. chrysosporium, GIST 2010, 26-27 December, 2010, Pune, India.

S. Murugavelh, K. Mohanty, Bioaccumulation of Cr(VI) by growing Phanerochaete chrysosporium: Growth Kinetics, National Conference on Biochemical Engineering: Present Scenario and Future Prospective, 12-13 March, 2010, Vallabh Vidyanagar, Guajarat.

Summary

Heavy metals are extremely toxic and remain persistent in natural environments. Human activities like mining and dumping industrial waste have resulted in heavy metal pollution in soil, surface water as well as ground water. Heavy metals cause a variety of acute and chronic effects in plants, animals, humans and other vertebrates. In a developing country like India due to industrial development, there is an increased trend in the amount of heavy metal pollution. It is essential to make a scientific assessment on heavy metal pollution and treat the heavy metal polluted wastewater. Chromium is one of the most toxic heavy metal which is discharged in to the environment through industrial activities. Chromium is released in to the environment by a large number of industries including chrome plating, petroleum refining, leather tanning, wood preservation, textile manufacture, and pulp processing. Chromium exists in two stable oxidation states, Cr(VI) and Cr(III). Cr(VI) is more soluble, mobile and most toxic. Cr(VI) is mostly found as oxyanions. Cr(III) is less toxic, less soluble and less mobile. It is found as oxides, hydroxides or sulfates, generally bound to organic matter. Cr(VI) is a strong oxidizing agent and mutagen. United States Environmental protection Agency (USEPA) has listed Cr(VI) as a carcinogen [6]. The discharge of Cr(VI) to the surface water is regulated below 0.05 mg L^{-1} by the USEPA. Therefore it is necessary to reduce the toxicity of the Cr(VI) before being discharged in to the environment. Conventionally Cr(VI) is treated by chemical reduction, precipitation, sorption on to different materials, membrane filtration and microbial treatment. All these methods have advantages and disadvantages. The major drawback of these conventional treatment methods for Cr(VI) contaminated water include high energy expenditure, use of expensive and toxic chemical reductants as well as inefficient removal of low concentrations of Cr(VI) in wastewater. The search for innovative methods thus lead to the development of biological methods for the treatment of Cr(VI). Biological treatment of wastewater is a more attractive option, the biological processes are relatively cheap and environmentally compatible.

Microbial detoxification is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. Significant advances in the understanding of microbe-metal interactions have been made in recent years. Microbes present in the Cr(VI) contaminated sites have their ability to reduce Cr(VI). It includes bacteria, fungi, algae, actinomycetes etc. Microbial reduction of Cr(VI) can be attained directly by microbial metabolism or by bacterial metabolites such as enzymes. Microbes develop resistance to Cr(VI) for their survival under chromium toxicity. One such resistance mechanism is the production of enzymes which reduces the toxicity of the Cr(VI) for the better survival. Bioreduction of Cr(VI) is a low-cost and environment friendly method which generally have a high public acceptance and can often be carried out easily.

Thus, based on the above brief discussion, it seems that there is enough scope to carry out further work on bioreduction of Cr(VI) using free cells, immobilized cells and metabolic products of the microbes. To achieve this, present work was carried out with the following objectives:

- To study the Cr(VI) bioreduction capacity of by live and active *Phanerochaete chrysosporium* and to optimize the physical parameters such as pH, temperature, substrate concentration, initial Cr(VI) concentration etc.
- Cr(VI) reduction using the immobilized live cells of *Phanerochaete chrysosporium*, optimization of substrate concentration, pH, temperature, biomass loading rate, initial Cr(VI) concentration and best suitable matrix.
- Evaluating the possible mechanism of Cr(VI) removal by *Phanerochaete chrysosporium* using various methods such as FTIR, SEM and TEM.
- To study the bioreduction capacity of free cells, cell free extracts (CFE) of *Halomonas sp*, the role of electron donors on the percentage reduction of Cr(VI), inhibitory effect of metabolic inhibitors on Cr(VI) bioreduction.
- To study the Cr(VI) reduction by immobilized cells of *Halomonas sp*, optimization of substrate concentration, pH, temperature, biomass loading rate, initial Cr(VI) concentration and best suitable matrix.

- Isolation, identification and characterization of Cr(VI) reducing microorganism from chromium contaminated soils, 16SrDNA sequencing, biochemical characterization, phylogenetic analysis, optimization of media and physical parameters such as pH and temperature. Evaluating the role of CFE of isolated culture on bioreduction is also planned.

- Packed bed reactor study using *Halomonas sp* with the optimized media. Evaluation of performance of the reactor under batch and continuous mode along with optimization of HRT, biomass (suspended and attached), DO and COD.

Thesis Outline

The outline of the thesis is presented below,

Chapter I: Introduction and Literature Review

Chapter II: Bioreduction of Cr(VI) by Live and Active *Phanerochaete chrysosporium*

Chapter III: Bioreduction of Cr(VI) using Live and Immobilized *Phanerochaete chrysosporium*

Chapter IV: Bioreduction of Cr(VI) by Free Cells and Cell Free Extracts of *Halomonas sp.*

Chapter V: Bioreduction of Cr(VI) by Immobilized Cells of *Halomonas sp*

Chapter VI: Isolation, Identification and Characterization of Cr(VI) Reducing *Bacillus cereus* from chromium contaminated soil

Chapter VII: Performance of *Halomonas sp* to Reduce Cr(VI) in Batch and Continuous Fixed Film Reactor

Chapter VIII: Conclusion and Future Scope

A brief description of the content of each of the chapters is furnished below:

Chapter I: Introduction and Literature Review

In this chapter a brief discussion on heavy metal pollution, the chemistry of chromium, the toxicity of the Cr(VI) and the conventional methods available for the treatment of Cr(VI) was discussed. The significance of biological methods for the treatment of Cr(VI) was discussed. From the literature study it was observed that the biological methods possess more benefits in treatment of Cr(VI) contaminated wastewater. The work plan was framed on the basis of the literature survey.

Chapter II: Bioreduction of Cr(VI) by Live and Active *Phanerochaete chrysosporium*

In this work the potential of live and active *Phanerochaete chryosporium*, a white rot fungi to remove lower concentration of Cr(VI) from aqueous solutions was reported. It was observed that the medium pH had significant role on the growth of the fungus and bioreduction of Cr(VI). Substrate inhibition on the growth of *P. chrysosporium* was evident beyond 20 g L^{-1} of dextrose concentration. A maximum biomass concentration of 15.64 g L^{-1} was obtained for an initial dextrose concentration of 20 g L^{-1} in metal free medium at pH 6.0. An increase in Cr(VI) concentration beyond 10 mg L^{-1} inhibited the growth of the fungi thereby reducing the chromium bioremoval efficiency. A maximum reduction efficiency of 98.92 % was reported for an initial metal concentration of 10 mg L^{-1}. A mathematical expression for the bioreduction of Cr(VI) considering the organic compounds in the cells was proposed. Thus *P. chrysosporium* can be suitably used for the bioreduction of lower concentration of chromium ions from water and wastewater in an environment friendly way.

Chapter III: Bioreduction of Cr(VI) using Live and Immobilized *Phanerochaete chrysosporium*

In this chapter the bioreduction capacity of the live and immobilized *P. Chrysosporium* was investigated. The basidiospores of the white rot fungi *P. Chrysosporium* were immobilized in different matrices viz., Na alginate, Acryl amide and Agar. Of the various dosages of inoculums studied for each matrix 10% (v/v) (Na alginate, Acryl amide) was found to be optimum for growth and reduction of Cr(VI). The effects of the physical parameters such as glucose concentration, pH, and temperature on bioreduction were investigated. A maximum of 98.3% of bioreduction of Cr(VI) was obtained with an initial Cr(VI) concentration of 10 mg L^{-1} at pH 5 and temperature of 25 ^{o}C. The best suitable matrix was optimized to be Na alginate. An enzyme based model was also studied.

Chapter IV: Bioreduction of Cr(VI) by Free Cells and Cell Free Extracts of *Halomonas sp*

In this chapter the bioreduction of Cr(VI) from aqueous solutions by *Halomonas sp*, a halophillic bacteria was reported. Medium pH had significant effect on the bioreduction of Cr(VI). Glucose at 1000 mg L^{-1} was essential for growth of *Halomonas sp* as well as bioreduction of Cr(VI). A maximum specific Cr(VI) reduction rate of 0.74 mg g^{-1} h^{-1} was obtained for an initial Cr(VI) concentration of 40 mg L^{-1}. A maximum of 98.6 % reduction of Cr(VI) was reported for an initial concentration of 10 mg L^{-1}. Other initial Cr(VI) concentrations studied viz. 20, 30 and 40 mg L^{-1} resulted in 96.3%, 94.4% and 93.3% of Cr(VI) reduction respectively. pH 7 was found to be optimum for the bioreduction of Cr(VI) with free cells of *Halomonas sp*. Cell free extracts reported a maximum reduction efficiency of 89.65% for an initial metal concentration of 40 mg L^{-1}. The optimum pH for CFE on Cr(VI) reduction was 6.5. NADPH and NADH were found to be the best electron donors. EDTA, NaN_3, KCN, PCP inhibited the reduction of Cr(VI) by cell free extract.

Chapter V: Bioreduction of Cr(VI) by Immobilized Cells of *Halomonas sp*

In this chapter, the bioreduction of Cr(VI) by immobilized cells of *Halomonas sp* was reported. Ca alginate, acryl amide and agar were tested as the matrices for immobilization. Ca alginate was found to be the suitable matrix among the different matrices studied. Of the various dosages of inoculum studied 2 g L^{-1} was found to be the optimum. Glucose at 1 g L^{-1} was completely utilized by the immobilized *Halomonas sp* even in the presence of Cr(VI) at 40 mg L^{-1}. The optimum pH for the bioreduction of Cr(VI) by immobilized *Halomonas sp* was found to be pH 6. The mechanical strength of the beads plays an essential role in the bioreduction process. *Halomonas sp* entrapped in a alginate matrix reported a maximum of 98.9% of reduction for an initial Cr(VI) concentration of 10 mg L^{-1}. The alginate beads can be reused for 3 times with slight drop in the percentage reduction. The presence of other metals decreased the bioreduction percentage.

Chapter VI: Isolation, Identification And Characterization of Cr(VI) Reducing *Bacillus cereus* From Chromium Contaminated Soil

In this chapter the bioreduction of Cr(VI) was investigated using a bacterial strain isolated from soil samples contaminated with tannery wastewater. The isolated species was found to be *Bacillus cereus.* Glucose was found to be the suitable electron donor for the Cr(VI) reduction with free cells of the isolated culture. Glucose yielded a maximum specific reduction rate of 0.83 mg g^{-1} h^{-1} for an initial Cr(VI) concentration of 60 mg L^{-1}. Near complete reduction of Cr(VI) was reported for an initial Cr(VI) from 10-50 mg L^{-1}. An initial Cr(VI) concentration of 60 and 70 mg L^{-1} reported 96.7 and 72.1 % reduction of Cr(VI) with free cells of the isolated culture. pH 6 was the optimum pH for the reduction of Cr(VI) with free cells of *Bacillus sp*. The cell free extracts (CFE) reported a maximum of 96 % reduction for an initial Cr(VI) concentration of 60 mg L^{-1}. NADPH and NADH were the suitable electron donors for Cr(VI) reduction with CFE. The optimum pH for reduction of Cr(VI) with CFE was pH 6.5. KCN, PCP, NaN_3 and EDTA were the inhibitors of Cr(VI) reduction with CFE.

Chapter VII: Performance of *Halomonas sp* to Reduce Cr(VI) in Batch and Continuous Fixed Film Reactor

In this chapter the performance of fixed film bioreactor on bioreduction of Cr(VI) was evaluated. The reactor was operated under batch and continuous mode. Influent contained glucose as the single carbon source. The maximum specific growth rate obtained was 7.16 mg L^{-1}. The half saturation constant for COD was 475 mg L^{-1}. The yield coefficient was found to be 0.209. A maximum biomass of 457 h^{-1} was obtained for Cr(VI) free cells. Both the reactor performed better under an hydraulic retention time of 24 h. Near complete reduction of Cr(VI) was reported for an initial Cr(VI) concentration of 10 and 20 mg L^{-1}. The maximum number of suspended cells reported was 4.3 X 10^{14} at an Cr(VI) loading rate of 240 mg $L^{-1}day^{-1}$. The maximum amount of attached and suspended cells reported was 2013 and 238 mg. A maximum COD reduction of 84.1% was reported. The lag period for growth of cells under a Cr(VI) load ranging up to 40 mg L^{-1} was 3 h.

Chapter VIII: Conclusion and Future Work

This chapter summarizes the important results arrived from the present study. The later part of this chapter also focuses on the scope for the future work that can be carried out. Microbial reduction of Cr(VI) was performed using *Phanerochaete chrysosporium*, *Halomonas sp*, and the isolated cultures *Bacillus cereus*. The CFE *Halomonas sp* had a great potential in Cr(VI) reduction. Isolation of microbes from Cr(VI) contaminated soils was carried out. The isolated cultures *Bacillus cereus* was found to be gram positive. The free cells and CFE of the isolated cultures were tested for the ability to reduce Cr(VI). The final part of the work reports on the packed bed reactor studies conducted using *Halomonas sp*. It was found that HRT of 24 h was optimum for the reduction of Cr(VI). High cell concentration resulted in maximum reduction. The overall results indicate that the microbes have greater potential in Cr(VI) reduction.