Chromium Removal by *Tradescantia pallida* (Rose) D.R. Hunt: Batch and Continuous Studies

A THESIS

Submitted by

VIBHA SINHA

for the award of the degree

of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

GUWAHATI-781039, ASSAM, INDIA

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Dedicated to my Parents

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STATEMENT

I, hereby declare that the content embodied in this thesis entitled "**Chromium Removal by *Tradescantia pallida* (Rose) D.R. Hunt: Batch and Continuous Studies**" is the result of investigations carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Prof. Kannan Pakshirajan (Supervisor) and Prof. Rakhi Chaturvedi (Co-Supervisor).

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: ……. **Vibha Sinha**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Chromium Removal by *Tradescantia pallida* (Rose) D.R. Hunt: Batch and Continuous Studies**" by Miss. Vibha Sinha for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India, and this work has not been submitted elsewhere for a degree.

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Date: …… **Vibha Sinha**

ABSTRACT

Chromium (Cr) is one of the high priority heavy metal pollutants commonly present in soil, aqueous waste stream and groundwater and, therefore, it can cause serious health hazards. Water pollution by Cr is of considerable concern in many countries, including India as it is widely used in many industries. Hexavalent form of chromium [Cr(VI)] is considered highly hazardous for most organisms due to its potential mutagenic, carcinogenic and teratogenic effects. Therefore, it is essential to treat effluent from Cr utilising industries prior to its discharge into aquatic environment.

In comparison with the physico-chemical and biological processes employed to treat Cr containing wastewater, phytoremediation using indigenous and wild plant species is a more cost-effective, efficient and sustainable alternative. It also requires no special growth conditions particularly for treating large-scale, low-level Cr contamination.

The present thesis aimed to mitigate the environmental pollution due to the discharge of Cr(VI) containing industrial wastewater. The potential application of phytoremediation using indigenous *Tradescantia pallida* plants that can tolerate, absorb and significantly bioaccumulate Cr(VI) in their parts is demonstrated. This plant species could be used in constructed wetlands to enhance the Cr(VI) reduction and retention in the soil, thereby serving as an efficient system to treat Cr contaminated water.

The present research work is focused on studying the mechanism of Cr(VI) tolerance, uptake and transport, localization and storage in *T. pallida*. Kinetics, biochemical and factorial analysis of Cr uptake in a multi-ion system by *T. pallida* were further examined. Continuous studies employing a laboratory scale constructed wetland were carried out further to study the plants efficiency and its applicability to treat Cr contaminated water. Different indigenous plant species were initially screened for their Cr(VI) tolerance and uptake under batch hydroponic conditions. Among the different plants screened in this

study, *T. pallida* was selected based on its significant bio-concentration factor (40.8), translocation factor (0.759) and tolerance index (87%) towards Cr. In order to understand the mechanism of Cr uptake and tolerance by *T. pallida*, analyses of its antioxidant enzyme system and different biochemical parameters were carried out during its growth and in the presence of 5-20 mg/L Cr(VI) concentration in hydroponic environment for up to ca. 90 days. Maximum Cr(VI) bioaccumulation in *T.pallida* roots reached up to 536 μg/g dry weight after 60 days of hydroponic culture. Increased activities of antioxidant enzymes, particularly ascorbate peroxidase (9,823±25 U/mg protein) was found to be highly up-regulated, which showed their important role in overcoming the Cr-induced oxidative stress on the plant.

Many of the tannery and electrochemical industries discharge different types of wastewater into the environment primarily in the form of liquid effluents containing both organic matter and toxic chemicals. Among them, SO_4^{2-}, NO^{3-} and PO_4^{3-} ions are commonly present in industrial wastewater along with Cr oxyanions species that can effect each other removal. Thus, interaction effect and simultaneous removal of Cr(VI) in presence of co-ions viz SO_4^{2-}, NO^{3-} and PO_4^{3-} were evaluated. Removal of Cr(VI) was found enhanced in the presence SO_4^{2-} ions at a high initial Cr(VI) concentration (20 mg/L). At the optimized conditions of the process parameters, a maximum removal of 84% Cr(VI), 87% SO_4^{2-} , 94% NO^{3-} and 100% PO_4^{3-} was achieved with no visible phytotoxicity to *T. pallida*, thereby demonstrating an excellent tolerance of the plant towards Cr even in presence of the co-ions. Cr uptake mechanism and its subcellular distribution within *T. pallida* plant tissue were determined using different techniques, including atomic absorption spectroscopy (AAS), differential centrifugation and energy dispersive X-ray (EDX) analyses. Subcellular fractionation of Cr-containing tissues showed that most of the accumulated Cr was found in the root vacuoles and the plant cell

wall. Whereas the EDX spectra showed that the electron dense areas contained high Cr in these cell compartments. All these studies confirmed a very good potential of *T. pallida* plants in Cr(VI) removal from contaminated water.

Continuous Cr(VI) removal from synthetic wastewater by *T. pallida* was evaluated using a laboratory-scale vertical subsurface flow constructed wetland (CW) system. The effect of different parameters (pH, HRT and inlet Cr(VI) concentration) on the continuous Cr(VI) removal was carried out using the constructed wetland system. Best results were achieved for 2 d HRT and for an influent pH of 7 with a maximum Cr(VI) removal efficiency of 97.2-98.3%. This study showed the importance of influent pH which controls the Cr removal mechanism in the CW. At the same pH, a maximum total Cr removal efficiency in the range 86-88.2% was achieved. Distribution of Cr in CW revealed that the soil acts as the main sink for Cr, sequestering up to 40-68.41% of the total Cr supplied to the system. Thus, the present study successfully demonstrated the potential of *T. pallida* plants based vertical subsurface CW as an alternative sustainable remediation method for the removal of Cr(VI) from industrial wastewater.

Biosorption experiments aimed at reusability of the Cr(VI) accumulated *T. pallida* plant biomass were carried out. It serves as a value added step in the process from remediation to biomass disposal. Cr(VI) biosorption studies using plant biomass, showed that Cr(VI) exposed/unexposed *T. pallida* leaf biomass could remove 94% of chromium with a sorption capacity of 64.672 mg/g. Optimum Cr(VI) sorption conditions was achieved at pH 2, contact time (5 h), sorbent dosage (1g), Cr(VI) initial concentration (100 mg/L) and temperature 50°C. The significant biosorption capability of the Cr accumulated plant biomass revealed a very good potential application as well as safe disposal of generated toxic biomass following phytoremediation, thus reducing their environmental impact.

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ABBREVIATIONS AND NOTATIONS

Abbreviations

AAS	Atomic Absorption Spectrophotometer
ANOVA	Analysis of Variance
APX	Ascorbate Peroxidase
ATP	Adenosine Triphosphate
BCF	Bioconcentration Factor
CAT	Catalase
Cr(VI)	Hexavalant Chromium
CW	Constructed Wetland
DW	Dry Weight
EDS	Energy Dispersive Spectroscopy
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
$FADH_2$	Flavin Adenine Dinucleotide
FTIR	Fourier Transform Infra Red
FW	Fresh Weight
Fo	Organelle Fraction
Fv	Soluble Fraction (Vacuole and Cytoplasmic Fraction)
Fw	Cell Wall Fraction
GR	Glutathione Reductase
HRT	Hydraulic Retention Time
ICDA	International Chromium Development Association
MDA	Malondialdehyde
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate
POD	Peroxidase
PB	Plackett–Burman
ROS	Reactive Oxygen Species
RWC	Relative Water Content
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
TI	Tolerance Index
TF	Translocation Factor

Abbreviations

USEPA	United States Environmental Protection Agency
WHO	World Health Organization
$K_2Cr_2O_7$	Potassium Dichromate

Notations

b	Langmuir constant related to the relative energy of Cr(VI) adsorption (L/mg)
°C	Degree centigrade
C_f	Final metal ion concentrations
C*e*	Equilibrium concentration of Cr(VI) in solution (mg/L)
C*o*	Initial metal ion concentrations
g	Gram
ΔG^0	Change in free energy (kj/mol)
ΔH^0	Change in enthalpy (kj/mol)
h	Hour
mg/L	Milligram per liter
mg/kg	Milligram per kilogram
min	Minute
ml/min	Milliliter per minute
*Q*e	Equilibrium loading of Cr(VI) on biosorbent (mg/g)
Q_0	Langmuir constant related to maximum Cr(VI) adsorption capacity (mg/g)
q_e	Amount of metal ion (mg/g) at time equilibrium
q_t	Amount of metal ion (mg/g) at time (t)
q_{max}	Maximum Cr(VI) sorption capacity (mg/g)
R^2	Regression coefficient
ΔS^0	Change in entropy (kJ/mol)
U/mg	Units per milligram
X_0	Initial biomass concentration (g/L)
β_i	Linear coefficient

n	Freundlich constant related to Cr(VI) sorption intensity
µg/g	Microgram per gram

CHAPTER 1 INTRODUCTION

1.1. Background / Generalities

With the increasing globalization at an unprecedented pace in industrialized as well as in developing countries, environmental protection is a major challenge. Heavy metals and metalloids are the major inorganic contaminants in the environment released from different industrial sectors. Chromium (Cr) as a transition metal is of great industrial importance, but poses a major threat to the environment. Its widespread use and unregulated release is a major environmental concern due to its direct negative impact on humans and environmental ecosystem. Consequently, environmental safety regulations such as soil and ground water contamination levels, management of industrial wastes and other pollution problems related to industrial development need to be strictly addressed.

Chromium (VI) contaminants are commonly found in soil, sediment and water. A key factor to the remediation of Cr(VI) is that it is persistent and non-biodegradable, but can be transformed through sorption, reduction, complexation and changes in its valence state. Recent researchers have found that certain plants can effectively reduce the mobility and reactivity of Cr(VI) in the environment. These plants can, therefore, be used for the remediation of Cr loaded industrial wastewater. Currently, more research is being performed on the use of plant based processes to clean up contaminated sites.

This thesis aims to provide an insight into the potential of *Tradescantia pallida*, an indigenous plant, for Cr(VI) removal and its application in constructed wetlands for secondary or tertiary wastewater treatment.

1.2. Chromium

In the current industrial scenario, Cr is widely used for various applications, but poses a major threat to the environment owing to its persistent nature and toxic effects. Chromium has a wide range of applications mainly in the metallurgical, leather tanneries,

Cr plating, wood processing, textile dyeing and pigment production sectors. Due to its versatile use in the industries, large quantities of Cr are discharged as liquid, solid and gaseous wastes into the environment that pose significant adverse effects on the receiving environment. Chromium (VI) is one of the most toxic oxidative state that has been classified as a Group A contaminant by the Environmental Protection Agency (EPA). Chromium (VI) contamination in natural water is a great threat to millions of people in many regions of the world including India, which is the world's third largest producer of chromite ore, the main source of Cr metal with an annual production of about 3.5–4 metric tonnes (Murthy et al., 2011). According to the data from the International Chromium Development Association (ICDA), global chromite use was 29.4 metric tons in 2014 (Prasad, 2016). Recently, many countries have strict regulations for Cr discharge standards. Current efforts are being made to develop an economical and sustainable remediation method that can efficiently treat the Cr containing waste generated mainly from industrial setups.

1.3. Treatment of Cr Contaminated Wastewater

Many physical and chemical methods have been employed for the removal of Cr from industrial wastes. However, the large capital investment, energy demand and generation of toxic wastes limit the techno-economic feasibility of these treatment processes (Dhal et al., 2013). At present, many studies are being carried out throughout the globe to find the best treatment method (Barrera-Díaz et al., 2012). In this respect, phytoremediation using native and wild plant species is interesting as no special growth conditions are required in the process. It provides an effective, low cost and environmentally sustainable approach to achieve the desired results (Hannink et al., 2002). It is a general term for several processes in which plants are used to remediate contaminated sites. Specific plants can

transform or uptake and stabilize heavy metal contaminants by acting as filters or traps. For the cleanup of contaminated industrial effluents, selective wetland plants are particularly suited for secondary treatment as they can be grown directly on wastewater streams near industrial areas and can provide in situ remediation. The potential application of constructed wetlands in the treatment of Cr bearing wastewaters has been reported recently (Sultana et al., 2014). It involves a combination of physicochemical and biological processes including biological reduction, sedimentation, binding to porous media, plant uptake and precipitation as insoluble forms (mainly sulfides and (oxy-) hydroxides).

In view of the above facts, it is considered worthwhile to use a plant species that can tolerate and effectively remove Cr oxyanions from contaminated sites. This research was aimed at evaluating the potential of an indigenous plant species for the removal of Cr(VI) ions, and study the underlying mechanism.

1.4. Problem Statement

Most of the methods used to treat Cr containing industrial effluents in emerging countries are adaptations of technologies originally developed in industrialized countries. These methods were designed for areas with different geographical features.

Constructed wetlands have been implemented as wastewater treatment facilities in many parts of the world, but till date, this technology has been largely ignored in developing countries where effective, low cost wastewater treatment strategies are critically needed.

In developed countries, CWs are used for treating various wastewater types e.g. domestic wastewater, acid mine drainage, agricultural wastewaters, landfill leachate and urban storm-water, and for polishing treated wastewater effluents for return to freshwater resources (Wu et al., 2015; Zhang et al., 2014). The potential application of wetland

technology in the developing world is enormous. Most of the developing countries have warm tropical and subtropical climates that are conducive for a high biological activity and productivity, and, hence, best suited for wetland treatment systems (Zhang et al., 2015). Tropical and subtropical regions are known to sustain a rich diversity of biota that may be used in wetlands. However, there is limited information on the level of development of wetland technology in developing countries. Research on the suitability of local and indigenous wetland emergent macrophytes for removal of nutrients and heavy metals is the recent focus in several countries. There are more than 650 natural and CW-systems in North America and more than 5000 sub-surface flow CWs in Europe for wastewater treatment (Zhao et al., 2004). Extensive research is being carried out on the structure, function and application of these systems for treatment of different industrial wastewater. However, these established guidelines may not be directly transferable to tropical countries. For instance, aquatic plants optimal for use in European and North American CWs may not be well suited to tropical regions.

Clearly, developing countries interested in implementing this technology must identify specific research needs and develop appropriate strategies based on local environmental conditions. A clear understanding of the biological, hydraulic and chemical processes involved is essential. Further, investigations are needed to identify and characterize tropical plant species in terms of their tolerance to high metal concentration and wastewater types.

In both developed and developing countries, large amount of lands are contaminated mostly by more than a single pollutant. These multiple pollutants could be of the same form, e.g., land contamination with different kinds of heavy metals, or could be of different forms, e.g. land contamination with organic as well as inorganic pollutants (e.g. metals). Research in this area is very limited and sufficient gaps in knowledge on the use

of plants to remediate industrial wastewater still exist. Hence, this research work is an attempt towards addressing environmental contamination due to discharge of Cr(VI) containing industrial effluent and its mitigation using an indigenous plant species. Detailed studies on Cr uptake, tolerance and distribution by the plant species along with their function and application in a constructed wetland system were carried out to accomplish the aim of this thesis.

1.5. Aims and Objectives

The main aim of the present research work was to evaluate the potential of an indigenous plant species, *Tradescantia pallida*, for Cr removal from industrial wastewater and to demonstrate its application using a constructed wetland system.

To achieve the aim, the following investigations were carried out:

1. Screening of indigenous Cr hyperaccumulator plant species
2. Biochemical characterization and enzymatic analysis of *Tradescantia pallida* for Cr tolerance and bioaccumulation
3. Bioaccumulation, subcellular distribution and localization of Cr in *T. pallida*
4. Effect of co-ions on Cr uptake by *T. pallida*: kinetics, biochemical and factorial analyses.
5. Continuous removal of Cr(VI) from synthetic wastewater using *T. pallida*, in a laboratory scale constructed wetlands and its performance evaluation
6. Feasibility study on the disposal of Cr accumulated *T. pallida* plant biomass

1.6. Organization of the Thesis

The entire thesis work has been divided into five chapters. The current **Chapter 1** gives a general introduction, objective and scope of the present work. The **Chapter 2** of this

thesis consists of a thorough literature review concerning the present work. It outlines the main concept of industrial water pollution due to Cr and treatment methods. It also briefly describes mechanisms adopted by plants responsible for Cr phytoextraction. Details of the materials and methods followed in this research are described in **Chapter 3**. It essentially details the procedure followed for screening, tolerance, bioaccumulation, enzymatic and biochemical analyses, transport, localization and distribution mechanism of Cr(VI) in *T. pallida* under hydroponics conditions. The description of biosorption and kinetic studies in batch shake flasks and continuous Cr removal studies in a lab scale CWs are emphasized. It also details the analytical methods followed in the above investigations. **Chapter 4** contains the results and discussion, wherein the results of Cr(VI) tolerance mechanism, bioaccumulation and localization by *T. pallida* and effects of multi-ions on the Cr removal are presented and thoroughly discussed. This chapter also discusses the results of Cr(VI) biosorption study, optimization of process parameters, biosorbent characterization and sorption mechanism for enhancing the Cr(VI) removal. Finally, the results of Cr(VI) removal using *T. pallida* plants along with their distribution in a laboratory scale CWs operated under continuous mode are presented and discussed. **Chapter 5** draws summary and appropriate conclusions based on this work. This chapter also provides some useful recommendations for researchers to carry out further work in this field.

Chromium(VI) is a highly toxic transition metal, that is considered as a priority pollutant. In the current industrial scenario, chromium (Cr) as a metal is of great industrial importance, but poses a major threat to the environment. Due to its widespread use in various industries, the demand of Cr is increasing and, as a consequence, the load of Cr pollution on environment is on a rise. Treatment of Cr contaminated wastes by phytoremediation has gained significant interest. It provides an environmentally sustainable, ecofriendly, cost effective approach for environmental cleanup of Cr. This chapter mainly presents the current status of phytoremediation research with particular emphasis on cleanup of Cr contaminated soil and water systems. It gives a detailed account of the work done by different authors on the Cr bioavailability, uptake pathway, toxicity and storage in plants following the phytoextraction mechanism. This chapter also describes recent findings related to Cr localization in hyperaccumulator plants. It gives an insight into the processes and mechanisms that allow plants to remove Cr from contaminated sites under different environmental conditions. These detailed knowledge on changes in metabolic pool of plants in response to Cr stress would immensely help to understand and improve the phytoextraction process. Further, this chapter provides a detailed understanding of Cr uptake and detoxification mechanism by plants that would undoubtedly help in their potential application.

2.1. Chromium

2.1.1. Physico-chemical Properties of Chromium

Chromium is a heavy metal belonging to the transition group (VI-B) of the modern periodic table. It is a steel-gray, lustrous, hard metal that takes a high polish and has a high melting point. It has various oxidation states ranging from Cr(0) to Cr(VI). The most stable and common forms in the environment are the trivalent Cr(III) and the hexavalent Cr(VI) species, both having different physico-chemical and biochemical properties (Dhal

et al., 2013). The intermediate oxidation states are metastable and do not occur naturally. Chromium constitutes about 0.037 percent of the crustal rock and ranks 21st in relative natural abundance. Chromium (III) is the most common naturally occurring state and forms complex with organic matter present in soil and aquatic environments. It occurs as chromic oxides (Cr_2O_3), hydroxides ($Cr(OH_3)$) or sulphates ($Cr_2(SO_4)_3$•$12(H_2O)$ (Gill, 2014). In contrast, Cr(VI) is considered the most noxious form of Cr with a strong oxidizing potential. It is more mobile than Cr(III) and is usually associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) ions (Sultana et al., 2014). Chromium (VI) is more water soluble and, thus, more bioavailable than Cr(III). It forms stable complexes with organic matter which further increases the Cr(VI) tendency to become persistent (Langård and Costa, 2015). Chromium (VI) can be transformed to Cr(III) under acidic conditions, and this reduction process is favoured in acidic soils with a high proportion of organic matter. Further, Cr(III) may also be oxidized to Cr(VI) in the oxygenated environment. Ratio of Cr(VI)/Cr(III) is a function of pH, dissolved oxygen concentration, presence of reducing agents and complexing factors in the environment. Under anoxic conditions, only Cr(III) predominates. Chromium (VI) is predominant at a pH above 7 and Cr(III) predominates at less than 6 pH. Chromium (III) precipitates under neutral to basic pH and, conversely, it is soluble in acidic media. Chromium (VI) salts are soluble at all pH, but may get co-precipitated with divalent cations (Stanin and Pirnie, 2004). Table 2.1 lists the properties of Cr.

Table 2.1: Properties of chromium.

Group	6	Melting point	1907° C
Period	4	Boiling point	2671° C
Block	d	Density (g/cm^3)	7.15
Atomic number	24	Relative atomic mass	51.996
State at 20°C	Solid	Key isotopes	^{52}Cr

2.1.2. Uses of Chromium

Chromium is widely used in many industrial applications such as in leather tanning, metallurgical, Cr plating, wood processing, anodizing aluminium, cleaning agents, catalytic manufacture, organic synthesis, textile dyeing and textile pigment production, wood preservation and alloy preparation (Alloway, 2013). Out of the total world production of $24{,}000 \times 10^3$ metric tons (gross weight of marketable chromite ore), about 60-70% is consumed in stainless steel and alloy preparation. Leather tanning, pigment production, electroplating and other chemical industrial processes use above 15% (Papp and Lipin, 2000). Presently more than 4000 tanneries are involved in chrome tanning processes. In India, tannery industries account for about 2,000-3,000 tons per year of elemental Cr discharged into the environment. Around 80-90% of leather industry uses Cr as a tanning agent. Effluents from these tanneries is loaded with about 40% of Cr used in the form of Cr(VI) and Cr(III) salts (Sundaramoorthy et al., 2010).

2.1.3. Sources and Concentration of Chromium in the Environment

Chromium occurs naturally in the form of crustal rocks but the main source is from various industrial units. It occurs predominantly as ferrochromite ($Fe_2Cr_2O_4$) and other minerals present in the earth's crust. The main ecological toxic burden is anthropogenic source concerned with industrial operations using Cr, as mentioned earlier in section 2.1.2.

Chromium concentration varies from 0.1 to 0.5 mg/L in fresh waters and from 0.0016 to 0.05 mg/L in sea waters (Kumar and Puri, 2012). As recommended by WHO, the maximum permissible limits for the discharge of Cr(VI) into inland surface and drinking water are 0.1 mg/L and 0.05 mg/L, respectively. Cr is ranked as the 21st most abundant element present in the earth's crust (Förstner and Wittmann, 2012). It is reported that Cr concentration in the soil ranges from 5 to 3000 µg of Cr per gram (Polti et al., 2011).

Besides natural rocks, major sources of Cr are effluent from various industries, ferrochromium slag, and solid wastes containing Cr as by products, leachates and dust particles where Cr concentration is found strikingly above permissible limits.

2.1.4. Health Effects of Chromium

The health hazards of exposure to Cr(VI) and Cr(III) are well documented by the World Health Organization (WHO, 1988) and the Agency for Toxic Substances and Disease Registry (ATSDR, 1991). Cr(VI) is listed by the United States Environmental Protection Agency (USEPA) among seventeen chemicals posing greatest threat to humans (Cheung and Gu, 2007). It has been classified as a Group A contaminant by the Environmental Protection Agency (EPA). Cr(VI) species, namely $Cr_2O^-_2$, $Cr_2O_7^{2-}$ and CrO_4^{2-} are the most mobile and bioavailable anionic forms in the aqueous environment. Cr(VI) oxyanions are considered as highly lethal for most organisms due to its mutagenic and carcinogenic properties (Li et al., 2013). Owing to a very high positive redox potential, Cr crosses cell membranes damaging the cellular and molecular components of the cell leading to membrane disruption, protein degradation and DNA alterations in humans, animals and plants (Oliveira, 2012). Figure 2.1 summerises the toxic effects of Cr on the ecosystem. Cr(VI) induces mutation by interfering with DNA protein cross-links and causes single-strand breakage (Shanker and Venkateswarlu, 2011). Exposure of Cr(VI) above the permissible limit (0.05 mg/L in drinking water) is known to cause lung cancer. It damages kidney and liver and may cause epigastric pain, nausea, vomiting, allergic reactions, stomach ulcers, and hemorrhage (Gad, 2014; McCarroll et al., 2010).

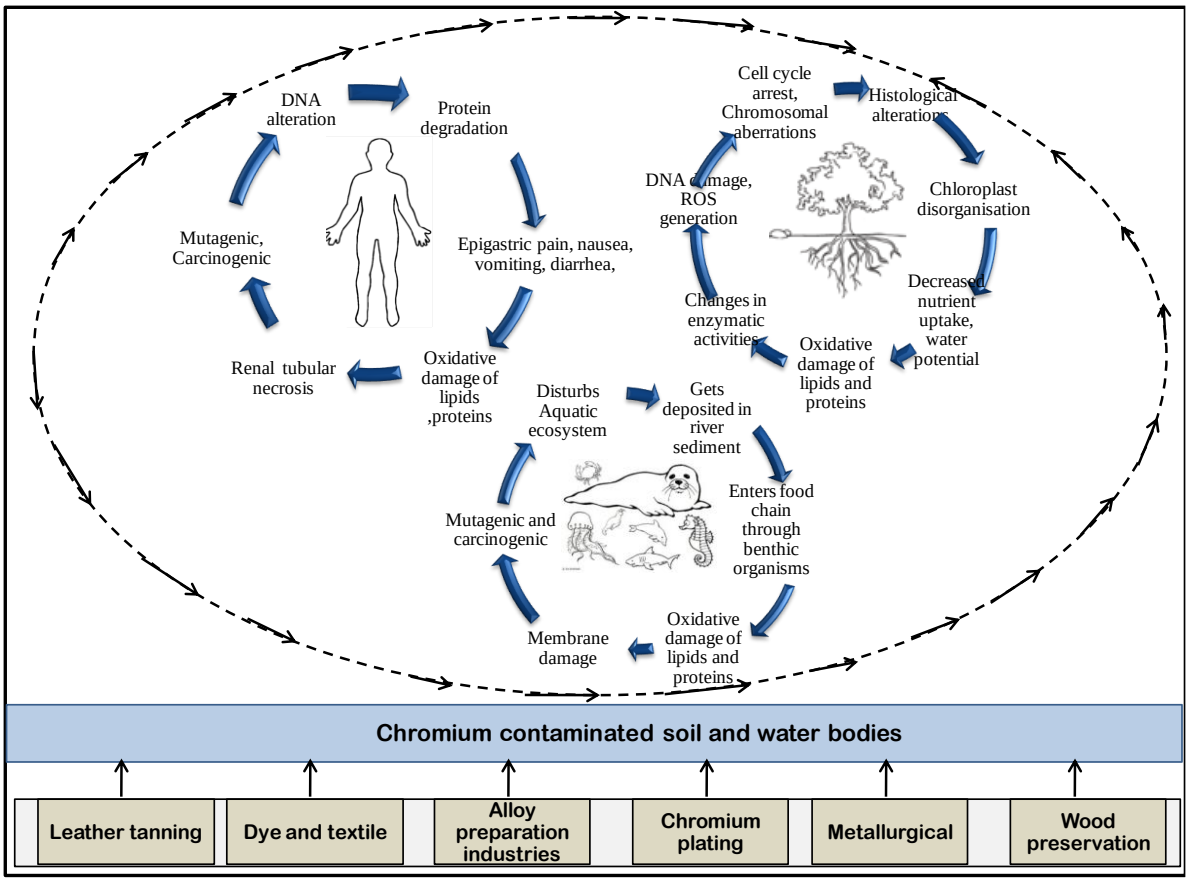

Figure 2.1: Chromium toxic effects on the ecosystem

In plants and many other organisms, reducing agents, such as NAD(P)H, $FADH_2$, several pentoses and glutathione in the cell pool, reduce Cr(VI) to Cr(III) (Hossain et al., 2012). During this conversion, transient formation of Cr unstable states occurs leading to free radicals formation, which induces oxidative stress conditions in plants (Sharma et al., 2012). Chromium is toxic for most agronomic plants at a concentration of about 0.5-5.0 mg/L in nutrient media and 5-100 mg/g under soil condition. In general, concentration of Cr in plants is usually less than 1 μg/g (Oliveira, 2012).

2.2. Chromium Removal Methods

Emission of Cr contaminated toxic wastes (solid, liquid and gaseous discharge) from various industries has led to an immense load on the environment, in many countries. Unlike organic compounds which are mostly biodegradable, Cr cannot be degraded, and decontamination usually requires their containment. To preserve soil, aqueous streams

and groundwater systems, different methods of removal using physiochemical and biological processes are being evaluated, among which the latter has the ability to provide more efficient and affordable technological solution (Kamaludeen et al., 2003; Ranieri and Gikas, 2014).

2.2.1. Physio-Chemical Methods of Chromium Removal

Current remediation strategies of Cr is primarily based on physico-chemical technologies which are meant primarily for intensive *in situ* or *ex situ* treatment of relatively highly Cr polluted sites, and thus are not very suitable for the remediation of vast, diffusely polluted areas where Cr occur at relatively low concentrations and superficially (Rulkens et al., 1998).

Table 2.2 lists the conventional and advanced treatment technologies reported in the literature for Cr removal. These conventional, physio-chemical remediation processes are briefly described as follows: (1) **Adsorption**: In this process, Cr is concentrated on the surface of an adsorbent (Barrera-Díaz et al., 2012). Suzuki (1990) described and Cr adsorption with respect to the adsorption equilibrium, kinetics and influence of parameters such as pH, temperature, surface area, etc. Adsorption of Cr is highly related to its solution pH. At low pH, a high adsorption of Cr(VI) is commonly observed, depending on the Cr species and the adsorbent surface. At acidic pH, the predominant species of Cr are $Cr_2O_7^{2-}$ and $HCrO_4^-$. Above pH 8, only CrO_4^{2-} is stable whereas when the pH decreases to 3.0–6.0 and the equilibrium shifts to $Cr_2O_7^{2-}$, and at pH values below 3.0, $Cr_3O_{10}^{2-}$ and $Cr_4O_{13}^{2-}$ species predominates. Similarly, a maximum Cr(VI) removal at pH 2.0 was observed by Wu et al. (2008).

Table. 2.2 Conventional and advanced treatment technologies reported for Cr removal.

Treatment Methods	Initial CrVI conc. (mg/L)	Cr(VI) Removal Efficiency	Advant-ages	Disadvantages	Method used	References
Adsorption	<10	55%-100%	Most effective adsorbent; high surface areas and rate of adsorption	Performance is dependent on the type of carbon used, adsorbent, progressively deteriorating in capacity as number of cycles increases, secondary toxic waste generation	Activated carbon	Dimos et al., 2012; Bayazit and Kerkez,2014; Baral et al., 2009; Owlad, 2009
			Low cost, natural polymer, high metal binding capacities	Nonporous sorbent, sorption capacity depends on the origin of the polysaccharide and the degree of N-acetylation	Chitosan	
			Low-cost, easy operating conditions, possibility of Cr recovery	Sensitivity to the presence of organic or inorganic ligands, replacement after 5 to 10 sorption-desorption cycles	Biosorbents (seaweeds, algae, bacteria, fungi, and agricultural products)	
			High chemical stability and thermalstabili y	High cost	Inorganic membrane	
Chemical precipitation	>10	3,680 to less than 0.2 mg/L	Low capital cost, simple operation	Sludge generation, extra operational cost for sludge disposal	Montmorillo nite-supported magnetite nanoparticle	Barakat, M. A., 2011; Graham et al., 2006
Ion exchange	<100	98.5%	Provide high flow rate of treated water, energy	High cost of ion exchange resins and high operational costs. Solution	Basic macro reticular anion exchange resin	Alvarado et al., 2013; Rivero et al., 2004; Mukhopadhyay et al., 2007

			efficient	concentration must be monitored		
	>10		High separation selectivity, energy efficient	High operational cost resulting from membrane fouling and energy consumption	Membrane Electrolysis	Owlad, 2009
Membrane separation	>10	Approximately 98% removal efficiency	Space requirement is unimportant, low pressure, high separation selectivity	High operational cost as a result of membrane fouling	Nano filtration composite polyamide membranes	Muthukrishnan and Guha, 2008; Hawley et al., 2004; Geckeler et al., 1996
Redox processes	-	High	Retention of contaminants within a confined zone, and low power consumption	Generation of toxic sludge	Pectin-stabilized nanoscale zero-valent iron	Chen et al., 2015; Lytle et al., 1998
Coagulation, cementation, electro-winning, reverse osmosis	>10	95% to 99%	High separation eficiency	Expensive and operating cost high.	-	Ozaki et al., 2002

(2) **Electrochemical precipitation (ECP)**: In this process, for precipitating Cr(VI) from wastewater, the solution pH is brought down to 2–3 and Cr(VI) is reduced to Cr(III) using a suitable reductant. To maximize the removal of heavy metal from contaminated wastewater, electrical potential has been utilized to modify the conventional chemical precipitation (Kurniawan et al., 2006). Kongsricharoern and Polprasert (1995) investigated electrochemical precipitation (ECP) for the removal of Cr(VI) from real electroplating wastewater. On treatment of electroplating wastewater (Cr(VI) 215–3860 mg/L) using an ECP unit consisting of an electrolytic cell made of two steel plates as anode and cathode, only < 0.2 mg/L of Cr(VI) remained in the effluent under optimum ECP conditions. To overcome the problem of sludge generation with the conventional

treatment techniques, EC appears to be a better alternative, as it can remove the smallest colloidal particles and produce very less amount of sludge. (3) **Ion exchange**: This technique is based on a reversible interchange of ions between the solid and liquid phases where an insoluble substance (resin) removes ions from an electrolytic solution and releases other ions of like charge in a chemically equivalent amount without any structural change of the resin (Vigneswaran et al., 2004). In many studies, various kinds of ion exchange resins were used to study the uptake of Cr(VI) (Lin and Kiang, 2003). It is widely used in hydrometallurgy for the recovery and purification of a number of metals, including Cr (Agarwal et al., 2006). In a study, 99.4% Cr(VI) removal in the pH range of 1–5 was obtained by Shi et al. (2008) while evaluating the adsorption capacity of various resins for Cr(VI) removal from aqueous solutions. In another study, >95% removal of Cr(III) by using IRN77 and SKN1 cation-exchange resins was achieved by Rengaraj et al. (2001). (4) **Membrane filtration**: Membrane processes are employed for the separation of metals by using a semi-permeable membrane. The driving forces for the separation are hydrostatic pressure, concentration gradient, electrical potential, and pressure and concentration gradients. Various types of membranes including inorganic, polymeric and liquid membranes can be employed for Cr(VI) removal (Kiril Mert and Kestioglu, 2014). Depending on the membrane characteristics (i.e., the porosity, hydrophilicity, thickness, roughness, and charge of the membrane), reverse osmosis works effectively at a wide pH range of 3–11 and at 4.5–15 bar of pressure (Kurniawan et al., 2006). In a study by Covarrubias et al. (2008), >95% Cr(III) rejection by Faujasite-type zeolite membranes was demonstrated. These methods suffer from one or more serios drawbacks, such as high cost, high –energy demand or toxic sludge production (Kurniawan et al., 2006).

2.2.2. Biological Methods of Chromium Removal

In the recent years, much attention has been given towards the biological approaches of Cr(VI) remediation. Chromium (VI) compounds cannot be biologically degraded and thus, bioremediation of Cr(VI) containing effluents involves an interplay of several biological processes. Reduction of Cr(VI) by several microorganisms has been reported under a number of conditions and by a variety of bacterial, fungal, yeast and algal strains viz. *Acinetobacter* and *Ochrobactrum* (Francisco et al., 2002), *Arthrobacter* (Megharaj et al., 2003), *Pseudomonas sp.* (Rajkumar et al., 2005), *Serratia marcescens* (Campos et al., 2005), *Ochrobactrum sp.* (Thacker and Madamwar, 2005), *Bacillus sp.* (Elangovan et al., 2006), *Desulfovibrio vulgaris* (Goulhen et al., 2006), *Cellulomonas spp*. (Viamajala et al., 2007). These microorganisms in order to protect itself from Cr(VI) toxicity, adopts a combination of several mechanisms, which include sorption, uptake, precipitation, methylation or reduction of Cr(VI) to lesser toxic valence state. Compounds of Cr(VI) are transformed enzymatically through reactions that take place as a part of their metabolic processes in the presence of large amounts of electron donors (Tripathi et al., 2011). Redox reactions are catalysed by a combination of several mechanisms, including enzymatic extra-cellular reduction, nonmetabolic reduction by bacterial surfaces and intra-cellular reduction and precipitation. However, the practical application are limited by the fact that high initial concentration of Cr(VI) can cause significant deactivation of the introduced microorganism that act efficiently only under optimum temperature, pH and geochemical conditions. Under aerobic environment, rate of microbial Cr(VI) reduction is more than that under anaerobic environment (Fendorf et al., 2001). Also, Cr(VI)- polluted streams and soils often contain additional toxic compounds, which severely inhibits Cr(VI) reduction.

Phytoremediation, on the other hand offers a cost-effective, nonintrusive, and safe alternative to conventional cleanup techniques (Glick, 2003). For Cr treatment,

phytoremediation appears as a valid option since it is best suited for the remediation of diffusely Cr contaminated sites and at much lower costs than other methods (Kumar et al., 1995). The idea of using plants to remove Cr from soils came from the discovery of different wild plants, often indigenous to naturally mineralized soils that accumulate high concentrations of metals in their parts (Baker, 1987; Raskin et al., 1997). This remediation approach provides a permanent in situ remediation and has got a high public acceptance.

2.3. Phytoremediation Overview

Phytoremediation is an emerging clean-up technology which uses plants to remove metals and other pollutants from contaminated sites (EPA, 2000). This approach includes overall biological, chemical and physical processes that enable uptake, sequestration, degradation and metabolization of contaminants by plants (Sebastiani, 2004). It is an emerging area of green technology and is an efficient method to treat pollutants present in soil, water or the air (Gratao et al., 2005). Utilizing the ability of certain trees, shrubs, and grass species to remove, degrade, or immobilize harmful chemicals can reduce risk from contaminated soil, sludges, sediments, and ground water through contaminant removal, degradation, or containment (Zavoda et al., 2001). Further, to improve the applicability of phytoremediation for real wastewater treatment, indigenous plants need to be screened which are better adapted to grow in a particular region and also survive under metal stressed condition. Table 2.3 enlists the advantages and limitations of phytoremediation**.**

Table 2.3: Advantages and limitations of phytoremediation (Ghosh and Singh, 2005; Tangahu et al., 2011; Pilon-Smits, 2005).

Advantages	Limitations
Amendable to a range of toxic metals compounds and radionuclides	Plant must be able to grow in the contaminated soil or material
Provides permanent in Situ / Ex Situ remediation with effluent/soil substrate respectively.	The contaminant must be within the rooting zones of remediative plants thus, applicable to areas with shallow and low level contamination
Low input costs & a clean technology	Process can take years for contaminant concentrations to reach regulatory levels
Soil stabilization & reduced leaching of water and transport of inorganics in the soil	Climatic conditions are a limiting factor
Generation of a recyclable metal rich plant residue which can be further utilized as bio-ore of heavy metals.	Introduction of non-indigenous species may affect biodiversity
Minimal environmental disturbance due to in-situ applications	
Reduces risk of secondary air or water-borne wastes	
Enhanced regulatory and public acceptance	

2.3.1. Removal of Chromium by Phytoremediation

Phytoremediation of Cr depends on five main mechanisms which operate simultaneously: (i) Phytoextraction – efficient Cr accumulator plants concentrate toxic metals into their roots and translocate it to above-ground plant parts (Kumar et al., 1995), (ii) Phytovolatilization – process of transformation of toxic compound into volatile state and evaporation from aerial parts of the plant, (Emamverdian et al., 2015) (iii) Phytostabilitzation- the use of plants to immobilize metals in soil by adsorption onto roots or precipitation in the rhizosphere (Prasad and de Oliveira Freitas, 2003), (iv) Rhizofiltration – process of absorbion and precipitation surrounding plant root system (Dushenkov et al., 1995), (v) Phytosequestration – phytochemical complexation in the root zone that can precipitate or immobilize metals in the root and such complexes are

then mobilized to store in the vacuolar space of plant cells (Panda and Choudhury, 2005). The toxic compound also gets transferred in different cells facilitated by transport proteins (Pinto et al., 2014). In general, phytoextraction and rhizofiltration are considered as the main options for the removal of heavy metals such as Cr, whereas phytodegradation and phytostabilisation are applied mostly to organic contaminants (Meagher, 2000; Guerinot and Salt, 2001).

Phytoremediation has proved to be an efficient process for the remediation of Cr(VI) contaminated soil and wastewater owing to its simplicity in operation and high efficiency of removal. It provides a sustainable treatment method utilising solar energy. In the recent years, a lot of research investigations to understand the process mechanism has been carried out. Field applications in the form of constructed wetlands (CWs) have been established near industrial setups to process effluents loaded with toxic Cr wastes and other organics. It uses Cr hyperaccumulators with their associated microbial flora and their innate mechanisms for Cr removal. Recent research studies revealed that selected plant species bioaccumulate substantial amount of Cr through their unique metabolic and absorption pathways from soil, sediments, sludges and aquatic systems. Plants adopt various Cr-resistance mechanisms including bioaccumulation, biosorption and precipitation (Chen et al., 2010). Plants possess the potential to reduce toxic Cr(VI) into the less toxic Cr(III). Further, some plants utilize chromate efflux mechanism which can effectively serve as a method to reduce Cr pollution (Jabeen et al., 2009). Figure 2.2 shows possible fates of chromium during the phytoremediation processes.

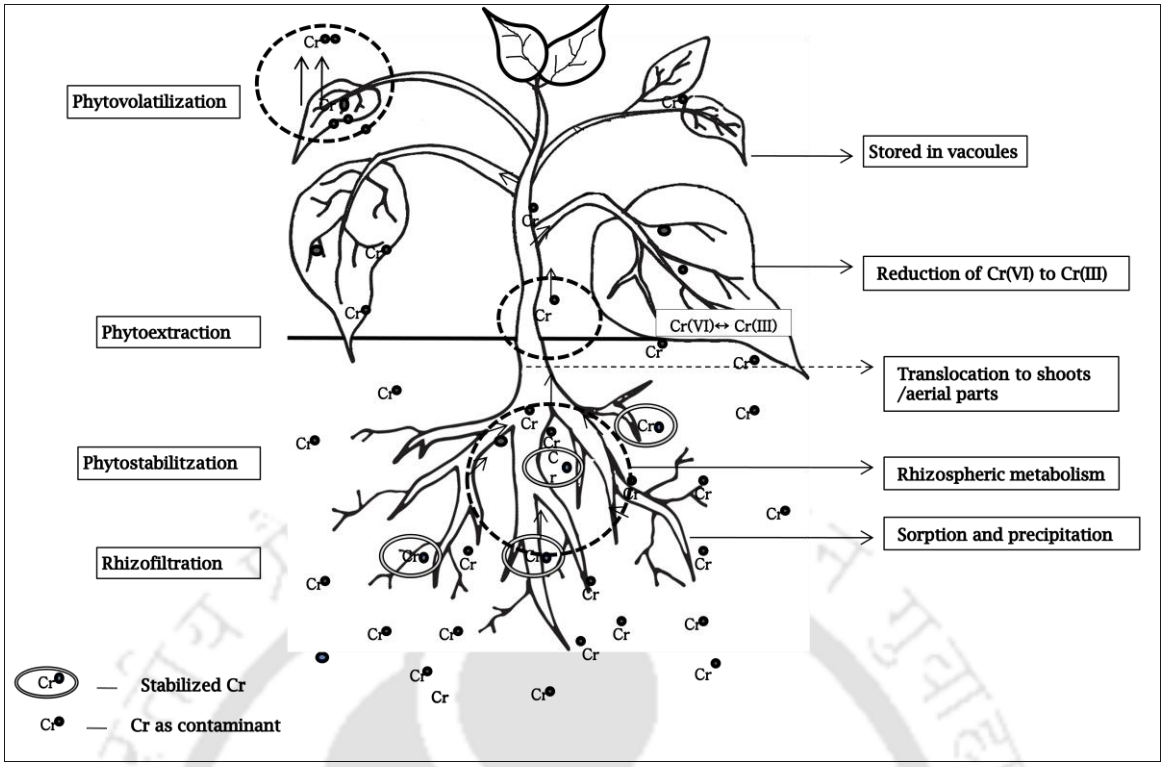

Figure 2.2: Schematic showing possible fates of chromium during the phytoremediation process.

Phytoremediation of Cr contaminated soil is primarily based on phytoextraction method that uses a specific hyperaccumulator to extract the pollutant through its roots which are then translocated to other plant parts (Hsiao et al., 2007). In order to use this as a suitable remediation method, plants should be able to uptake significant amount of Cr, with a high translocation factor so that it gets accumulated in aerial parts and, thereafter, be capable of producing large biomass for an even higher Cr bioaccumulation to take place. Whereas rhizofiltration is the main process which operates in the wetlands near an industrial set up of polluted waters (Chaudhuri et al., 2008).

2.3.2. Plants with Potential of Chromium Phytoremediation

Hyperaccumulating plants have the potential to transform contaminants, e.g. Cr, into less toxic trivalent state with reduced mobility. It utilizes the plants innate mechanism to bioaccumulate and store high levels of Cr in their roots, shoots and leaves. Cr

hyperaccumulator plants can accumulate more than 1,000 mg Cr/kg dry weight (DW) in their tissues (Zhang et al., 2007). Phytoremediation efficiency depends on many factors such as the soil's physical and chemical properties, Cr speciation, its mobilization and subsequent uptake, Cr bioavailability, plant and microbial exudates, plant's ability to extract, accumulate, translocate, sequester and store Cr (Hooda, 2007; Shanker et al., 2005).

2.3.2.1. Use of Hydroponics as a Method for Plant Growth in Chromium Removal

Hydroponics is a useful method for plant growth which utilises static solution culture, with plants grown in containers (plastic buckets, tubs, or tanks) containing nutrient solution (Nath et al, 2008). The solution is usually gently aerated but may be unaerated. If unaerated, the solution level is kept low enough that enough roots are above the solution and hence get adequate oxygen.

The nutrient solution is changed either as per afixed schedule, such as once in a week, or when the solution level drops below a certain limit. Commonly used chemicals as macronutrients for plant growth include potassium nitrate, calcium nitrate, potassium phosphate and magnesium sulphate. Various micronutrients are typically added to hydroponic solution to supply essential elements; among these are Fe, Mn, Cu, Zn, B, Cl, and Ni. Many variations of the nutrient solutions used by Arnon and Hoagland have been styled 'modified Hoagland solutions' and are widely used. One of the key advantages of hydroponics culture is that no soil is required and thus, eliminating the risk of soil-borne pathogens. It is a low cost, stable system that provides complete control of root environment. Moreover, nutrition ratios can be precisely regulated to match and control physiological growth stages (Torabi et al., 2010; Savvas, 2003).

In hydroponics conditions treating Cr laden effluents, several plant species have been studied for their Cr removal efficiency and translocation ability (Table 2.4). Most of these

plants do not meet the hyperaccumulator criteria, as their translocation efficiency to above ground parts does not meet the requirement. However, at present these plants are reported to accumulate substantial amount of Cr and classified as significant Cr phytoremediators.

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Table. 2.4: Efficient chromium accumulator plants under hydroponics culture conditions and removal mechanism

Cr accumulator plants	**Habitat**	**Family**	**Cr(VI) removal mechanism**	**Mode of opera-tion**	**Max % removal/ Bioaccumul-ation capacity**	**Exp. period**	**Influent conc.**	**References**
Amaranthus viridis (Green amaranth)	Perennial broadleaf herb	Amaranthaceae	Increased activity of antioxidative enzymes	Batch	Cr accumulation: Roots : 2624.39 µg/g Cr(VI) (dw) at 5.2 mg/ L	20 days	0.052-5.2 mg/L Cr(VI)	Liu, Zou et al., 2008
Azolla (Water fern)	Aquatic fern	Salviniaceae	Not Reported	Batch	Cr accumulation: 356 and 964 mg/kg dm Cr(VI) and Cr(III) at 1 mg /dm	12 days	1-20 mg/L Cr(VI)	Arora et al., 2006
Bacopa monnieri (Smooth water hyssop)	Perennial, creeping herb	Plantaginaceae	Not Reported	Batch	319.5 mg/kg DW for Cr at 10 µg/ml	8 weeks	0.01, 0.1, 1.0, 2.5, 5.0, 10 mg/L Cr	Shukla et al., 2007
Callitricha cophocarpa (Water-starwort)	Aquatic macrophyte	Callitrichaceae	Cr(VI) reduction	Batch	Cr accumulation: 1000 mg/kg (dw)	3 weeks	2.6 -36.4 mg/L Cr(VI)	Augustynowicz et al., 2010
Eichhornia crassipes (Water hyacinth)	Free-floating perennial aquatic plant	Pontederiaceae	Increased activity of Antioxid-ative enzymes Cr(VI) reduction	Batch	Maximum Cr accumulation : 2.52 X 10^3µg/g of water hyacinth in 20 mg/L Cr removal efficiency : 91%	42 days	3, 5, 7, 10 and 20 Cr(VI) mg/L	Zewge et al., 2011

			Plants exposed to 520 mg/L Cr(VI) for 4 days did not survive 52 mg/L Cr(III) for 2 days stimulated growth	Batch	Maximum Cr accumulation 1258 mg/kg (dw) 520 mg/L Cr(III) for 2 days	2-4 days	52 and 520 mg/L Cr (III) and Cr(VI)	Mangabeira et al., 2004 Paiva et al., 2009
Genipa americana L (Genipap)	Wood plant	Rubiaceae	Not Reported	Batch Batch	- Reduction of 79 and 90% for 15 and 30 mg/L of Cr(VI)	5 months 15 days	0, 5, 10, 15, 20, 25 and 30 mg/L Cr(III) 15 and 30 mg /L Cr(III) and Cr(VI)	Barbosa et al., 2007 Santana et al., 2012
Gynura pseudochina (Purple passion)	Herb	Asteraceae	Cr(VI) reduction	Batch	Cr accumulation: Tubers : 823.1 mg/kg Cr(VI) (dw) Shoots: 787.9 mg/kg Cr(VI) (dw)	2 weeks	100 mg/L Cr(VI)	Mongkhonsin et al., 2011
Hydroctyle umbellata (Marshpenny wort)	Anchored hydrophyte	Araliaceae	Not Reported	Batch	Cr accumulation: 18,200 mg /kg	90 days	Semi-solid tannery (wet) sludge at 0, 20, 40, and 60% total Cr	Khilji, 2008

							concentrations.	
Leersia hexandra (Southern cutgrass)	Perennial herb (grow in swamps)	Poaceae	Cr(VI) reduction and sequestration	Batch	Highest bioaccumulation coefficients for leaves :486.8 for Cr(III) and 72.1 for Cr(VI) Cr accumulated in leaves was 4868 μg Cr(III) / g and 597 μg Cr(VI)/ g	45 days	10 mg/L Cr(VI) and 60 mg/L Cr(III)	Zhang et al., 2007
Lemna sp. (Duckweed)	Free-floating aquatic plants	Araceae	Not Reported	Continu-ous	4.423 mg Cr(VI)/g	7 days	5.0 mg/L Cr(VI) pH 4.0	Uysal, 2013
Miscanthus sinensis (Chinese silver grass)	Herbaceous perennial plant	Poaceae	Altered vacuole sequestration, nitrogen metabolism and lipid peroxide-tion	Batch	-	3 days	0, 2.6, 5.2, 10.4, 15.6, 26, 39 or 52 mg /L Cr (VI)	Sharmin et al., 2012
Nymphaea spontanea (Water lilies)	Aquatic rhizomato-us perennial herbs	Nymphaeaceae	Not Reported	Batch	Cr accumulation: 2.119 mg/g (dw)	9 weeks	1, 2.5, 5 and 10 mg/L Cr(VI)	Choo et al., 2006
Penisetum purpureum (Napier grass)	Perennial tropical grass	Poaceae	Not Reported	Continu-ous	78.1% removal	8 weeks	10 and 20 mg Cr/dm^3	Mant et al. 2005
Polygonum hydropiperoid	Rhizomat-ous perennial	Polygonaceae	Not Reported	Batch	Cr accumulation:	10 days	1 mg/L Cr(VI)	Qian et al., 1999

s (Swamp smartweed)	aquatic herb				Shoots: 44 mg/kg (dw)			Mei et al., 2002
Pteris vittata (Chinese brake)	Fern species	Pteridaceae	Not Reported	Batch	Cr accumulation: Fronds 234 mg/kg (dw) Roots:12,630 m g/kg (dw) at 2.6 mg/L Cr(VI)	14 days	0, 2.6, 13 and 65 mg/L	de Oliveira et al., 2014
Salvinia minima (Water spangl es)	Aquatic macrophyte(Free floating fern)	Salviniaceae	Cr(VI) reduction	Batch	Maxima Cr accumulation: Submerged leaves 2210.1 µg/g Cr(VI) (dw) Floating leaves 484.5 µg/g Cr(VI) (dw) at 10 mg /L Cr concentration	6 days	2, 5, and 10 mg /L Cr (VI)	Prado et al., 2010
Salvinia molesta (Kariba weeds)	Aquatic fern	Salviniaceae	Not Reported		Cr removal ranged from 40-99%	7 days	-	Shiny et al., 2004
Tradescantia pallida (Wandering jew)	Succulent perennial herb	Commelina-ceae	Increased activity of antioxidative enzymes	Batch	Max Cr accumulation: 536 mg/ kg dw	60 days	10 mg/L Cr(VI)	Sinha et al., 2014
Vetiveria zizanoides	Perennial grass	Poaceae	Not Reported	Batch	77–78% for Cr uptake ability	-	5-20 mg/L	Singh et al., 2015

(Khas-khas)					Max Cr accumulation: Stem (28.3 g/kg)			

2.3.3. Chromium: Phytotoxicity, Accumulation and Translocation in Plants

Yu et al. (2007) reviewed the several studies and concluded that Cr phytotoxicity, accumulation rate and translocation to shoots and leaves depend on the plant species, Cr speciation, bioavailability and initial media concentration. Soil pH, organic matter content and chelating agents are among the different parameters which play an important role in Cr absorption and translocation (Zhang et al., 2010).

A high Cr(VI) concentration showed adverse effects in plants, causing reduction in seed germination of crop plants, plant growth parameters, photosynthetic rate, nitrate reductase activity (Liu et al., 2008; Sangwan et al., 2014) and soluble protein content and damage to chlorophyll structure (Singh et al., 2013). Vernay et al. (2007) found that in leaves, a high dichromate concentration caused chlorosis, a symptom often associated with detrimental effects of heavy metals in *Raphanous sativus* (Vernay et al., 2007). Seed germination may get affected due to interference of Cr on plants enzymatic activities such as amylase which affects the sugar transport (Santana et al., 2012).

Doses of Cr(VI) greater than 100 μM inhibited or caused a significant decrease in root growth of plant species such as *Arabidopsis thaliana* (Eleftheriou et al., 2015), *Salix viminalis* (Ranieri and Gikas, 2014) , *Caesalpinia pulcherrima* (Rai et al., 2006), *Triticum aestivum* (Subrahmanyam, 2008) and *Vigna radiata* (Diwan et al., 2010). This root growth inhibition due to dichromate toxicity was attributed to decreased root cell division or due to the arrested cell cycle.

In crop species such as *Oryza sativa* (Zeng et al., 2011; Schiavon et al., 2012), *Triticum aestivum, Avena sativa* and sorghum (Lopez-Luna et al., 2009), Cr affected the biomass yield, thereby negatively affecting the crop production. Shanker and co-workers (2011) proposed that this may be due to nutrient imbalance, which resulted in stunted growth and lowered biomass production. Chromium shares structural and chemical similarity with

some essential oxyanions including sulphate and phosphate, due to which it can affect the plant mineral nutrition through competitive uptake by common transport proteins (Martínez-Trujillo et al., 2014). Chromium (VI) adversely alters most of the plants biochemical and physiological parameters. Boonyapookana et al. (2002) reported that at a high concentration besides reducing the plant growth rate and crop yield production, Cr adversely affects the vital photosynthetic pigments production channel (e.g., chlorophyll, anthocyanin). In contrast, Henriques et al. (2010) reported pigment damage due to Cr(VI) by performing a photochemical experiment with irradiated chloroplasts. It was proposed that Cr caused reduction in Ca and Mn availability, resulting in pheophytinization of the chlorophyll molecules causing disruption in its function. It is hypothesised that Cr at higher doses might even inhibit chlorophyll biosynthesis by inhibiting δ-aminolaevulinic acid dehydratase, an essential enzyme in pigment synthesis (Gill et al., 2015).

Recently, Cr effect on the Calvin cycle enzymes has been studied in detail. Dhir et al. (2009) suggested that Cr caused oxidative damage to RuBisCO (ribulose-bisphosphate carboxylase oxygenase) enzyme complex which enhanced its oxygenation activity instead of decarboxylation. This may be due to the substitution of Mg^{2+} in the active site of RuBisCO subunits by Cr ions. In some Cr tolerant species, Cr induced the expression of ATP synthase, RuBisCO small subunit and coproporphyrinogen III oxidase. Bah et al. (2010) performed proteomic analysis of *Typha angustifolia* exposed to Cr and found that exposure to Cr increased the expression of ATP synthase, RuBisCO small subunit and coproporphyrinogen III oxidase, all of which play a protective role against Cr stress. The authors suggested that the enhanced expression of ATP synthase was due to increased energy demand under such stressed conditions. Metabolic changes in relation to energy demands as induced by Cr stress are an unfocussed area which needs to be explored. Sugars are the main source of energy metabolism and it plays a key role as signaling

molecules (Rosa et al., 2009). Understanding changes in the soluble sugars and starch accumulation in leaves is very crucial which will help in characterization of Cr-induced phytotoxicity. Rodriguez et al. (2012) found that in *Pisum sativum*, following exposure to Cr(VI) upto 2000 mg/L, soluble sugars and starch concentration increased, but sucrose (transport sugar) and glucose concentration decreased. Tiwari et al. (2009) reported decrease in the amount of non-reducing sugars while the reducing sugars amount increased. In contrast to these reports, Prado et al. (2010) observed an increase in sucrose levels whereas the concentration of glucose decreased in Cr treated plants. Najafian et al. (2012) found that in *Brassica napus L*. the dissolved sugar in root and aerial parts significantly raised upon Cr accumulation. This can be attributed to the plant's defence mechanism in dealing with the Cr toxicity.

High Cr(VI) concentrations lowered the uptake of essential elements, viz. Fe, K, Mg, Mn, P and Ca in *Salsola kali* (Oliveira, 2012). Tiwari et al. (2013) reported that in *Raphanus sativus*, a high concentration of Cr (20.8 mg/L) induced toxic effects on plant's metabolic activity and translocation of nutrients. As a result, iron concentration in leaves was severely affected (from 134.3 to 71.9 µg/g dw) and it negatively affected the translocation of sulphur, zinc and phosphorus.

Thus, it can be inferred from the afore-mentioned studies that in the advent of Cr toxicity, plants respond by specific physiological and biochemical changes thereby enabling the plants to adapt and protect against oxidative stress conditions. Exposure to a high concentration of Cr ions exerts oxidative stress that affects basic metabolism, transport processes, membranes and cellular structure in plants. Such defence mechanisms, therefore, play a major role in protecting cellular and metabolic machinery in plants.

It is reported for most aquatic plants that Cr concentration in the shoots is considerably lower than in the roots (Gil-Cardeza et al., 2014). Cr(VI) accumulation potential is about

10 to 100 times lower than that of Cr(III), probably due to higher Cr(VI) toxicity (Kováčik et al., 2014). Liu et al. (2008) performed experiments with *Amaranthus viridis* at different concentrations of Cr(VI) under hydroponic condition (Liu et al., 2008) and reported roots as the primary site for Cr accumulation. Corroborating these results are the findings of several authors (Vernay et al., 2008). Vernay et al. (2007) showed that *Lolium perenne* roots accumulated 10 times more Cr than its leaves when grown in the presence of 500 μM of Cr(VI). Further, Ghani et al. (2011) found much lower accumulation of Cr in shoots as compared to that in roots signifying a low rate of translocation in *Brassica oleracea* grown on sand with 0.5mM Cr(III). *Spinacia oleracea* L. cv. "Banarasi" also showed a high Cr accumulation in the roots than in the aerial parts when grown in medium supplemented with Cr(VI) (Sinha et al., 2007). *Monochoria vaginalis* accumulated the most Cr in its underground parts and *Eclipta prostrata* accumulated the most in its aboveground parts. Similar results were obtained in celery seedlings grown in the presence of Cr(III), where Cr was accumulated mainly in the roots (Scoccianti et al., 2006). In *Tradescantia pallida* plants, Cr(VI) accumulation was observed to be dose dependent with a maximum accumulation occurring in the *T. pallida* roots; a good amount was also translocated to the plant's shoots and leaves (Sinha et al., 2014). In another study performed by Lopez-Luna et al. (2009) the roots of wheat, oat, and sorghum were found to accumulate more Cr than the shoots of these crops.

Several studies have reported Cr complexation with organic compounds, to facilitate Cr availability in plants (Lopez-Luna et al., 2009). It has been found that exposure of *Triticum vulgare* to $CrCl_3$ supplemented with oxalic acid, malate or glycine resulted in more accumulation of Cr in roots than in plants exposed to Cr only (Cervantes et al., 2001). Furthermore, Kabata-Pendias (2010) reported 100 times more Cr accumulation in underground parts of several crops irrespective of Cr valance state with a reduced rate of

translocation from roots to shoots. These reports signify that in the majority of accumulator plants, Cr is accumulated mainly in roots, followed by that in stems and leaves; however, only small amounts of Cr are translocated to leaves.

Bioconcentration factors (BCF) and translocation factors (TF) are the two main parameters used to evaluate a plant's potential to remediate a particular metal. Gafoori et al. (2013) found high amounts of Cr in the aboveground parts of *Dyera costulata* and, thus, suggested that the plant species has a high phytoremediation potential. *Pluchea indica* also showed a significant translocation rate and Cr concentration in leaves, thus showing good potential as a phytoremediator (Sampanpanish et al., 2006). Pandey et al. (2012) reported a very good potential of *Azolla caroliniana* for phytoremediation of Cr with a BCF value of 11 in the leaves along with uptake of multiple heavy metals. Mellem et al. (2012) reported that *Amaranthus dubius* has a BCF value greater than 2 and a translocation factor of 1.1 at 25 mg/L initial Cr(VI) concentration. Furthermore, Gardea-Torresdey et al. (2005) found that *Convolvulus arvensis* L. species accumulated about 3800 mg Cr per kg dw tissue under hydroponics condition (20 mg/L initial Cr(VI) concentration).

2.4. Chromium Tolerance and Detoxification Mechanisms in Plants

The response to Cr stress involves a network of shared pathways that are involved in Cr detoxification which help in Cr tolerance by plants. In Cr accumulator plant species several mechanisms have been described to account for their resistance to chromate. The significant cellular pathways which are found to be upregulated in response to Cr stress are ROS signalling (Singh et al., 2013), increased antioxidant system (Valko et al., 2005), phytochelatins production (Prasad et al., 2004), phytosequestration and differential compartmentation (Oliveira et al., 2012) all of which help in increasing the Cr

bioaccumulation ability of the cells. Fig 2.3 shows a schematic of chromium tolerance and detoxification mechanisms adopted by Cr tolerant plants.

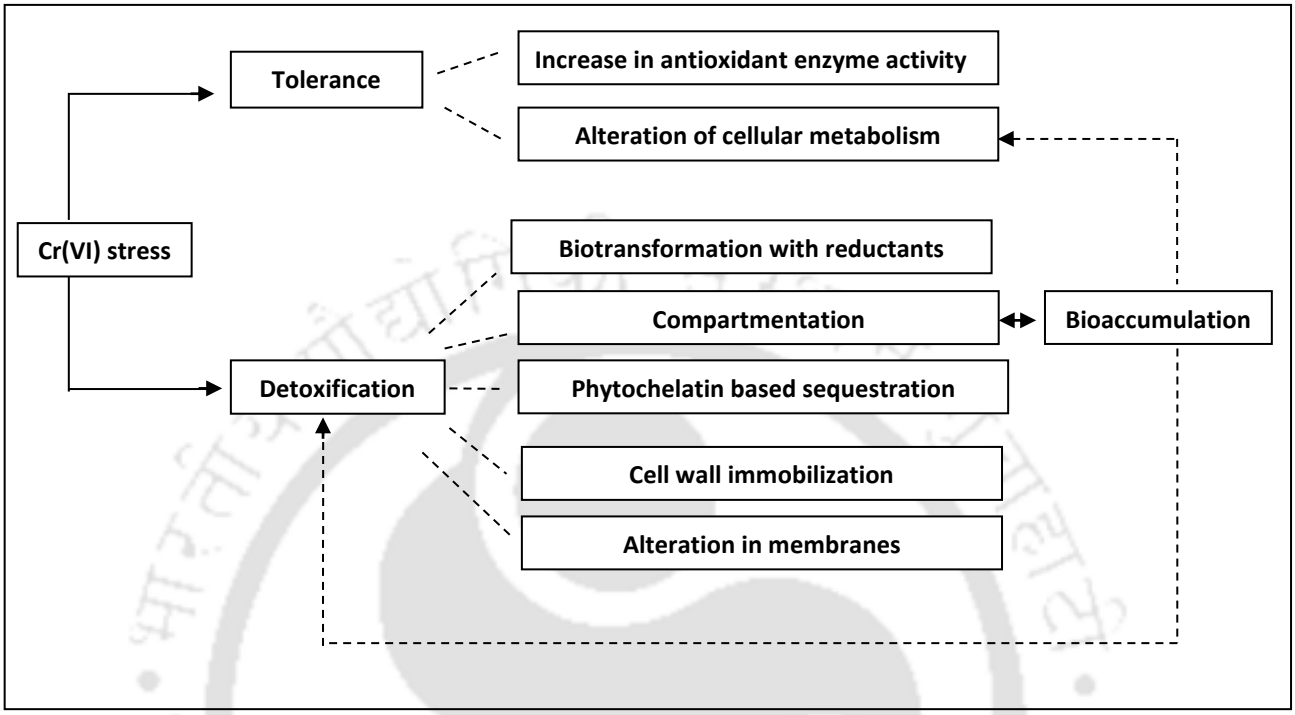

Figure 2.3: Schematic of chromium tolerance and detoxification mechanisms

2.4.1. Tolerance Mechanisms

Chromium tolerant plants can be categorised on the basis of their ability to survive in the presence of Cr at concentrations that are in general inhibitory or lethal to plants. Cr accumulator plants develop several tolerance and detoxification mechanisms that helps these plants to tolerate high levels of Cr. Increase in antioxidant enzyme activity is considered a vital defence mechanism against Cr stress. Costa et al. (2010) reviewed the tolerance mechanism of Cr concluding that Cr concentration at toxic level can produce ROS via the Fenton and Haber–Weiss reactions and indirectly by inhibiting antioxidant enzymes (Costa et al., 2010; Ganesh et al., 2008; Gill and Tuteja, 2010) reported elevated activity of antioxidant enzymes, such as catalase, peroxidases, superoxide dismutase, ascorbate peroxidase, which protects the plants from reactive oxygen species (ROS)

generated under Cr stress by activating scavenging machinery of the plant system. These enzymes inhibit or reduce the rate of the oxidative processes by interrupting the free radical chain reaction.

It has been reported that plants undergo changes at the gene expression level which alters the metabolic pool in response to oxidative stress induced by Cr (Shao et al., 2008). Glutathione reductase (GR) plays a key role in Cr tolerance. It acts as a ROS scavenger, metal chelator and as a substrate for phychelatins biosynthesis. Increase in the synthesis of GR, which is one of the main enzymes of Ascorbate–Glutathione pathway, has been reported by many authors (Anjum et al., 2012; Foyer and Noctor, 2005). GR protein (which prevents the formation of HO• radical) has been characterized and used in transgenic plants such as *Brassica juncea, Helianthus annuus* and *Liriodendron tulipifera* for reducing ROS load in plants (Shanker et al., 2005; Sarangi et al., 2009). In *Miscanthus sinensis*, 36 proteins including oxidative stress-related proteins, metabolism-related proteins, molecular chaperone proteins and others were found to be overexpressed in response to 50–1,000 μM Cr concentration (Sharmin et al., 2012).

2.4.2. Detoxification Mechanisms

A key mechanism for reducing Cr toxicity in plants is the reduction of Cr(VI) to Cr(III) by chemical or enzymatic processes. Whitacre (2010) found chemical reduction of Cr(VI) is mediated by cysteine, glutathione, sulphite and thiosulfates present in the plant cell. Enzymatic reduction of Cr(VI) is carried out by a diverse range of rhizospheric bacteria, for example by *Staphylococcus arlettae* spp. (Sagar et al., 2012), *Ochrobacterium intermedium* (Masood and Malik, 2005), *Pseudomonas sp.* (Dogan et al., 2011), *Bacillus* spp. (Das et al., 2014), *Mesorhizobium* spp. (Wani et al., 2008) and *Cellulosimicrobium cellulans* $KUCr_3$ (Chatterjee and Mukherjee, 2009). These bacteria contain soluble and membrane-bound reductases such as flavin reductase, cytochromes and hydrogenases that

can use chromate as the terminal electron acceptor in their electron transport system (Soni et al., 2013). In addition to biotransformation, these bacteria also release plant growth promoting substances that further enhances Cr(VI) reduction.

To prevent toxicity upon Cr ions uptake, plant system seems to have adopted different mechanisms to store the Cr ions in metabolically inactive organelles. Liu et al., (2009) reported that *Leersia hexandra* accumulated and preferentially stored Cr in the root cell walls and the leaf vacuoles. They reported Cr sequestration inside the cell wall as a primary site and secondarily in internal organelles, mainly inside the vacuoles and plastids. Lahouti et al., (2008) reported Cr accumulation as inclusion bodies in the cell wall of root cortical cells in *Raphanus sativus*. Eleftheriou et al., (2015) reported membrane integrity, particularly of plasma membrane and tonoplast as another cellular defence mechanism in *Arabidopsis thaliana* to attenuate Cr toxicity.

Mangabeira et al. (2011); Volland et al. (2012) and Leitenmaier and Kupper (2013) reported compartmentalization of Cr(VI) in the cytoplasm or in the vacuole of plants. This subcellular compartmentaliza-tion of Cr inside the vacuoles represents a key mechanism of Cr detoxification by hyperaccumulator plant cells (Zeng et al., 2011). In this detoxification mechanism, Cr ions are prevented from entering into metabolically active compartments (e.g. chloroplast and mitochondria), which act as the most vital organelles for carrying out photosynthesis and respiration. Such a process helps in maintaining a low cytoplasmic Cr concentration, thereby acting as a possible detoxifying mechanism (Liu et al., 2009). Table 2.5 lists the Cr localization and toxicity effects based on ultra structural studies carried out on different plant species.

Table. 2.5: Chromium localization and toxicity effects based on plant ultra structural studies.

Plant Species	Cr conc.	Main Cr accumulation sites	General Effects	Toxic effects	Method Used	References
Alternanthera philoxerides (Alligator-weed) Family: Amaranthaceae *Borreria scabiosoides* Family: Rubiaceae *Polygonum ferrugineum* (Knotweed) Family: Polygonaceae *Eichhornia Crassipes* (Water hyacinth) Family: Pontederiaceae	Cr(III) at 0.25, 50 mg/L	Cr accumulated principally in the roots of all the four macrophytes (8.6–30 mg /kg dw)	Cr was present mainly in the vacuoles of root parenchyma cells and cell walls of xylem and parenchyma	Alterations in the shape of the chloroplasts and nuclei in *A. philoxeroides* and *B. scabiosoides*	Inductively coupled plasma mass spectrometry (ICP-MS), Transmission Electron Microscopy (TEM) and Secondary Ion mass Spectrometry (SIMS)	Mangabeira et al., 2011
Allium cepa (Onions) Family: Amaryllidaceae	Cr(III) at 5.2 mg/L	High amount of Cr was mainly accumulated in the cell walls and vacuoles of fourth or fifth cortical layer of	Large vacuolar precipitates surrounded by membranes inside vacuoles; increment of disintegrated organelles and high vacuolization in cytoplasm	Not Repoted	Transmission electron energy loss spectrometry	Liu and Kottke, 2003

		root cell.				
Arabidopsis thaliana (Mouse-ear cress) Family: Brassicaceae	Cr(VI) at 5.2 mg/L	Primarily in the cell wall and secondarily in internal compartments, such as vacuoles and plastids	Severely damaged plastids, mitochondria, golgi bodies and vacuoles Endoplasmic reticulum, cytoplasm and membranes the least affected Nuclei and cell walls were intermediately affected	High ROS production. A concentration-dependent decrease of root growth and a time-dependent increase of dead cells, callose deposition, hydrogenase and peroxidase activity	Light Microscopy (LM) TEM	Eleftheriou et al., 2015
Borreria scabiosoides Family: Rubiaceae	Cr(III) at 25, 50 mg/ L	Higher number of Cr deposits in cortical parenchyma, particularly in vacuoles and cell walls, compared to stellar tissue	Cr preferentially accumulated in cell walls and in vacuoles of cortical roots cells. Plant roots exhibited higher Cr concentrations than the aerial plants parts	Not Repoted	HRI-SIMS (High-resolution imaging secondary ion mass spectrometry) ICP-MS	Mangabeira et al., 2006
Callitricha cophocarpa (Water-starwort) Family: Callitrichaceae	Not Reported	Cr(III) accumulated solely in glands/hairs Cr(VI) accumulated mainly in vascular bundles	Cr uptake, transport and accumulation depended on the oxidative state of the element	Not Repoted	Micro X-ray fluorescence (μXRF) Electron probe X-ray microanalysis (EPXMA)	Augustynowicz et al., 2014
Eichhornia crassipes (Water hyacinth)	Not Repoted	Roots xylem cell walls	Not Repoted	Not Repoted	ICP-MS SIMS	Mangabeira, 2004

Family: Pontederiaceae						
Iris pseudacorus (Yellow flag) Family: Iridaceae	Cr(III) at 0.039 mg/L	Cr content was highest in cell walls of the root cortex and in the cytoplasm and intercellular spaces of the rhizome The Cr conc. in root tissues was in the order cortex >rhizodermis> Stele. Even Cr distribution in rhizome	Increased number of vacuoles and granules in rhizome cortex Cr co-occured with sulphur, indicating Cr sequestration by metal binding proteins	Ultrastructural alterations in the rhizodermis (cell wall disorganisation, thickening, plasmolysis, and electron-dense inclusions) Rhizome parenchyma showed (reduced cell size, cell wall detachment, vacuolation, and opaque granules)	TEM and Energy Dispersive X-Ray Analysis (EDX)	Caldelas et al., 2012
Leersia hexandra Swartz (Southern cutgrass) Family: Poaceae	Cr(III) at 60 mg /L	Most of the accumulated Cr was isolated to the cell walls in roots and the vacuoles in leaves	83.2% of the root Cr was localized in the cell wall fraction, while 57.5% of leaf Cr was localized in the vacuole and cytoplasm fraction	No phototoxic symptoms No significant decrease in biomass All organelles appeared normal	Differential centrifugation, TEM and EDX	Liu et al., 2009
Lycopersicum esculentum (Tomato) Family: Solanaceae	Cr(III) at 25, 50 mg/ L	Cr accumulated mainly in the roots, and walls of xylem vessels	No Cr was detected in epidermis, palisade parenchyma and spongy parenchyma cells of the leaves Transport of Cr is restricted to the vascular system of roots,	Not Repoted	HRI-SIMS (High-resolution imaging secondary ion mass spectrometry)	Mangabeiara and Gavrilov, 2006

			stems and leaves		ICP-MS	
Pteris vittata (Chinese brake) Family: Pteridaceae	Cr(VI) at 300 mg/L (22 days)	Roots (upto 7686 mg/ kg dry weight) Shoots (upto 2108 mg /kg dry weight)	Stunted growth with an increase in Cr concentration Dose-dependent inhibition	Water stress and collapse of internal structure (leaves and cellular breakdown of roots)	LM, Scanning Electron Microscopy (SEM) and TEM	de Oliveira et al., 2014 Sridhar et al., 2011
Raphanus sativus L. (Radish) Family: Brassicaceae	Cr(III) at 0-7 mg /L	Cr accumulated in periplasmic zone (cell wall) of root cortical cells and not in the matrix	Presence of Cr deposit inclusions in root cortical cells	Not Repoted	TEM	Lahouti et al., 2008
Taxithelium nepalense (Schwaegr.)(moss) Family: Sematophyllaceae	Cr(VI) at 0, 5.2 and 52 mg/L	Not Repoted	Not Repoted	Increase in H_2O_2 and O^-_2 radical. Distortion of the thylakoid, distortion of chloroplast membrane. Increase in the lipid peroxidation.	TEM	Choudhury and Panda, 2005
Transgenic cotton cultivars (J208, Z905)	Cr(VI) at 0.52, 2.6, 5.2 mg/L (7days)	Cr accumulated more in roots	Increase in number of nuclei and vacuoles Presence of Cr dense granules in	Significant reduction in root/shoot length, number of secondary roots, and fresh root and	Multiple biomarkers approach	Daud et al., 2014

			dead parts of the cell (vacuoles/cell wall). Upregulation of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), total soluble proteins, superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) with elevated levels of Cr.	dry biomass at 5.2 mg/L		
Ocimum basilicum (Basil) Family: Lamiaceae	Cr(III) at 4, 6, and 8 mg/L	Cr accumulated maximally in the roots	Dense granular metal deposits in the periplasmic zone along the cell walls in root cortex cells Cr was mainly deposited in the cytoplasm of root cortex cells and enlarged periplasmic zone along the innermost layer of the cell wall.	-	TEM, X-ray microanalysis	Bishehkolaei et al., 2011

Oliveira (2012) reported Cr tolerance in hyperaccumulator plants through chelation with suitable high-affinity ligands and biotransformation with reductants. Plants enrich their cytoplasmic pool with an increased production of phytochelatins, histidine, glutathione, ascorbic acid and other biochemically similar molecules which further help protect the plant metalloenzyme systems against Cr damage and combat the stress situation (Diwan et al., 2010; Yadav, 2010). Studies were conducted using four salix species to identify Cr-stress responsive genes involved in the regulation of Cr tolerance and accumulation (Quaggiotti et al., 2007).

Some plants restrict the uptake of Cr ions within root tissue and prevent its entry into the active cellular pool and its further translocation. The binding of Cr ions onto the plant cell walls is frequently reported in Cr accumulating aquatic and terrestrial plants spp. (Elangovan et al., 2008; Mangabeira et al., 2011). Cell wall provides abundant pectic sites, and secretes extra-cellular carbohydrates such as callose and mucilage that reduces Cr translocation into cytosol. Pectins are polysaccharides rich in carboxyl groups (–COOH) which enable the binding of Cr ions. Lignin, another polymer rich in hydroxyl (–OH) and phenolic groups, plays a crucial role in Cr binding onto secondary cell wall (Miretzky and Fernandez Ciirelli, 2010). Zeng et al. (2014) reported the up regulation of two proteins related to cell wall structure, NAD-dependent epimerase/dehydratase and reversibly glycosylated polypeptide in response to Cr stress in *Oryza sativa* L. Their enhancements along with callose accumulation under Cr stressed condition suggest that cell wall is an important barrier for rice plants to reduce its translocation as a resistance mechanism. Further, transport of Cr ions occur using active efflux pumps present in membranes to apoplastic regions like cell wall where it binds with the cell wall components and precipitates.

Hence, it could be concluded that plants strategies in response to Cr toxicity are genotype-specific. The underlying mechanism of plant's tolerance is a combination of process that enables certain plants to survive Cr stress and adapt to maintain its growth and development without any toxicity symptoms.

2.5. Chromium Transport and Uptake Mechanism in Plants

Very few studies have been conducted to illustrate Cr uptake pathway in plants. Cr valence state is one of the main factors affecting Cr transport inside the plant cell (Banks et al., 2006). Since Cr is a nonessential element, plants lack any specific mechanism or transporters for its uptake. It has been reported that for its entry inside the plant cell, reduction of Cr(VI) to less harmful Cr(III) takes place on plant root surface. Plant cell constituents such as NAD(P)H, glutathione, several pentoses, $FADH_2$, ascorbic acid, cyanocobalamin, cytochrome P-450, and the mitochondrial respiratory chain are involved in the reduction process (Cheung and Gu, 2007). In contrast, Davidian et al. (2010) suggested that Cr(III) forms water insoluble compounds in non-acidic aqueous solutions and, are, therefore, membrane impermeable to biomembranes. Buchner et al. (2004) have further demonstrated that Cr(VI) uptake in plants occurs without undergoing reduction. Cr(VI) compounds structurally resemble SO_4^{2-} ions and enters the cell through carriers of essential anions such as sulphate and phosphate transporters which are essential plant nutrients that easily cross the plant cell membranes.

Figure 2.4 shows Cr uptake, transport and antioxidant defence mechanism adopted by plant cells.Chromate transport across biological membranes is reported to be an active process (Schiavon et al., 2012; Marieschi et al., 2015). Following uptake and inside the cell cytoplasm, Cr(VI) detoxification pathway follows reduction of Cr(VI) to Cr(III) via intermediate formation of the unstable Cr(V) and Cr(IV) states which leads to ROS

generation (Gupta and Ballal, 2015). Contrary to this no differences in the uptake of either Cr(III) or Cr(VI) were observed in *Phaseolus vulgari* (Nath et al., 2009) and *Triticum aestivum L*. (Subrahmanyam, 2008).

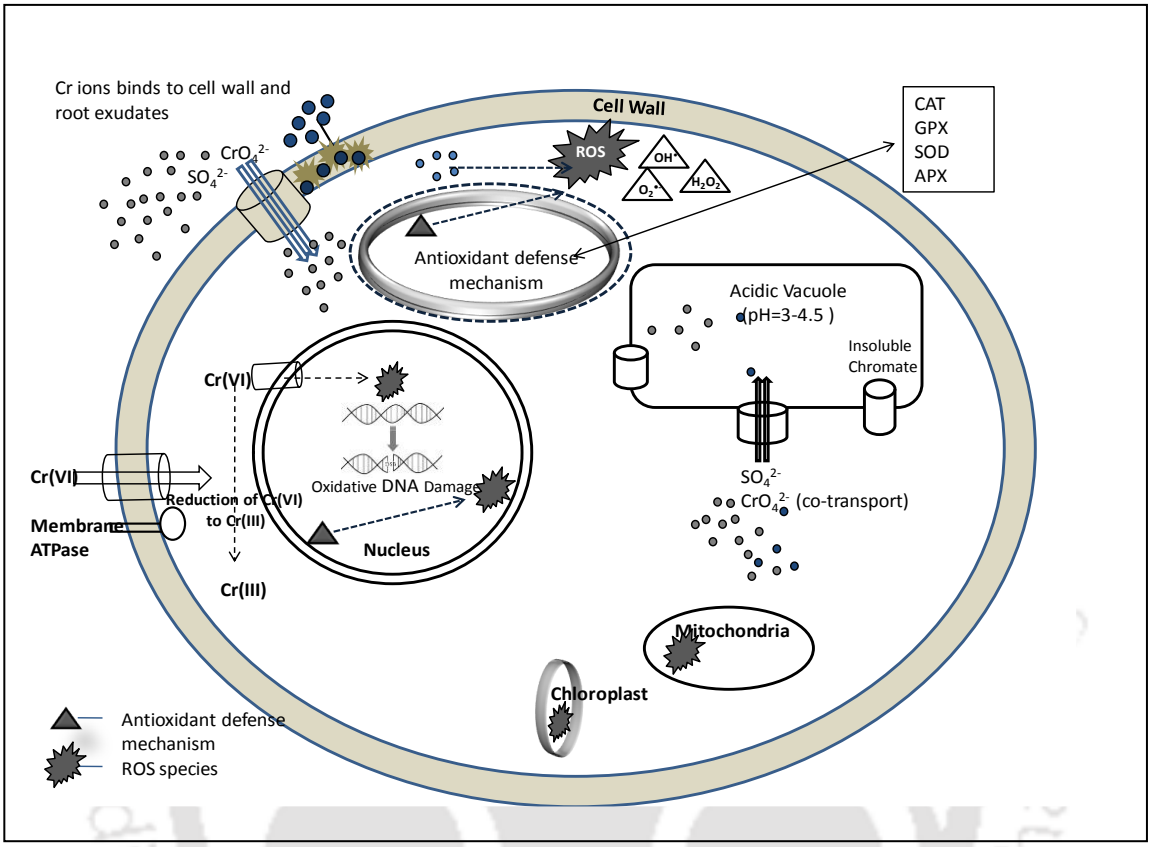

Figure 2.4: Chromium uptake, transport and antioxidant defence mechanisms adopted by plant cells

It has been shown that addition of multidentate chelating agents like EDTA and vermicompost enhance the Cr bioavailability and thus increases the uptake of Cr by plants (Jean et al., 2008). This is ascertained by the fact that chelating agents possess functional groups capable of Cr absorption and conversion. Supplementation of the contaminated soil with vermicompost was reported to further enhance the plant biomass growth, thus favouring the plant bioaccumulation potential as found in *Sorghum* (Revathi et al., 2011) and *Helianthus annus* (Jadia and Fulekar, 2009).

2.6. Chromium Removal in Constructed Wetland Systems

Constructed wetland is a mimic of natural wetland that combines physicochemical and biological treatment mechanisms in removing pollutants from wastewater as it flows through the wetland. Among the different treatment systems, constructed wetland offers better hydraulic control and management over natural wetlands (Lim et al., 2001). Recently, CWs have been successfully applied to be efficient and low cost treatment method for secondary treatment of industrial effluents loaded with heavy metals and other contaminants (Wu et al., 2016; Ge et al., 2016). The removal mechanisms concerning Cr removal in a wetland system are: adsorption to the bed media, sedimentation, cation and anion exchange, complexation, precipitation as insoluble forms, plant uptake, and microbial metabolism (Kadlec and Wallace, 2009 and Yadav et al., 2010).

2.6.1. Types of Constructed Wetlands

Fig 2.5 is a schematic showing characteristics of constructed wetlands into different types according to the water flow mode and the dominant aquatic plant species (Vymazal Jan., 2007). For heavy metals removal, vertical subsurface flow CWs has been efficiently employed by many researchers (Arivoli et al., 2015; Hua et al., 2013; Kamarudzaman et al., 2012).

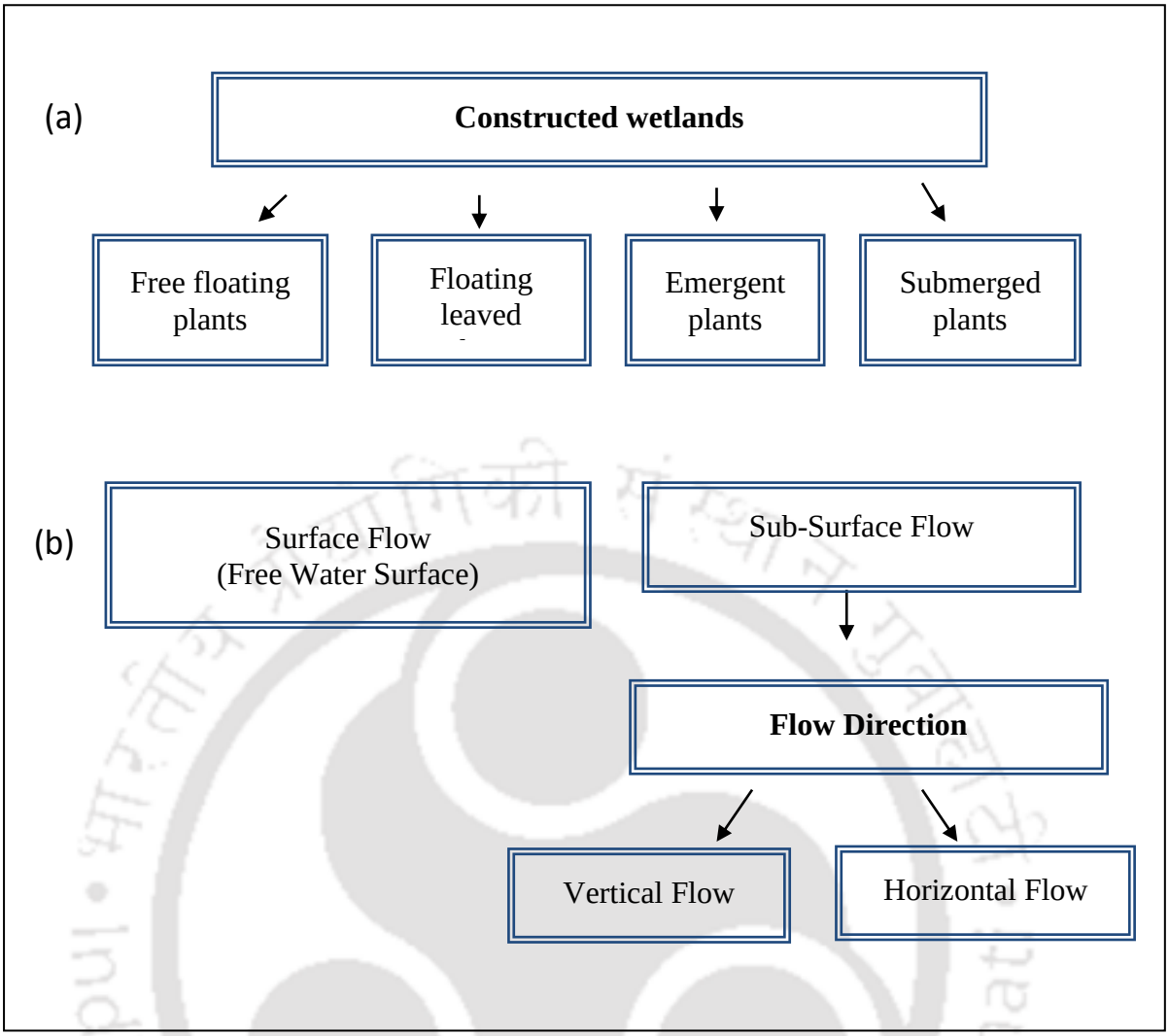

Figure 2.5: Classification of constructed wetlands based on (a) life form of the dominating plant species (b) wetland hydrology (Vymazal Jan., 2007)

It consists of one or more layers of a granular medium such as soil, coarse sand and/or gravel. Contaminated wastewater is supplied over the top surface, which gradually percolates down through the permeable filter layers and is then collected at the bottom by means of drainage pipes. The above-mentioned types of CWs can be combined with conventional wastewater treatment systems, to treat pollutants in wastewater (EPA, 2000; Shutes, 2001). Fig 2.6 shows vertical and horizontal flow constructed wetland.

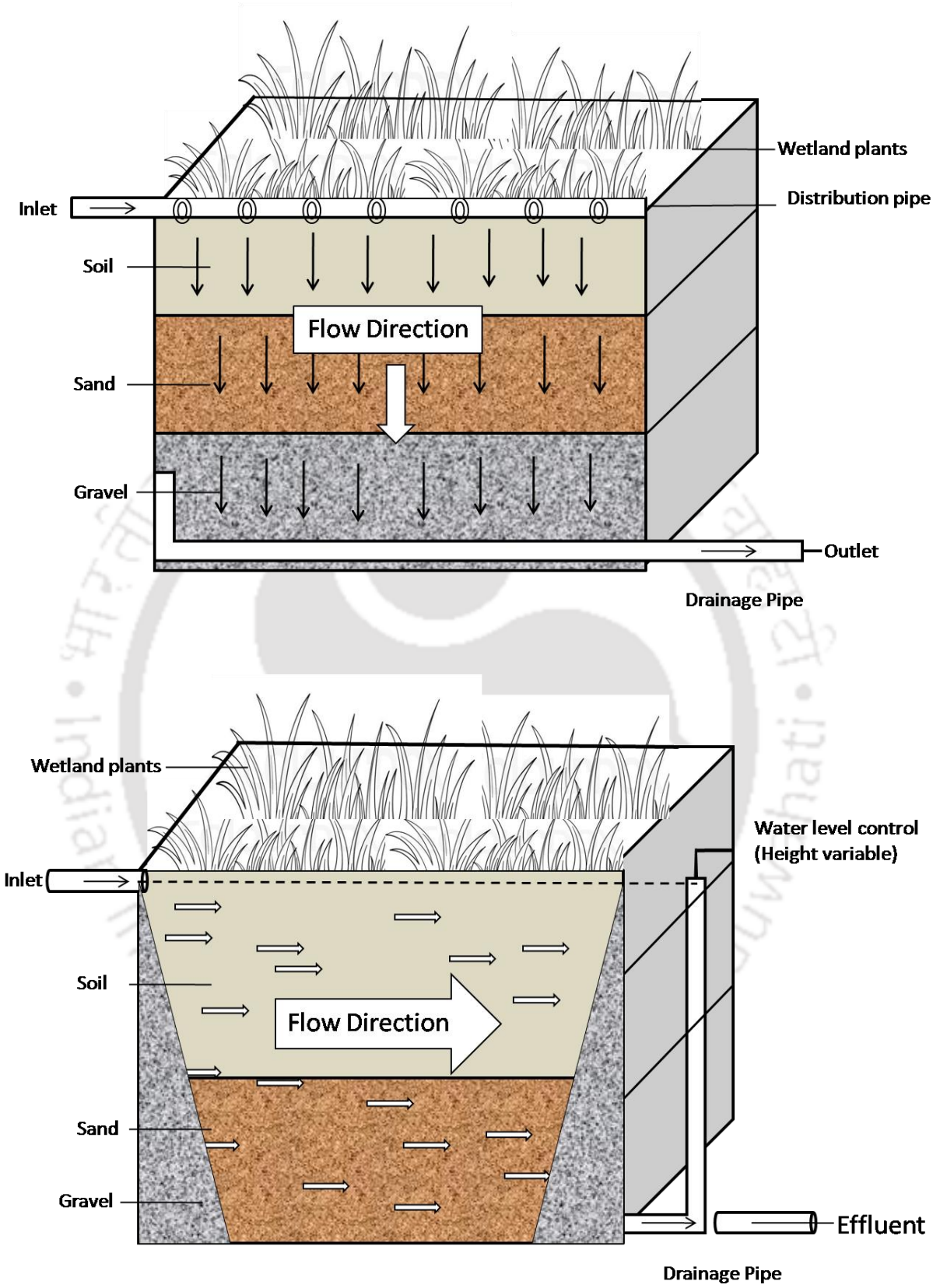

Figure 2.6: Schematic of the (a) Vertical flow constructed wetland (b) Horizontal flow constructed wetland.

It has been shown that the wetland systems had remarkable removal efficiencies on treating Cr contaminated wastewater. Several studies have shown significant removal of Cr from wastewater using horizontal subsurface flow (HSF) CWs (Aguilar et al., 2008; Calheiros et al., 2009; Dotro et al., 2012; Fibbi et al., 2012; Rai et al., 2015; Sultana et al., 2015; Alemu et al., 2016; You et al., 2016) and vertical subsurface flow (VSSF) CWs (Mant et al., 2006, Yadav et al., 2010; Sochacki et al., 2014).

Table 2.6 lists the recent phytoremediation research carried out by chromium accumulator plants using constructed wetlands and soil system. In recent years, many research studies have been carried out to understand Cr tolerance, accumulation and uptake mechanism processes by Cr accumulator plants in CWs and soil systems. Currently many phytoremediation experiments have been performed using CWs, fed with different concentrations of Cr, under controlled environmental conditions have been carried out. The most commonly used Cr accumulator plant species used in CWs for Cr removal are the *Phragmites australis*, *Typha latifolia* (Vymazal and Brezinova, 2016), *Leersia hexandra* (Liu et al., 2015), *Brassica juncea* (Bluskov et al., 2005) and *Dicoma niccolifera* (Banach et al., 2012).

Table. 2.6: Efficient chromium accumulator plants investigated using constructed wetlands and soil system.

Cr accumulator	Habitat	Family	Cr(VI) removal mechanism	Culture condition	Mode	Max % removal/ Bioaccumulation capacity	Exp. period	Influent conc.	Referen-ces
Brachiaria mutica (Paragrass)	Perennial grass	Poaceae	Not Reported	Soil field study (mine wastewater)	Continuous	Transportation index (TI): 6.16 Total accumulation rate (TAR) :8.2 mg/ kg/day	100 days	0.65 mg/L and 0.74 mg/L for Cr(VI)	Mohanty and Patra, 2012
Brassica juncea (Indian mustard)	Annual growing perennial herb	Brassicaceae	Not Reported	Soil condition	Batch	Cr accumulation: 48 and 58 μg Cr per plant from Cr (III) and Cr(VI) treated soils	69 days	Soil amended with 100 mg/ kg of Cr (III or VI)	Bluskov et al., 2005
Callitricha cophocarpa (Water-starwort)	Aquatic macrophyte	Callitrichaceae	Cr(VI) reduction	Wetland		Cr(VI) storage vascular bundles Cr accumulation: Cr(III) 28,385 mg/kg (dw) Cr(VI) 7,315 mg/kg (dw) Cr(III); 98.8 % removal	7 days 5 days	5.2 mg/L Cr(III) and Cr(VI) 26-208 mg/L Cr(III)	Augustyno wicz et al., 2014
Dicoma niccolifera	Terrestrial	Asteraceae	Not Reported	-	-	Cr accumulation: >1000 mg/kg Cr	-	-	Banach et al., 2012
Helianthus annuus (Sunflower)	Annual forb	Asteraceae	Not Reported	Cr contaminat-	Continuous	70 % chromium removal	90 days	10 mg /L Cr(VI)	Ranieri et al., 2013

				ed soil		Cr accumulation: Roots (2,730 mg Cr/kg dry tissue)			
Jatropha curcas (Barbados nut)	Perennial plant	Euphorbiaceae	Not Reported	Greenhouse experiment (Soil and compost based media)	Batch	50 % removal	30 days	10, 30, 50, 70 and 90 mg Cr(VI)	Mangkoedihardjo et al., 2008
Leersia hexandra (Southern cutgrass)	Perennial herb (grow in swamps)	Poaceae	Facilitates microbial growth Cr(VI) reduction and sequestration	CWs (Lab-scale)	Continuous	99.7%	120 days	5 mg/L Cr(VI)	Liu et al., 2015
Medicago sativa (Alfalfa)	Perennial flowering plant	Fabaceae	Not Reported	Soil pot conditions	Batch	60-74%	50 days	0, 4, 8,10 mg Cr(VI) /kg soil	Karimi 2013
Phalaris arundinacea (Reed canarygrass)	Perennial grass	Poaceae	Not Reported	Horizontal subsurface flow CW	Continuous	Cr accumulation: 14.7 mg/ kg dry mass Roots: 18.5 mg/kg Cr .	4 years	Municipal sewage with 0.5- 4 mg/L Cr	Vymazal et al., 2007
Phragmites australis (Common reed)	Perennial grass	Poaceae	Cr(VI) reduction Cr(III) precipitation	Soil pot conditions Horizontal subsurface flow CW	Continuous Continuous	54 % removal Cr(VI) respectively	90 days 2 years	10 mg/L Cr(VI) 5.5 µg /L Cr(VI) and Cr(III)	Ranieri et al., 2013 Fibbi et al. (2012)

Spartina argentinensis (Cordgrass)	Perennial grass	Poaceae	Not Reported	Glasshouse experiment	Batch	Cr accumulation: 15.1 mg/ g Cr(VI) (dw) at 1040 mg/ L	15 days	0-1040 mg/ L Cr(VI)	Redondo-gomez et al., 2011
Typha latifolia (Cattails) *Phragmites australis* (Common reed)	Aquatic grass Perennial grass	Typhaceae Poaceae	Not Reported	Horizontal subsurface flow CW	Continuous	Maximum removal efficiency of 73%	17 months	Synthetic tannery waste water	Calheiros et al., 2007

The studies have revealed that all types of CWs have been used with most systems being horizontal or vertical subsurface CWS with emergent vegetation have been successfully used for removal of Cr from contaminated wastewater. Thus, Cr remediation method using CWs has been widely applicable which is nowadays successfully used in many developed countries.

2.6.2. Disposal and Reuse of Chromium Accumulated Plant Biomass

Following Cr bioaccumulation, however, proper waste disposal management of Cr loaded plant biomass is needed. One viable solution to this problem could be their reuse as biosorbent for removal of the same metal. This way waste plant biomass can serve as a material source as well as reduce the cost of waste disposal.

Biosorption has emerged as a very promising method for treatment of heavy metal containing industrial effluents. The process involves passive uptake mechanism and depends mainly on the "affinity" between a biosorbent and sorbate which can result from complexation, metal chelation, ion exchange, adsorption and microprecipitation (Sud et al., 2008). Performance of an adsorbent depends on many factors, such as pH, the ionic form of the metal to be removed and competition from other organic or inorganic ions for the available binding sites on the adsorbent (Anwar et al., 2009). Different types of biomass have been studied for the removal of Cr(VI), which includes agricultural wastes, plant biomass, and industrial by-products (Pakshirajan et al., 2013). It is well known that cellulosic waste materials can be obtained and employed as cheap adsorbents. Thus, the application of biosorbents obtained from plant wastes can serve as a replacement for costly conventional methods for removing Cr ions, particularly considering the economic and environmental advantages.

Other disposal strategies could be pyrolysis of the plant biomass which is carried out under anaerobic conditions yielding pyrolytic fluid oil and coke in the product stream. Coke concentrated with the Cr can be further used in a smelter. A pilot scale reactor study reported 98.5% metal recovery (Ni, Zn, Cu, Co or Cr) in the char formed by pyrolysis and gasification of hyperaccumulating plant biomass (Koppolu et al., 2003). Incineration under controlled condition, in which ash with a high metal content is recovered, is another option available for disposal of such plant biomass (Revathi et al., 2011). Furthermore, volume reduction processes, such as composting and compaction of Cr accumulated biomass have been proposed as a post harvest biomass treatment (Shukla et al., 2009).

This Chapter highlights the significance of phytoremediation technology for treating Cr(VI) contaminated water as a sustainable, cost-effective, nonintrusive, and safe alternative to conventional cleanup techniques. In particular, the application of specific Cr accumulator plants in CWs to treat Cr contaminated wastewater has been identified as a promising method for remediation. However, there is very little understanding of the plant's tolerance to Cr(VI), its uptake mechanism, detoxification, and translocation. Further, application of CWs using hyperaccumuting Cr(VI) plants are not well-reported.

CHAPTER 3 MATERIALS AND METHODS

3.1. Chemicals and Reagents

All chemicals and solvents used in this study were of analytical grade and procured from Sigma, Hi-Media Pvt. Ltd., India and Merck India Ltd. Stock solution of hexavalent chromium (1000 mg/L) was prepared by dissolving 0.283 g of potassium dichromate (Merck, India) in 100 ml de-ionized water. The stock solution was suitably diluted to achieve a desired working initial Cr(VI) concentration in the experiments. For adjusting the pH of Cr(VI) containing solution of desired concentration,1 N HCl /1 N NaOH was used.

3.2. Collection and Maintenance of Plant Species

Table 3.1 presents the details of different plant species selected to study their Cr(VI) tolerance. All experiments to examine Cr accumulation and tolerance by different plants in this study were carried out under hydroponic conditions. These plants were collected from a non-polluted site in North Guwahati, India. These plants were selected based on their minimal nutrient requirement, non-medicinal or non-edible use, easy propagation within a small time period and a profuse root system. Following collection of the whole plants, they were immediately transferred to polystyrene bags containing 1 L of tap water and stored for 3 days under outdoor condition (recuperation period). For hydroponics experiment, healthy plants with uniform weight were selected, thoroughly rinsed with running tap water in order to eliminate any remains of sediment and finally transferred to clean plastic containers (15 plants per container) with 2 L each of 50% Hoagland's solution. Plants cultivated in Hoagland solution without Cr(VI) served as the control. All the hydroponic culture experiments were carried out under controlled environmental conditions with a temperature regime of 25°C day/night, 14/10 h light/dark period (1,800 lux) and a relative humidity of 70-80%. Composition of Hoagland's solution used in the

hydroponic studies was: (mM in tap water): 2.4 Ca $(NO_3)_2$, 1.0 KH_2PO_4, 3.0 KNO_3, 1.0 $MgSO_4$ and 0.5 NaCl and (μM) 23.1 H_3BO_3, 4.6 $MnCl_2$, 0.38 $ZnSO_4$, 0.16 $CuSO_4$, 0.052 H_2MoO_4 and 44.8 Fe-EDTA complex.

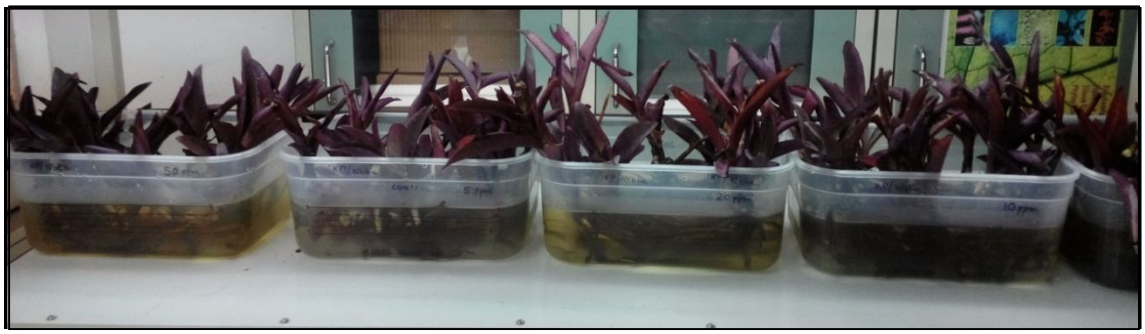

Figure 3.1: *Tradescantia pallida* plants exposed to different concentrations of Cr(VI) in hydroponic culture

3.3. Chromium (VI) Tolerance, Accumulation and Localization

3.3.1. Screening of Different Plants Species

Table 3.1: Different plant species screened for Cr bioaccumulation and tolerance.

Scientific name	Common name	Family	Type	Habitat
Tradescantia pallida	Wandering jew	Commelinaceae	Herbaceous perennial	Tropical and semi-tropical areas
Gnaphalium luteoalbum	Jersey Cudweed	Asteraceae	Annual herb	Cosmopolitan
Alternanthera philoxeroides	Alligator weed	Amaranthaceae	Perennial aquatic herb	Water bodies
Chlorophytum comosum	Spider plant	Asparagaceae	Herbaceous perennial	Tropical areas
Ageratum conyzoides	Billygoat weed	Asteraceae	Annual herb	Tropical areas
Blumea fistulosa	Cutleaf Blumea	Asteraceae	Annual herb	Tropical and sub-tropical areas

The different plant species (Table 3.1) were tested for the effect of different initial concentration of Cr(VI) ranging from 5 to 20 mg/L on their Cr tolerance capability. All

the plant species were grown under hydroponics conditions and maintained under conditions as detailed previously in section 3.2.

Tolerance index (TI) based on plants wet weight was calculated using equation 3.1 with an overall objective of selecting a plant with maximum (TI) value.

$$\text{Tolerance Index} = \frac{\text{mean biomass of plants treated with Cr(VI)}}{\text{mean biomass of control plants}} \times 100 \quad (3.1)$$

Cr(VI) phytoextraction potential of *T. pallida* was measured by calculating its bioconcentration factor (BCF) and translocation factor (TF) at the end of the experimental period (Ali et al., 2013). BCF was calculated according to Eq. 3.2.

$$BCF = \frac{C_{T.pallida}}{C_{Medium}} \quad (3.2)$$

where; C_{Plant} = Cr concentration in *T. pallida* (mg Cr/kg of *T. pallida* biomass) and C_{Medium} = Cr concentration in the medium.

Translocation factor (TF) was expressed as the ratio of Cr(VI) concentration in the plant shoot and root biomass and was calculated according to Eq. 3.3.

$$TF = \frac{C_{Shoot}}{C_{Root}} \quad (3.3)$$

where; C_{Shoot} = concentration of Cr in *T. pallida* shoots (mg Cr/kg) and C_{Root} = concentration of Cr in *T. pallida* roots (mg Cr/kg).

3.3.2. Chromium Accumulation in *T. Pallida*

Based on the previous screening experiment, *T. pallida* was investigated further for Cr(VI) bioaccumulation and tolerance. All experiments to examine Cr tolerance and accumulation by *T. pallida* in this study were carried out under hydroponics conditions.

Different concentration of Cr(VI): 0 (control), 5, 10 and 20 mg/L was supplied as potassium dichromate ($K_2Cr_2O_7$) from its stock solution (100 mg/L). The nutrient solution in each container was replaced with a fresh medium every 4 days during the experiments. However, the plants leaves were kept away from contact with the nutrient culture solutions to avoid Cr precipitation on the same.

For analyses of different *T. pallida* plant parts for Cr accumulation, plants were harvested after 30 days of treatment, and its leaves, shoots and roots were separated. The plant parts were then blotted dry on a filter paper before final drying at 70 °C for 2 days to determine their dry weight (dw). For estimation of total Cr content, plant parts were harvested after 30 and 60 days of Cr treatment, thoroughly rinsed with distilled water to remove any adsorbed metal on the root surface prior to digestion and analyses as detailed further.

3.3.2.1. Extraction and Estimation of Total Chromium

For estimation of total Cr, Cr(VI) and Cr(III) content, *T. pallida* plant parts were harvested after 60 days of Cr treatment, thoroughly rinsed with distilled water to remove any adsorbed Cr on the root surface. The washed plants were separated into leaves, shoots and roots. They were dried at 105°C for 30 min, and then at 70°C for 48 h to constant weight. Dried biomass was digested with HNO_3/ $HClO_4$ in the ratio 3:1 at 110 °C for 1hour (USEPA 3051 method). The digested extract was suitably diluted with de-ionized water and analysed by Atomic absorption spectrometry (AA240, Varian, the Netherlands) as per Standard Methods (USEPA 1995 method).

3.3.3. Separation and Determination of Chromium in Different Subcellular Fractions of *T. pallida*

3.3.3.1. Plant Growth in Presence of Cr

For this experiment, healthy *T. pallida* plants of uniform height (8 ±3 cm) were grown from stem cutting under hydroponics culture conditions. Different concentration of Cr(VI): 0 (control), 10, 20 and 30 mg/L was supplied. The solutions were refreshed every 7 days to maintain the constant Cr(VI) concentration during the 30 days experimental period. Plants were harvested after 30 days of treatment, and its leaves, shoots and roots were separated.

3.3.3.2. Separation of the subcellular fractions

Fresh leaf, shoot and root tissues were homogenized using a chilled mortar and a pestle in a pre-cold extraction buffer containing 50 mM Tris–HCl (pH 7.5), 250 mM sucrose and 1.0 mM dithioerythritol. The homogenate was then transfered into a 50 ml centrifuge tube and centrifuged at 300×g for 5 min using a high speed refrigerated centrifuge. The pellet was considered as cell wall fraction (Fw). The supernatant was further centrifuged at 12000×g for 30 min to sediment the cell organelles. The pellet obtained was taken as organelle fraction (Fo). The resultant supernatant solution was referred as soluble fraction including macromolecular organic matter and inorganic ions in the cytoplasm and vacuoles (Fv). All homogenizations and subsequent fractionations were performed at 4°C. The pellets from different fractions were dried and the dried plant tissues were ground with pestle and mortar to pass through a 40-mesh screen. The plant tissues (about 0.5 g) of dried pellet and 5 ml supernatant were digested. After cooling and dilution using 0.2% HNO_3, total Cr concentration were measured by atomic absorption spectrophotometry as mentioned earlier in section 3.3.2.1. In order to determine the total Cr concentrations in

different tissues percentage recovery of Cr was calculated according to according to Eq. 3.4.

$$\text{Recovery (\%)} = \frac{(\text{Cell wall} + \text{Soluble fraction} + \text{Organelle})}{\text{Total}} \times 100 \qquad (3.4)$$

Organelles sedimented by centrifugation at increasing speeds are listed in Table 3.2. Schematic showing steps followed in separation of organelles sedimented by differential centrifugation in presented in Fig. 3.2.

Table 3.2: Organelles sedimented by centrifugation at increasing speeds.

	Speed	**Fraction**	**Organelles**
1.	300 x g (5 min)	Fw	Cell wall fraction
2.	12,000 x g (30 min)	Fo	Cell organelles fraction
3.	12,000 x g Supernatant	Fv	Cytoplasm and vacuole fraction

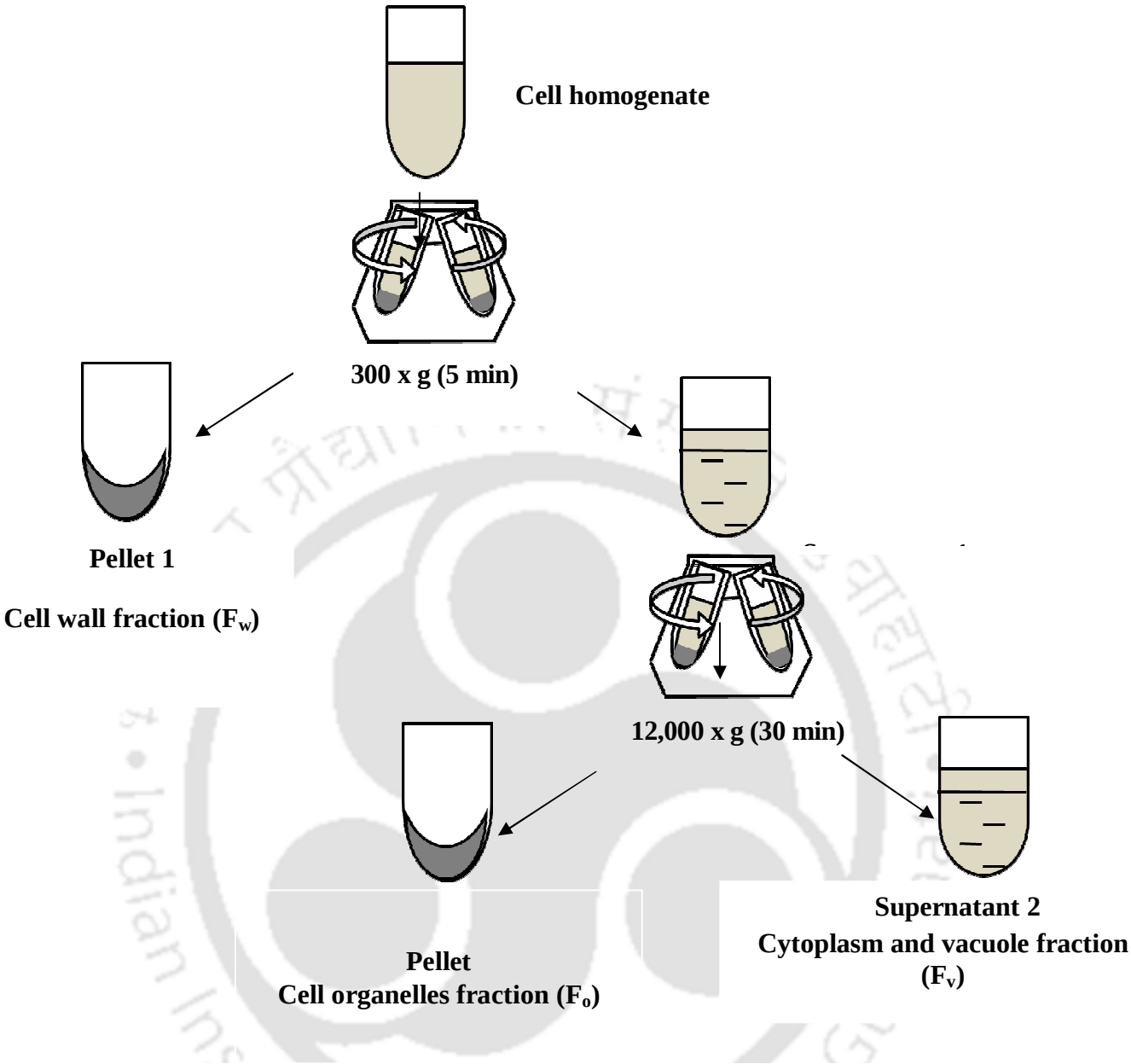

Figure 3.2: Schematic of steps followed in separation of organelles sedimented by differential centrifugation

3.3.3.3. Energy Dispersive X-Ray Analyses

To observe Cr accumulation, EDX study was performed. Dried and powdered plant biomass of cytoplasm and vacuolar fraction (F_v) was mounted on copper grids for observation under electron microscope (JEOL TEM-1230EX) at 60.0 KV voltage. They were analysed by EDX with a 35 nm spot at 200 kV using an energy dispersive detector (JEOL TEM-1230EX). The C peaks in the spectra were caused by the carbon film. In

order to eliminate the interference of Cu-grid, Cu information was deducted from the spectra.

3.4. Antioxidant Enzyme and Biochemical Changes in *T. pallida* due to Chromium Exposure

For estimation of antioxidant enzyme activity and biochemical parameters due to Cr exposure, *T. pallida* plant parts were harvested after 30, 60 and 90 days treatment and analysed for catalase, peroxidase, ascorbate peroxidase and lipid peroxidase. Protein, carbohydrate, proline and chlorophyll content were determined to observe biochemical changes in the plant parts.

3.4.1. Estimation of Lipid Peroxidase Activity

The lipid peroxidase activity was determined following the method described by Health and Packer (1968). Briefly, 0.5 g of powdered plant tissue was homogenised in 20 % trichloroacetic acid (TCA), containing 0.5 % 2-thiobarbituric acid and heated at 95 °C for 30 min. The thiobarbituric acid reactive substances (TBARS) concentration was measured as malondialdehyde (MDA; ε = 155 mM/cm), which was determined at OD_{532} and corrected for nonspecific turbidity at OD_{600} using a ultraviolet (UV)–visible spectrophotometer (Cary 100, Varian, Australia).

3.4.2. Estimation of Catalase Activity

Catalase activity was determined according to a method described by Aebi et al. (1984). About 0.5 g fresh leaf samples were homogenised with 5 mL of cold 200 mM sodium phosphate buffer (pH 7.8), using chilled mortar and pestle. The homogenates were centrifuged at 10,000×g for 20 min at 4 °C, and the supernatant was analysed for catalase activity by using a UV–visible spectrophotometer (Cary 100, Varian, Australia). The

reaction mixture (2.8 mL) contained 1.5-mL 200-mM sodium phosphate buffer (pH 7.8), 1.0-mL deionised water and 0.3-mL 0.1-M H_2O_2 prepared afresh prior to its use. The reaction mixture was then added with 0.5 mL enzyme extract, and the enzyme activity was measured by monitoring the decrease in absorbance at 240 nm due to H_2O_2 consumption. One unit of catalase activity was defined as change in absorbance of the mixture at 240 nm/min/g of fresh weight.

3.4.3. Estimation of Peroxidase Activity

The peroxidase activity was determined based on the oxidation of guaiacol, which was measured by an increase in the absorbance at 470 nm (Ambreen et al., 2000). The reaction mixture (total volume, 1 mL) contained 25 mM phosphate buffer (pH 7.0), 1.0 mM H_2O_2 (30%), 0.05% guaiacol and 0.1 mM EDTA and enzyme extract. The reaction was initiated by adding H_2O_2, and the increase in the absorbance was monitored at 470 nm (ε=26.6 mM/cm) at 1-min intervals up to 4 min. One unit of peroxidase activity was defined as the change in absorbance at 470 nm/min/mg fresh weight (fw) of leaf.

3.4.4. Estimation of Ascorbate Peroxidase Activity

Ascorbate peroxidase (APX) activity was measured according to a method described by Leonardis et al. (2000). Two-millilitre reaction mixture for this assay contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.3 mM ascorbate, 0.1 mM H_2O_2 and 100 µL enzyme extract. The reaction was initiated by the addition of H_2O_2, and oxidation of ascorbic acid was estimated by following the decrease in absorbance at 290 nm. APX activity was calculated using the molar extinction coefficient (ε =2.8 mM/cm) and was expressed as units per milligram of protein.

3.4.5. Extraction and Estimation of Proline Content

Proline was extracted and estimated by following the method of Bates et al. (1973). Fresh plant tissue weighing 0.5 g was homogenized in a mortar and pestle with 10 ml of 3% aqueous sulfosalicyclic acid. The homogenate obtained was then filtered through Whatmann No.1 filter paper. The residue was re-extracted and pooled, and thevolume was made upto 20 ml with aqueous sulfosalicylic acid before estimation of proline. To 2 ml of proline extract, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added. The mixture was incubated for an hour at 100°C in a boiling water bath and then transferred to an ice bath to terminate the reaction. 4 ml of toluene was added to the mixture and mixed vigorously using a test tube stirrer for 20 seconds, the toluene phase containing the chromophore was separated from the aqueous phase with the help of a separating funnel and the absorbance was measured at 520 nm in a spectrophotometer using a reagent blank. The proline content was determined from a standard curve obtained using pure proline, as shown in Fig. 3.3, and the results were expressed in milligram per gram fresh weight (Fig. 3.3).

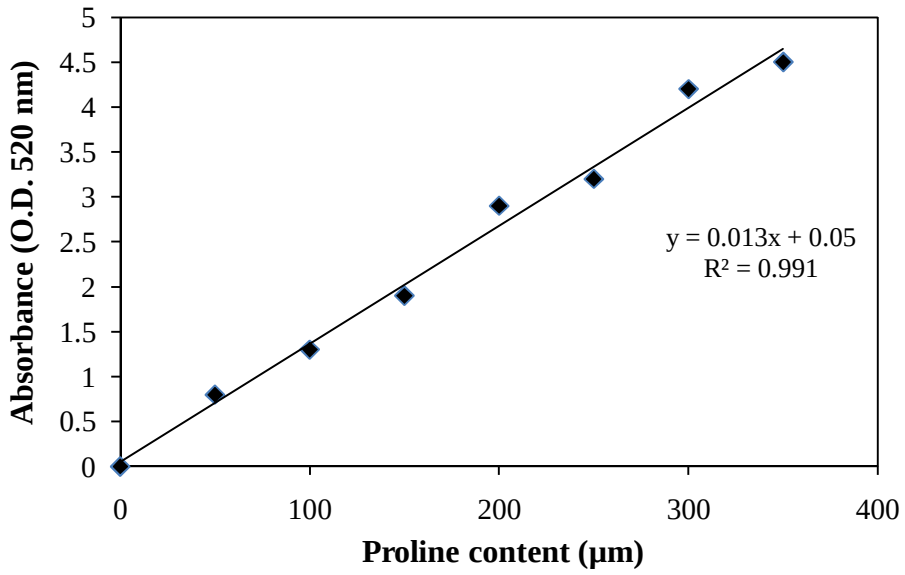

Figure 3.3: Standard curve of proline concentration vs. absorbance at 520 nm used in the estimation of proline content.

Proline content on fresh-weight-basis is calculated using the equation 3.5:

$$\mu\text{moles per g tissue} = \frac{(\mu\text{g proline})/\text{mL} \times (\text{mL toluene})}{115.5} \times \frac{5}{\text{g sample}} \qquad (3.5)$$

where, 115.5 is the molecular weight of proline

3.4.6. Estimation of Chloroplyll Content

Chloroplyll content was measured according to a method described by Arnon et al. (1949). About 0.1 g fresh leaf sample was homogenised in 10 mL of cold 80% acetone using chilled mortar and pestle and the leaf homogenate was filtered through (Whatman No. 1) a the filter paper. The extract was then analysed using a UV-visible spectrophotometer (Cary 100, Varian, Australia) and to measure its absorbance at 663 nm and 645 nm for estimating the Chl concentration. Chlorophyll concentration was calculated using Arnon's equation (Eq. 3.6- 3.8) to convert the absorbance measurements to mg Chl/g leaf tissue.

Chl a (mg/g) = $[(12.7 \times A_{663}) - (2.6 \times A_{645})] \times$ ml acetone / mg leaf tissue (3.6)

Chl b (mg/g) = $[(22.9 \times A_{645}) - (4.68 \times A_{663})] \times$ ml acetone / mg leaf tissue (3.7)

Total Chl = Chl a + Chl b. (3.8)

3.4.7. Estimation of Total Carbohydrate Content

Total carbohydrate content was measured according to anthrone method (Raunkjer et al. 1994) using glucose as the standard and as detailed further. About 100 mg plant tissue sample was hydrolyzed for 3 hrs in water bath with 5 ml of 2.5N HCl. The reaction mixture was cooled to room temperature and neutralised with sodium carbonate powder till effervescence ceased. Final volume was made upto 100 ml with Milli Q water and centrifuged at 10000 ×g for 10 minutes. Supernatant was collected and 0.5 ml aliquot was used for analyses.

For Anthrone method, 2.5 ml of anthrone reagent was mixed with 0.5 ml of a suitably diluted sample taken in a series of test tubes and heated in a boiling water bath for 15 min. A suitable blank prepared by mixing 2.5 ml of Anthrone reagent with 0.5 ml of Milli Q water and heated in a boiling water bath for 15 min.. The intensity of the colour developed was measured at 630 nm using a UV–visible spectrophotometer (Cary 100, Varian, Australia). A calibration curve prepared using known concentrations of glucose (0.02 to 0.2 g/l), as shown in Fig. 3.4, was used for estimating the total carbohydrate estimation in the samples.

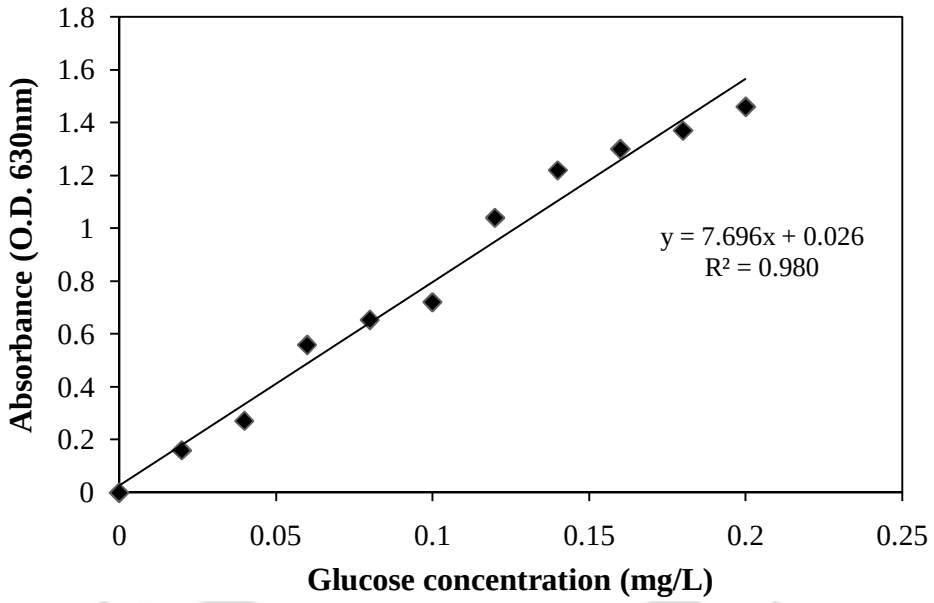

Figure 3.4: Standard curve of glucose concentration vs. absorbance at 630 nm used in the estimation of total carbohydrate content.

3.4.8. Estimation of Total Protein Content

The soluble protein content in the samples was measured according to Lowry's method (Lowry et al. 1951) using bovine serum albumin (BSA) as the standard protein**.** 2 ml of alkaline copper sulphate reagent was mixed with 0.5ml of a suitably diluted sample taken in a series of test tubes. The test tubes were mixed well and incubated at room

temperature for 10 minutes. Then 0.2 ml of Folin Ciocalteau solution (1N) was added to each test tube and incubated for 30 min. The intensity of the colour developed was measured at 660 nm using a UV–visible spectrophotometer (Cary 100, Varian, Australia). A calibration curve prepared using known concentrations of BSA (0.05 to 1 mg/ ml), as shown in Fig. 3.5, was used for estimating the total protein concentration in the samples.

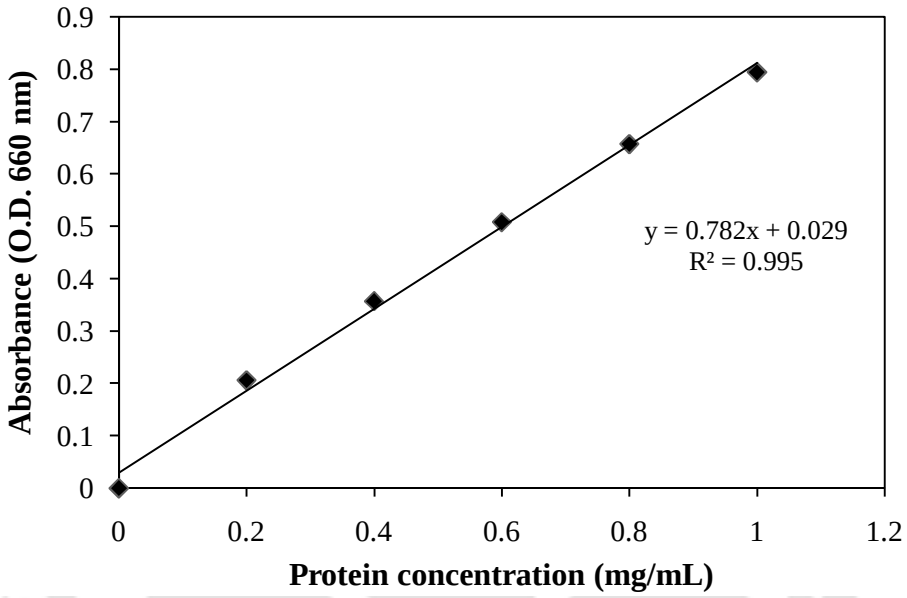

Figure 3.5: Standard curve of protein concentration vs. absorbance at 660 nm used in the estimation of total protein content.

3.5. Effect of Co-ions on Chromium (VI) Uptake by *T. pallida*

The effect of co-ions on Cr(VI) removal by *T. pallida* was studied under batch hydroponics conditions. Prior to the Cr(VI) removal experiments, the plants were acclimatized in 50% (v/v) Hoagland solution (HS) for two weeks. Water lost due to natural evaporation was replenished through periodic addition of distilled water. To study the effect of co-ions on Cr(VI) uptake by *T. pallida*, a Plackett-Burman design comprising 12 experimental runs with four variables was chosen.

3.5.1. Plackett–Burman (PB) Design of Experiments

The Plackett–Burman design, an efficient technique for medium-component optimization (Yong et al., 2011), was used to select concentrations that significantly influence the response (% Cr removal) and insignificant ones were eliminated to obtain a smaller, more manageable set of co-ions factors. The Plackett–Burman design is based on a first-order polynomial equation (Tasharrofi et al., 2011) of the form (Eq. 3.9):

$$Y = \beta_0 + \Sigma \beta_i X_i \qquad (3.9)$$

where; Y is the response (% Cr removal), β_0 is the model coefficient, β_i is the linear coefficient, and X_i = is the level of the independent variable. This model identifies the main ions and their concentrations required for maximal % Cr removal. Table 3.3 presents the different combination levels of the factors viz. Cr(VI), SO_4^{2-}, NO_3^- and PO_4^{3-} in this multicomponent study. The low (-1) and high (+1) levels of each factor were chosen based on the concentration range in which these ions are commonly found in industrial effluent.

Table 3.3: Plackett-Burman experimental design matrix showing combination of levels of Cr(VI) and co-ions.

Exp. no.	Cr(VI) (mg/L)	SO_4^{2-} (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)
1	5	50	20.0	10.0
2	20	150	20.0	0.5
3	5	150	0.5	10.0
4	20	50	20.0	10.0
5	20	150	20.0	0.5
6	5	150	20.0	10.0
7	20	150	0.5	10.0
8	20	50	0.5	10.0
9	20	50	0.5	0.5
10	5	50	20.0	0.5
11	5	150	0.5	0.5
12	5	50	0.5	0.5

Stock solution of 1000 mg/L each of Cr(VI) and co-ions was prepared by dissolving (g/L) 1.631g KNO_3, 1.432g KH_2PO_4, 1.478g Na_2SO_4 and 2.828g $K_2Cr_2O_7$, respectively, in 50% (v/v) Hoagland solution. These stock solutions were suitably diluted to achieve a desired concentration of Cr(VI) and the co-ions in each experimental run (Table 3.3). Temperature, light/dark period and relative humidity were maintained the same as mentioned in section 3.2. Percentage removal of Cr(VI) and co-ions were estimated from samples taken at 1 day time interval. Response in each of the experimental runs was expressed as % removal of Cr(VI) and the co-ions and calculated using following Eq. 3.10.

$$\% \text{ Removal} = \frac{(Co - Ce)}{Co} \times 100 \qquad (3.10)$$

where; C_o= initial concentrations of Cr(VI) or the co-ions present in the solution (mg/L) and C_e = final concentrations of Cr(VI) or the co-ions present in the solution (mg/L).

The statistical software package Minitab^{TM} (version 16, PA, USA) was used for analyzing the experimental data.

3.5.2. Chromium (VI) Removal Kinetics

The kinetics of Cr(VI) uptake by *T. pallida* in presence of the co-ions was studied by fitting the metal removal results obtained at different intervals of time to the Lagergren's pseudo first-order, Ho's pseudo second-order and Langmuir type irreversible kinetic models (Table 3.4).

Table 3.4: Models applied for the estimation of biokinetic constants involved in Cr(VI) removal.

	Kinetic model equation*		Estimable kinetic parameters*
Lagergren's pseudo first-order	$log(q_e - q_t) = log\, q_e - \frac{k_1\, t}{2.303}$	(3.11)	k_1, q_e
Ho's pseudo second-order	$\frac{t}{q_t} = \frac{1}{k_2 {q_e}^2} + \frac{t}{q_e}$	(3.12)	k_2, q_e
	$C(t) = \frac{BC_0}{(AC_0 + B)\exp\{(B/V)(t - t_0)\} - AC_0}$	(3.13)	
Langmuir type irreversible	$A = \frac{k_3 V}{n}$		S_{M0}, $Y_{P/M}$, $Y_{X/M}$
	$B = k_3 q_{max} - k_3 q_0 - \frac{k_3 V C_0}{n}$	(3.14)	

*q_e = amount of metal ion (mg/g) at time t, q_t= amount of metal ion (mg/g) at time equilibrium, k_1 = rate constant of pseudo-first-order adsorption (min^{-1}), $k_{2=}$ rate constant of pseudo-second-order adsorption (g/ mg/ min), C (mg/L) is the residual Cr(VI) concentration in solution, q (mg/g) is the amount of Cr(VI) sorbed onto *T. pallida*, qmax (mg/g) is the maximum Cr(VI) sorption capacity, V (ml) is the liquid volume, n is the number of plants used, k_3 (g/mg/min) is the Cr(VI)sorption rate constant, and t (min) is the time. C_0 (mg/L), q_0 (mg/g) and t_0 (min) are the initial Cr(VI)concentration in solution, initial amount of Cr(VI) sorbed onto *T. pallida* and initial time (t=0) respectively.

For solving the equations (3.11-3.14) the mathematical software Matlab™ 6.1 (The Mathworks, Inc.) was used.

3.5.3. Estimation of Sulphate

Total sulphate content was measured according to the standard barium sulphate turbidity method (EPA 1983). Water samples were first filtered though filter paper (Whatman No. 1) and 50 ml of the filtrate obtained was mixed with 20ml of buffer solution in a 100 ml volumetric flask. The solutions were mixed well, and while stirring, 0.15 g of barium chloride was added to the sample and again stirred with the help of magnetic stirrer for about an hour. The intensity of the colour developed was measured at 420 nm using a

UV–visible spectrophotometer (Cary 100, Varian, Australia). A calibration curve prepared using known concentrations of sulphate (0.0 to 40 mg/L), as shown in Fig. 3.6, was used for estimating the total sulphate concentration in the samples.

3.5.4. Estimation of Nitrate

Total nitrate content was measured according to the standard salicylic acid method (Cataldo et al. 1975). 0.50 ml of sample was mixed thoroughly with 0.8 mL of 5% (w/v) salicylic acid in conc. H_2SO_4 in a 50 ml volumetric flask and incubated for 20 min at room temperature. 19 mL of 2 N NaOH was added to the sample to raise the pH above 12 followed by cooling to room temperature. The intensity of the colour developed was measured at 410 nm using a UV–visible spectrophotometer (Cary 100, Varian, Australia). A calibration curve prepared using known concentrations of nitate (0.0 to 60µg /20ml), as shown in Fig. 3.7, was used for estimating the total nitrate concentration in the samples.

3.5.6. Estimation of Phosphate

Total phosphate content was measured according to the standard ascorbic acid method (APA 1992). About 5 ml of sample was mixed thoroughly with 8 mL of combined reagent (Composition: 50 mL 5N H_2SO_4, 5 mL antimony molybdate solution, 4% 15 mL ammonium molybdate solution and 30mL 0.1M ascorbic acid) in a 100 ml volumetric flask and the volume was made upto 60 ml with distilled water.The reaction mixture was incubated for 10 min at room temperature. The intensity of the colour developed was measured at 880 nm using a UV–visible spectrophotometer (Cary 100, Varian, Australia). A calibration curve prepared using known concentrations of phosphate (0.0 to 10mg/L), as shown in Fig. 3.8, was used for determining the total phosptate concentration in the samples.

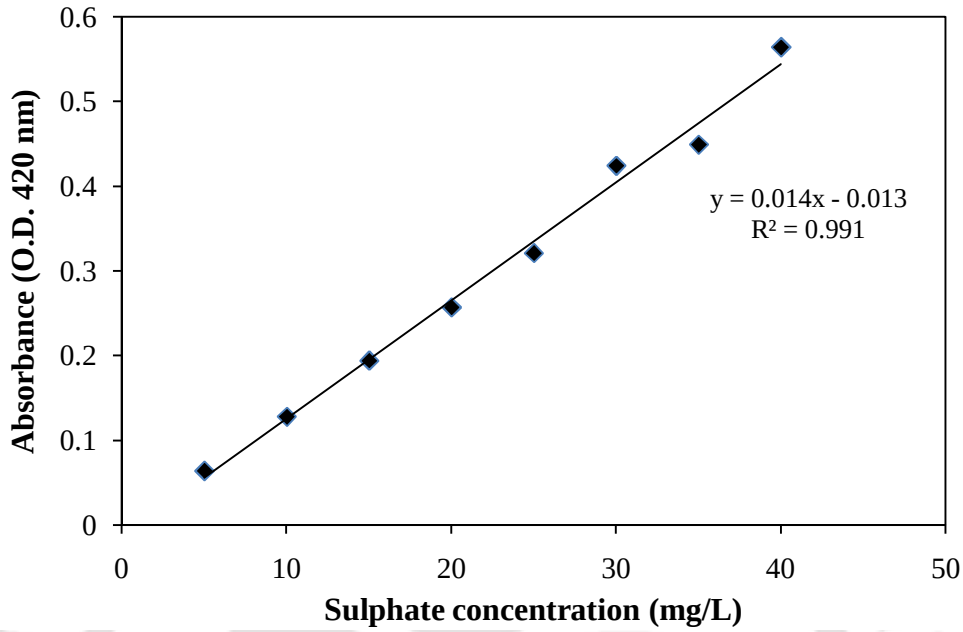

Figure 3.6: Standard curve of sulphate concentration vs. absorbance at 420 nm used in the estimation of sulphate content.

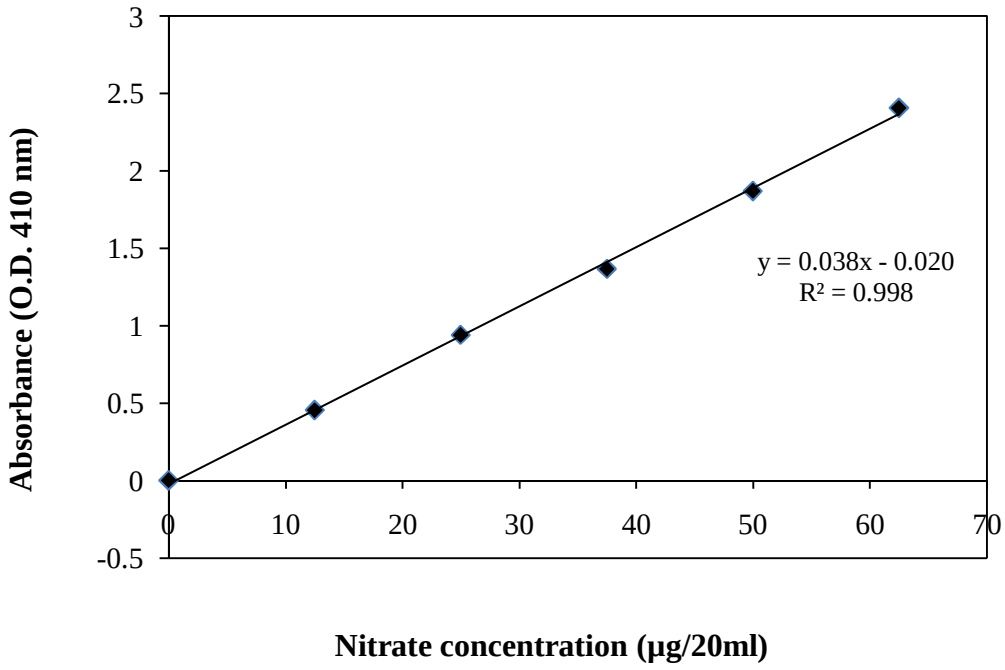

Figure 3.7: Standard curve of nitrate concentration vs. absorbance at 410 nm used in the estimation of nitrate content.

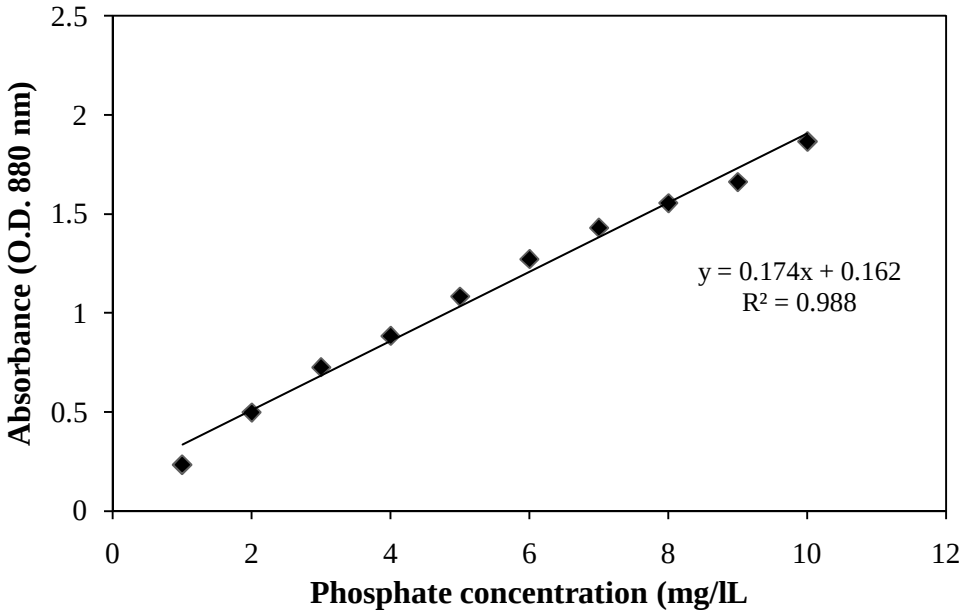

Figure 3.8: Standard curve of phosphate concentration vs. absorbance at 880 nm used in the estimation of phosphate content.

3.6. Continuous Chromium (VI) Removal from Wastewater by *T. pallida* Using Vertical Subsurface Flow Constructed Wetland System

Continuous experiments were carried out using the wetland system operated under vertical subsurface flow mode.The effect of different parameters (pH, HRT and inlet Cr(VI) concentration) on the continuous Cr(VI) removal was carried out using the VSSF CW system. The effect of different HRT, i.e. 1, 2 and 3 day HRT, was first studied at an influent Cr(VI) concentration of 20 mg/L. Later experiments were carried out at different influent pH (7 and 4) and at different influent Cr(VI) concentration (20 and 30 mg/L), but with the same HRT of two days. Further, Cr speciation and distribution in different fractions of CW was measured. At the end of the continuous Cr(VI) removal experiments *T. pallida* growth was analysed.

3.6.1. Constructed Wetland Design

Continuous experiments for Cr(VI) removal was carried out in a vertical subsurface flow constructed wetland system. Laboratory scale wetland system of dimensions 0.3 m length, 0.15 m width and 0.5 m depth was operated in vertical subsurface mode. The unit was constructed out of Perspex material. A schematic of the set up used is shown in Fig. 3.9. Beds were filled with soil (up to 15 cm), sand (up to 10 cm) and gravel (up to 10 cm) of two different particle sizes (5–7 mm and 3–4 mm). Sand and gravels were washed thoroughly with water prior to use. At the base of the unit, large size gravel was placed up to a height of 5 cm to prevent any clogging. The top of the wetland unit was left open. Three sampling ports (4mm internal diameter) at the height of 0 (P1), 20 (P2) and 30 (P3) cm from the bottom were provided for samples collection from different depths of the units. Void volume of the unit was measured by draining the unit and determining the water volume of each tank. Table 3.5 presents the characteristics of the wetland unit**.**

Table 3.5: Wetland design and characteristics.

	Dimension/ effective size(cm)	**Volume/Capacity**	**Area of vegetation (no.of stems)**	**Working volume**	**Porosity/root depth**
Main unit	30×15×50cm (l×w×d)	5.5 L	450 cm^2 24 plantlets	3.5 L 3.7 L	
		Treatment zone/ media type			
Plant	*Tradescantia pallida*	-	24		8-10 cms
Soil	Garden soil (Alluvial)	2.5 kg 3 kg	- -	-	
Coarse sand	125-150 µm				
Fine gravel	Size: passed through seive no. 6 retained by 4 (3-4 mm)	2 kg	- -	-	
Large	Size: passed through seive no. 10 retained	3 kg			

gravel	by 8 (5-7mm)

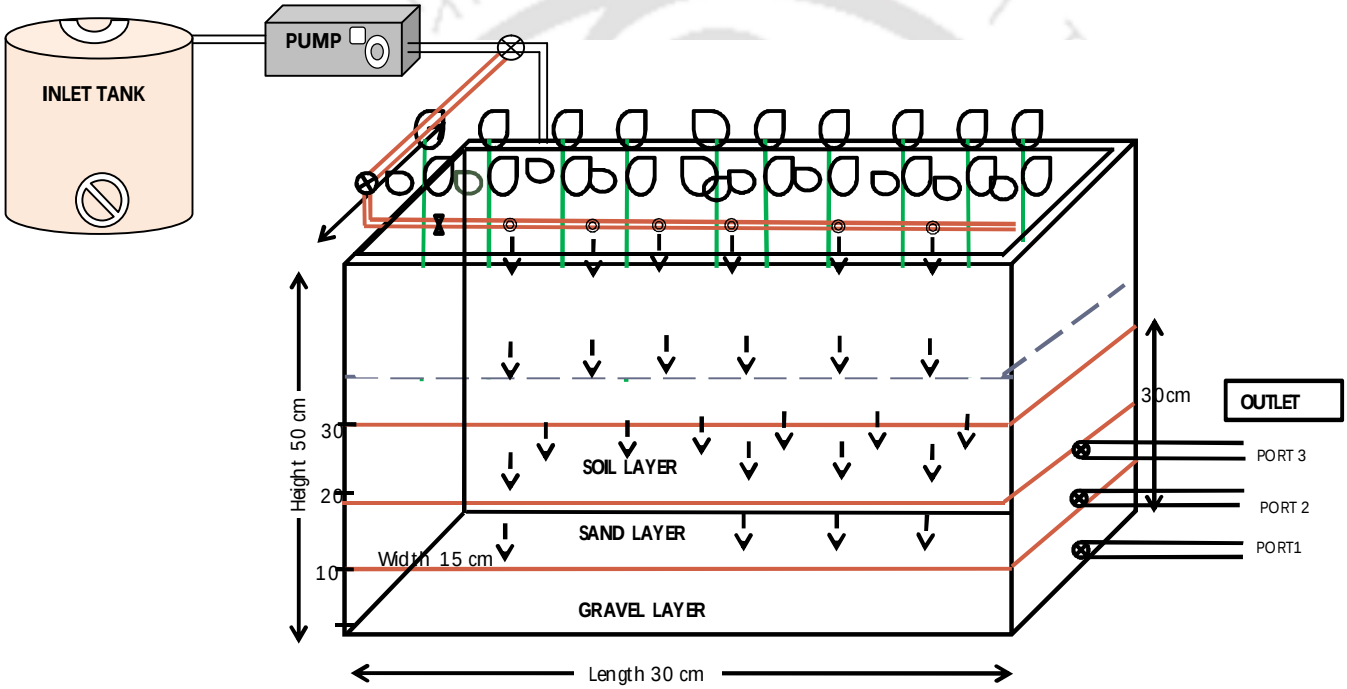

Figure 3.9: Schematic of the vertical flow constructed wetland experimental setup used in this study.

(a)

(b)

Figure 3.10: Photograph of the experimental setup showing VSSF CWs operated under continuous mode: (a) Planted unit (b) Control unit

3.6.2. Continuous Chromium (VI) Removal Experiments

Continuous experiments were carried out using the wetland system operated under different hydraulic retention time (HRT), pH and influent Cr(VI) concentration conditions. Freshly collected *T. pallida* plantlets of height 5 cm ±2 each were used for this continous experiment. The unit was initially supplied with only tap water to acclimatize the plants to the CW environment. Thereafter, Cr(VI) solution (pH = 5.6) at 20 mg/L concentration was continuously supplied into the unit using a peristaltic pump providing three different HRT (3, 2 and 1 days). The water level was maintained 5 cm below the soil surface in order to maintain a subsurface flow. Only the photoperiod was maintained at 16h , and the temperature was not controlled in the unit, but was maintained in the range 23–32 °C. Samples were collected periodically from the influent and the effluent points for Cr(VI) analyses. A similar unit of the same dimensions, containing only soil, sand and gravel, but without the *T. pallida* plant species served as the control.

To study the effect of influent pH on the Cr(VI) removal in the CW system, two identical CW units with the *T. pallida* plants, soil, sand, gravel, etc. were used. Whereas one of the units received influent at pH 4, the other unit received influent at pH 7. However, both the units were operated at 2-days HRT and influent Cr(VI) concentration of 20 and 30 mg/L. The initial pH of the Cr(VI) containing influent was adjusted by HCI (2 N) or NaOH (2 N) or both.

All these continuous experiments at the respective afore-mentioned conditions (HRTs and pH) were carried out for a period until at least three consecutive steady state Cr(VI) values in the effluent were achieved. Results reported are average of three steady state values and duplicate sample analyses.

3.6.3. *T. Pallida* Growth and Chromium Bioremoval

At the end of the continuous Cr(VI) removal experiments using the *T. pallida* based CW system, the plant growth was analysed in terms of its height, fresh weight, dry weight and chlorophyll contents. For measuring the plant height, measurements were taken from the root-stem intersection to the growing tip of the stem. Fresh weight and dry weight was measured according to the Standard Methods (Gowd and Govil, 2008), for which plants were air-dried and fresh weight was determined. To measure the dry weight, plants were dried at 80°C for 32 hours till constant weight was observed.

Water content of the plant was determined by measuring its relative water content (RWC), obtained using Eq. 3.15.

$$RWC = \frac{FW - DW}{FW} \qquad (3.15)$$

where; FW = fresh weight (g) and DW = dry weight (g) of plant at the end of the experimental period.

Cr(VI) phytoextraction potential of *T. pallida* was measured by calculating its bioconcentration factor (BCF) and translocation factor (TF) at the end of the experimental period as discussed earlier in section 3.3.1. Total chlorophyll content was measured according to the procedure discussed earlier in section 3.3.4.6.

3.6.4. Extraction and Estimation of Chromium (VI)

For the extraction of Cr(VI) from soil, sand and gravel, alkaline digestion method was followed. The samples (2.5 ± 0.10 g) were air dried and digested with 50 ml of 0.28M Na_2CO_3 /0.5M NaOH solution and heated at 95°C for 60 min following the USEPA 3060 method. For the effluent wastewater analyses, samples (10 ml) were neutralized with 25

ml extraction solution (2% NaOH/3% Na_2CO_3) and treated with 2 ml 0.1% $KMnO_4$ and 0.75 ml 6 N H_2SO_4 to remove any reducing agents. The samples were then acidified with 7.8 mL of 6 N H_2SO_4 following the addition of 2 ml of 0.25% 1, 5-diphenyl carbazide and final volume was adjusted to 100 mL. The solution was mixed well and kept for full color development for 10 min. Cr(VI) content in the samples was analysed by measuring the absorbance of a colour complex formed at 540 nm using a UV visible spectrophotometer (Cary 100, Varian, Australia). A calibration curve prepared using known concentrations of Cr(VI) (0.05 to 7 mg/ L), as shown in Fig. 3.11 was used for determining the total Cr(VI) concentration in the samples.

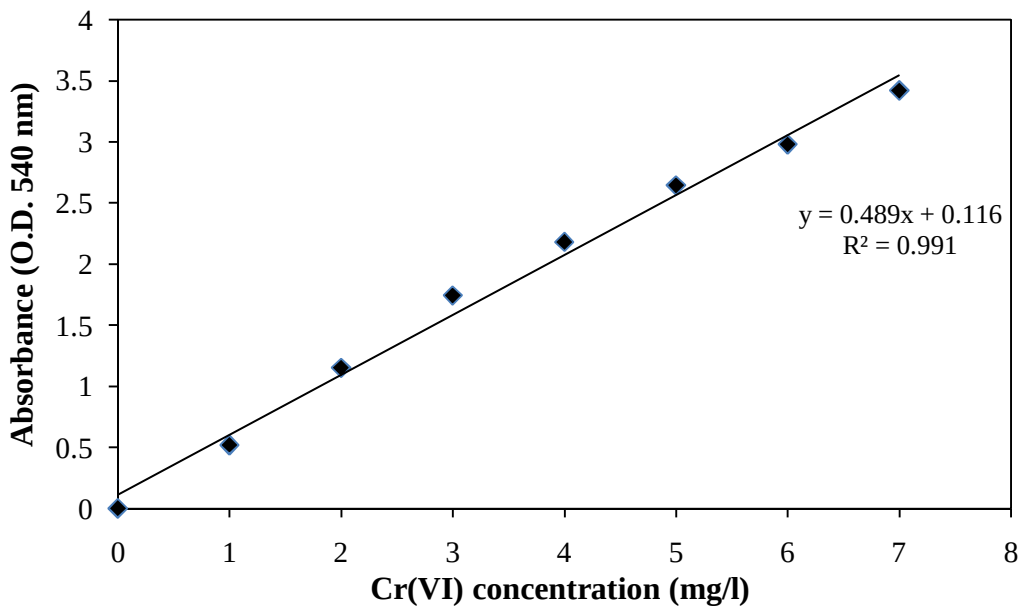

Figure 3.11: Standard curve of Cr(VI) concentration vs. absorbance at 540 nm used in the estimation of Cr(VI) content.

3.6.5. Extraction and Estimation of Total Chromium and Cr(III)

For extraction of total Cr, acid digestion method was used. For the estimation of total Cr content, *T. pallida* plant parts were harvested and thoroughly rinsed with distilled water to remove any adsorbed Cr on the root surface. The washed plants were separated into leaves, shoots and roots. They were dried at 105°C for 30 min and then at 70°C for 48 h to constant weight. 0.5 g (dw) of leaves, shoots and roots were digested in an acid mixture as mentioned further. Samples of soil, sand and gravel were air dried and digested with $HCl:HNO_3:H_2SO_4$ in the ratio 3:1:1 (v/v) at 95°C on a hot plate. The plant parts were thoroughly rinsed with distilled water, oven-dried at 70°C for 48 hr and digested and analysed for total Cr as discussed earlier in section 3.3.2.1. Cr(III) concentration was calculated by subtracting Cr(VI) concentration from the total Cr concentration.

3.7. Feasibility Study on the Disposal and Reuse of Chromium Bioaccumulated Plant Biomass

In order to check the feasibility of disposal of Cr accumulated *T. pallida* plant biomass, the plant was tested for Cr biosorption which is a simple and passive process as compared wih the active bioaccumulation process.

3.7.1. Preparation of *T. Pallida* Plant Biomass

T. pallida plants were grown under hydroponic conditions for 60 days with or without supplementation with 10 mg/L Cr(VI). These Cr(VI) exposed and unexposed plants were collected and subsequently separated into different parts (roots, shoots and leaves). These plant parts were washed extensively with water and then with de-ionized water to remove any adhering dirt and particulate material from its surface, followed by drying in an oven for 2 days at 70°C. The dried plant parts were finally ground to a desired size (100 mesh particle size) and subsequently used for the Cr(VI) biosorption experiments.

3.7.2. *T. pallida* Biomass Characterization for Chromium (VI) Biosorption

Characterization of the *T. pallida* (exposed/unexposed) leaf biomass for Cr(VI) biosorption was carried out by scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy analyses. The sample is irradiated by a broad spectrum of infra-red light and the level of absorbance at a particular frequency is plotted after Fourier transforming the data. The resulting spectrum is characteristic of the functional groups of organic molecules present in the sample and is absolutely specific. The surface morphology of *T. pallida* Cr(VI) loaded leaf biomass was elucidated using SEM (500× and 10 K X) (LEO, 1430 vp, Germany). Prior to the observation under SEM, the samples were coated with a thin, electric conductive gold film. In order to determine the functional groups in the plant biomass for Cr(VI) binding, biomass samples taken before (control biomass) and after Cr(VI) biosorption (loaded biomass) with 50 mg/Linitial Cr(VI) solution (pH = 2) were analysed using FTIR (IR Affinity, Shimadzu, Australia) within the range 400–4000 cm^{-1}. KBr was taken as the background material for this FTIR analyses.

3.7.3. Chromium (VI) Biosorption Experiments

All Cr biosorption experiments using *T. pallida* biomass were performed under batch with a known plant biomass dose in a 250 mL Erlenmeyer flask containing 50 mL solution of known Cr(VI) concentration. All these experiments were conducted by agitating the biosorption flasks at 250 rpm in an orbital incubator shaker till the equilibrium was established. The temperature was maintained within ±1°C of the required values during the experiments.

3.7.4. Influence of Chromium (VI) Biosorption Process Parameters

The effect of different process parameters, viz. solution pH, contact time, biosorbent dose, temperature and initial concentration on Cr(VI) biosorption by *T. pallida* , was first investigated by varying these parameters one-at-a-time and by maintaining the other parameters at a constant level. The following range of these parameters was tested to examine their effect: pH 2–3.5, temperature 30–50°C, biosorbent dose 0.5–2 g, initial Cr(VI) conc. 50–200 mg/L. At the end of each biosorption experiment, samples were taken from the respective test solutions to determine the residual Cr(VI) concentration. Prior to the Cr(VI) analyses, the biosorbent was separated by filtration with Whatman filter paper number 1 followed by centrifugation at 4,000 ×g for 10 min. Experiments were carried out in triplicate, and the mean value was used for calculations. Cr(VI) removal was expressed as either % Cr(VI) removal or mg Cr(VI) removed per g of the biosorbent (mg/g) as represented by equations (3.16) and (3.17), respectively.

$$\%\ \mathrm{Cr(VI)\ removal} = \frac{C_i - C_f}{C_i} \times 100 \qquad (3.16)$$

$$\mathrm{Cr(VI)\ Uptake}\ (q) = \frac{C_i - C_f}{m} \times V \qquad (3.17)$$

Where; C_i = initial Cr(VI) concentration, C_f = final Cr(VI) concentration, V = volume of test solution (L) and m = biosorbent mass (g).

3.7.5. Chromium (VI) Sorption Kinetics and Isotherm

For determining the Cr(VI) sorption kinetics, aqueous solution containing different Cr(VI) ion concentration (50–200 mg/L) was taken with 0.5 g of *T. pallida* leaf biomass (exposed) as the biosorbent. Other initial conditions were: pH 2, temperature 35°C and

agitation speed 250 rpm. Samples were taken from the solution at definite time intervals until equilibrium time was achieved. In order to determine the Cr(VI) sorption isotherm parameters in this study, aqueous solution (pH=2) containing different initial Cr(VI) concentration (50-200 mg/L) was added with the plant leaf biomass and incubated at a temperature in the range 30-50°C. Experimental data obtained at different time intervals were fitted to the pseudo first and pseudo second-order kinetic models as detailed earlier in Table 3.4. Table 3.6 presents the isotherm models applied in this study along with the estimable isotherm parameters from these models.

Table 3.6: Adsorption models applied for the estimation of isotherm parameters involved in Cr(VI) removal by biosorption using *T. pallida.*

	Isotherm model equation*		**Estimable isotherm parameters***
Langmuir Isotherm	$\frac{1}{Qe} = \frac{1}{Qo} + \frac{1}{bQoCe}$	(3.18)	b, Q_o
Freundlich Isotherm	$\log Qe = \log K_f + \frac{1}{n}\log Ce$	(3.19)	n, K_f

*Ce= equilibrium concentration of Cr(VI) in solution (mg/L), Qe=equilibrium loading of Cr(VI) on biosorbent (mg/g), Q_0=Langmuir constant related to maximum Cr(VI) adsorption capacity (mg/g), b = Langmuir constant related to the relative energy of Cr(VI) adsorption (L/mg), K_f = Freundlich constant related to Cr(VI) sorption capacity, n = Freundlich constant related to Cr(VI) sorption intensity.

3.7.6. Chromium (VI) Sorption Thermodynamics

The changes in free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) related with Cr(VI) sorption in this study were determined using the relationships shown in Table 3.7

Table 3.7: Equations applied for the estimation of thermodynamics parameters for the Cr adsorption process by *T. pallida.*

Equation*		**Estimable thermodynamics parameters***
$\Delta G° = -RT \ln K$	(3.20)	ΔG^0
$K = \frac{C_{ae}}{C_e}$	(3.21)	K
$\Delta G° = \Delta H° - T\Delta S°$	(3.22)	
$\log K = \frac{\Delta S°}{2.303R} - \frac{\Delta H°}{2.303RT}$	(3.23)	ΔH°, ΔS°

*ΔG^0 = change in free energy (kJ/mol), R = universal gas constant (8.314 J/mol K) and T = absolute temperature (K), K = equilibrium constant, C_{ae} = equilibrium concentration (mg/L) of Cr(VI) on the biosorbent, and C_e = equilibrium concentration (mg/L) of Cr(VI) in the solution, ΔH^0 = change in enthalpy (kJ/mol), ΔS^0 is the change in entropy (kJ/mol).

CHAPTER 4 RESULTS AND DISCUSSION

Concerns regarding the discharge of toxic Cr(VI) containing industrial effluents into the environment have led to continuous research for sustainable remediation methods. Cr(VI) is one of the transition metal that possess high mutagenic and teratogenic properties. Cr(VI) oxyanions are persistent in the environment and their contamination is becoming prevalent due to increased industrialization. Recently, many researchers have tried to investigate the phytoremediation potential of different plant species to remove Cr(VI) from contaminated wastewaters. Wetland plants are particularly suited for environmental detoxification of heavy metals as they can be grown directly on wastewater streams near industrial areas and can provide in situ bioremediation. However, there is a need to explore the potential of indigenous plants to remove Cr(VI) and other heavy metals, particularly in developing countries where environmental conditions favour the application of CWs. Hence, the main goal of this study is to demonstrate the potential of indigenous plant species for Cr(VI) removal from contaminated system, under batch and continuous conditions. This chapter first discusses the tolerance mechanism, bioaccumulation efficiency, uptake and storage mechanism of Cr(VI) by *T. pallida*. The efficiency of the plant to phytoremediate Cr(VI) using a vertical subsurface flow constructed wetland system is detailed further. Finally, Cr(VI) removal by biosorption using the waste plant biomass as a feasible and safe method for their disposal is discribed in detail.

4.1. Chromium (VI) Tolerance, Accumulation and Localization by *T. pallida*

In the present work, tolerance index (TI) of different plant species was determined for screening and selection of Cr(VI) accumulating plant species. Phytoextraction capability of the *T. pallida* was evaluated as a function of bioconcentration factor (BCF),

translocation factor (TF) and bioaccumulation values of Cr(VI) within the plant tissues. Localization within the subcellular fractions was carried out to understand Cr(VI) tolerance and storage mechanisms.

4.1.1. Screening of Different Plants for Chromium Tolerance

Screening of indigenous Cr(VI) tolerant plant is one of the main step for application in CWs system to remediate Cr(VI) contaminated sites. However, important aspects which must be considered when choosing the best phytoremediator species for a specific location and level of contamination are the biomass production ability and the ecology of the species. In this respect, phytoremediation using native and wild plant species becomes imperative as no special growth conditions are required in the process. Plants selected for phytoremediation should be tolerant to pollutant, fast growing and non-edible with fibrous root system and easily harvestable. Therefore, different indigenous plants were initially screened based on these criteria and further evaluated for Cr(VI) tolerance and accumulation efficiency.

In the preliminary screening experiments, six indigenous plant species, *Tradescantia pallida*, *Alternanthera philoxeroides*, *Gnaphalium luteoalbum*, *Chlorophytum comosum*, *Ageratum conyzoides* and *Blumea fistulosa* were choosen to find a Cr(VI) tolerant plant species. The plants were hydroponically cultured and grown in 50% Hoagland solution supplemented with Cr(VI) at an initial concentration of 10 mg/L over 30 days experimental period.

Figure 4.1 shows the Cr tolerance index for the plant species investigated, in which the horizontal line indicates a tolerance index of 50% that is generally considered the minimum desired biomass production for plants growing under heavy metal-stressed condition.

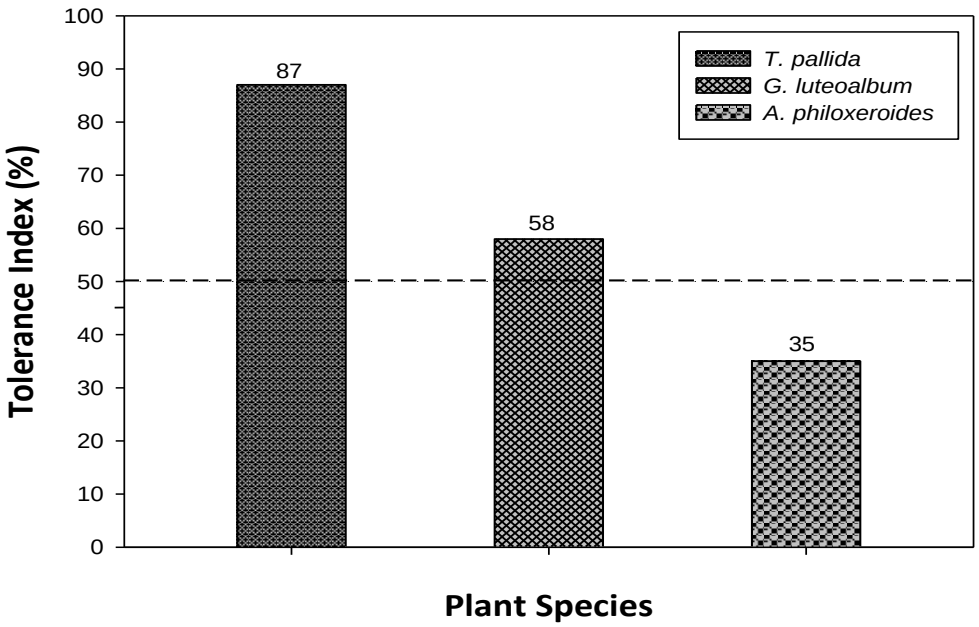

Figure 4.1: Cr(VI) tolerance index of different plant species (Cr(VI) initial concentration = 10 mg/L and Cr(VI) exposure period = 30 days).

Among different plant species investigated, *T. pallida*, *A. philoxeroides* and *G.luteoalbum* were found to tolerate and uptake Cr(VI). Severe growth inhibition and toxic symptoms in the form of leaf necrosis as revealed by yellowish, dark brown spots and acute burning on the edges of the leaves were observed in *C. comosum*, *A. conyzoides* and *B. fistulosa* plant species under Cr(VI) stress based on which they were not selected. *T. pallida* showed the maximum TI (87%) to Cr(VI) without any toxicity symptoms among all the indigenous plants tested. *Alternanthera philoxeroides* and *Gnaphalium luteoalbum* showed some toxic symptoms at the end of the experimental period compared with the respective control plants. The Cr toxic effect was more pronounced in *A. philoxeroides* than *G. luteoalbum*. The TI was the lowest for *A. philoxeroides* (35%). Although *G. luteoalbum* (58%) showed tolerance value higher than 50%, the aforementioned toxicity symptoms were still observed. Further, all the Cr(VI) treated plants (root and aerial parts) showed reduced dry weight (dw) as

compared with the respective control plants indicating its harmful effect on development and growth of the different plant species. This may be due to the inhibition of cellular division and damage to the root systems of the plants by Cr(VI). It has been reported that Cr causes acute decrease in photosynthesis rate and hinders its translocation in plants (Shanker et al., 2005). Therefore, only *T. pallida* that showed the highest TI with no toxicity symptoms was finally selected for further experiments. The plant *T. pallida* belongs to Kingdom Plantae - Plants, Subkingdom Tracheobionta (Vascular plants), Superdivision Spermatophyta - Seed plants, Division Magnoliophyta - Flowering plants, Class Liliopsida - Monocotyledons,Subclass Commelinidae, Order Commelinales, Family Commelinaceae - Spiderwort family, Genus *Tradescantia L.* - spiderwort, Species *Tradescantia pallida* (Rose) D.R. Hunt - purple queen (USDA classification).

Table 4. 1: Cr(VI) bioconcentration factor (BCF) and translocation factor (TF) values of *T. pallida* (exposure period = 30 days).

	Bio-concentration factor		
	5 mg/L	10 mg/L	20 mg/L
Leaves	30.4	36.8	8
Shoots	16	31	9.9
Roots	36	40.8	11
	Translocation factor		
	0.444	0.759	0.900

Table 4.1 presents the BCF and TF values of *T. pallida* at different initial Cr(VI) concentration. It represents the potential of *T. pallida* to extract Cr(VI) from the media. The BCF value was highest at an initial Cr(VI) concentration of 10 mg/L in the roots (40.8) followed by leaves (36.8) of *T. pallida*. The TF value estimates the ability of a plant to transfer metals from root to shoot. It is defined as the ratio of metal

concentrations in the shoots to the roots (Zacchini et al., 2009). In this study the significant TF value (0.7-0.9) shows the potential of *T. pallida* to transfer Cr from root to shoot. The values of TF increased with an increase in the initial Cr(VI) concentration in the medium.

4.1.2. Chromium accumulation by *T. pallida*

The selection of plants on the basis of maximum uptake potential is the key factor for phytoremediation application. The concentration of Cr in different plant parts of *T. pallida* indicates the tolerance capacity of the plant towards Cr(VI) and, therefore, its potential for phytoremediation. Fig. 4.2 shows the results of Cr ions accumulation by *T. pallida* at different initial Cr(VI) concentration and time of exposure are presented and discussed. Cr content in *T. pallida* revealed a high metal accumulation in the plant roots compared with that in the plant's shoots and leaves, which was not observed in the control plants (Fig. 4.2a). Cr accumulation also increased in all the plant tissues with an increase of Cr initial concentration in the nutrient solution upto 10 mg/L. Maximum Cr accumulation values for the plant's roots, leaves and shoots were 536, 449 and 432 µg/g dw, respectively, at 10 mg/L Cr initial concentration in the medium. Thus, Cr accumulation levels in different plant parts following exposure to 10 mg/L Cr(VI) were in the order: roots>leaves>stem. However, at 20 mg/L Cr initial concentration, the plant showed some phytotoxic symptoms with a reduced Cr accumulation value, particularly after 60 days exposure period. This is possibly due to a high energy demand of the plant tissues to maintain its metabolism under such adverse conditions, which in turn led to a reduced Cr uptake by the plant (Shankar et al., 2005). The present study thus showed significant tolerance and accumulation of Cr(VI) by *T. pallida*.

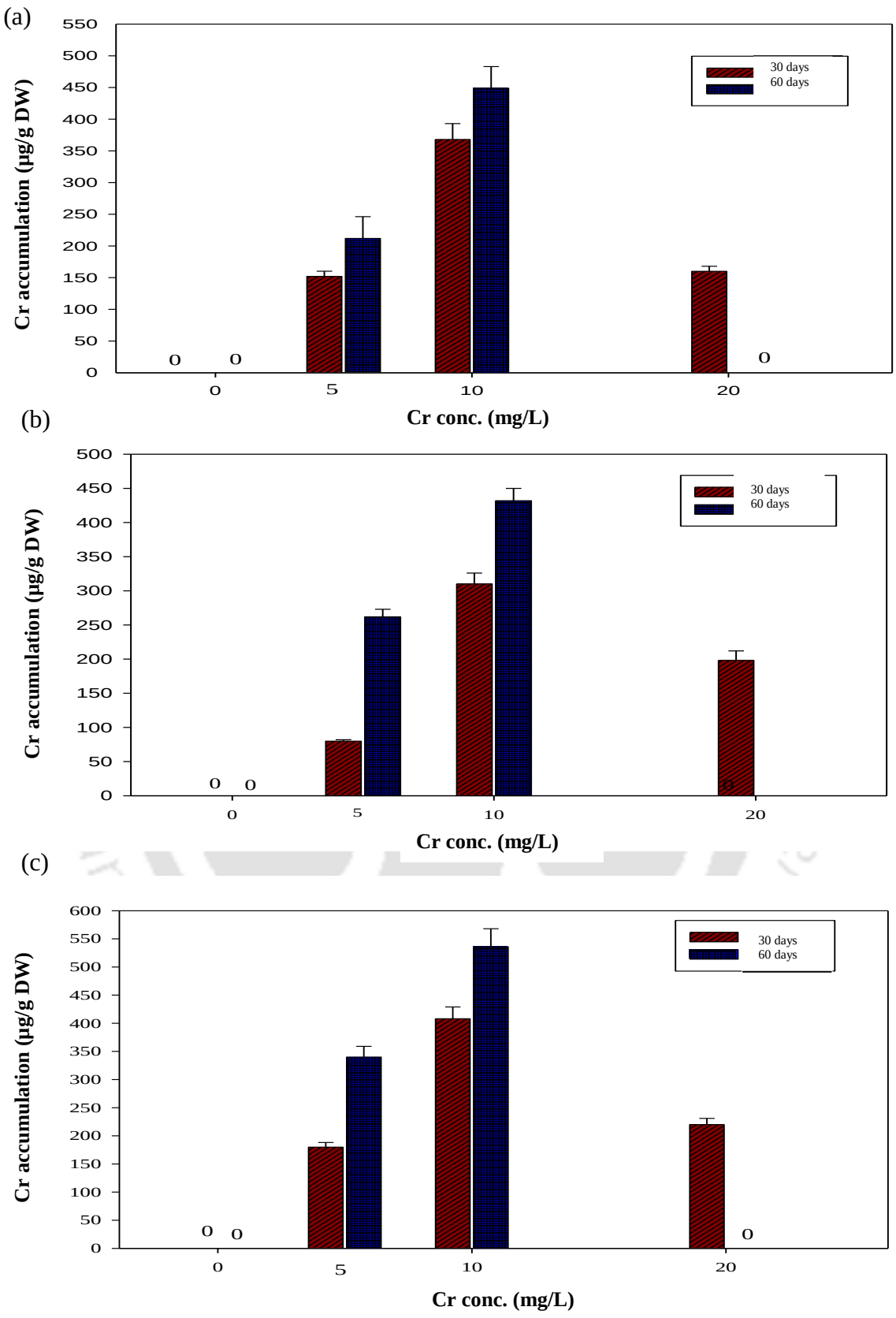

Figure 4.2: Total Cr uptake by *T. pallida* at different initial concentration over 30 and 60 days period: (a) leaves (b) shoots and (c) roots

4.1.3. Subcellular Distribution and Chromium Localization in *T. pallida*

The extent to which Cr gets accumulated and sequestered within different cell compartments of a plant plays an important role in the tolerance and survival of the plant. The localization studies were carried out to gain an insight into the possible sites of Cr sequestration in *T. pallida* plants exposed to Cr. In order to achieve this, sequential extraction and energy dispersive spectroscopy (EDS) for qualitative and quantitative determination of Cr in plant tissues were carried out. The specific objective of this study was to identify the potential sites for Cr localization and detoxification in *T. pallida*.

Figure 4.3 shows the bioaccumulation of Cr ions by *T. pallida* at different initial Cr(VI) concentration at the end of experimental period. In this study, the Cr contents in different tissues of *T. pallida* plant parts showed very high bioaccumulation of Cr, particularly in the roots. Concerning the translocation factor, a trend to a lower percentage of Cr accumulation in leaves and roots was found with increasing initial Cr concentration in the medium. A high Cr concentration in the roots than in other tissues could be considered as an important tolerance mechanism of *T. pallida*, an important strategy adopted by certain metal tolerant plants as reported in the literature (Sarma et al. 2011).

In order to elucidate the subcellular localization of Cr in *T. pallida*, sequential extraction analyses of Cr accumulated *T. pallida* plant tissues (leaves and roots) were performed the relative distribution of Cr in the different fractions of the plant parts was examined. Table 4.2 shows subcellular distribution of Cr ions present in different plant fractions at different initial Cr(VI) concentration. .

The distribution of Cr in different subcellular fractions was different in leaves and root tissues. These results show that in the leaves, the maximum accumulation of Cr was observed in the soluble fraction (F_v) at all initial Cr(VI) concentration; maximum 52% of

the total Cr content was found to be present in the soluble fraction (F_v) (vacuole and cytoplasmic fraction) and 39.8% in the cell wall fraction (F_w).

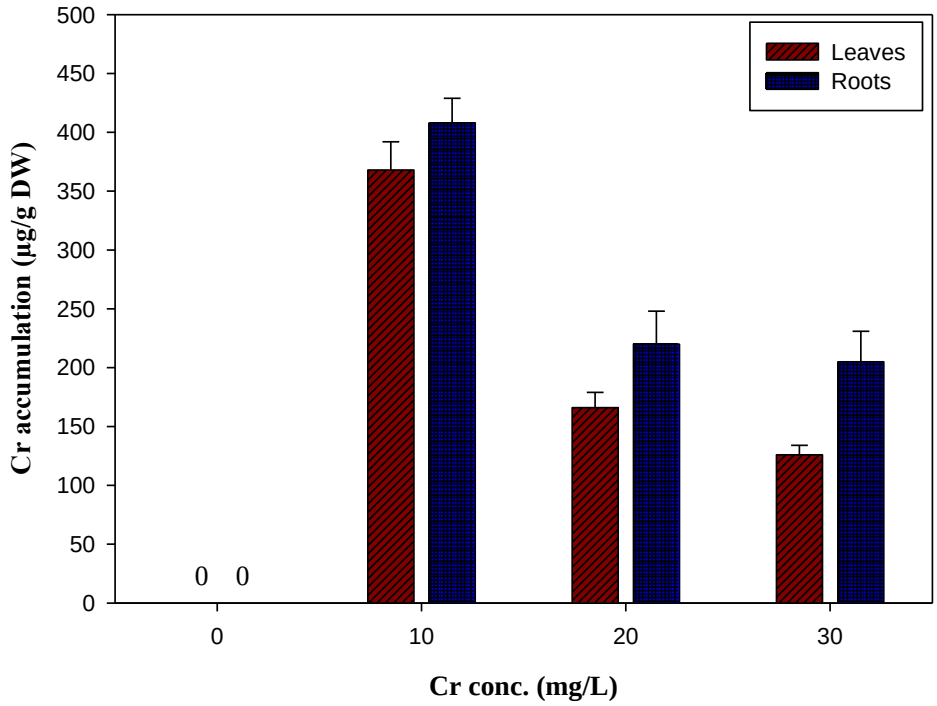

Figure 4.3: Total Cr uptake by *T. pallida* at different initial concentration over 30 days period

Whereas, in the root tissue, maximum accumulation i.e. 63.2% of the total Cr was found to be present in the root cell wall fraction (F_w), whereas 28% of Cr was present in the soluble fraction (F_v). At all initial Cr(VI) concentration (10, 20 and 30 mg/L), the organelle fraction (F_o) contained the least level of Cr in both the leaves and the roots. Thus, Cr content in the intracellular compartment was found to be relatively low. These results indicated that following uptake of Cr by *T. pallida* roots, Cr gets preferentially stored in the cell walls and vacuoles of the roots, which is then transported to aerial parts of the plant. This in part as well explains the high accumulation of Cr(VI) by the roots as compared with that by plant leaves.

Table 4. 2: Subcellular distribution of Cr (µg/g FW) in different fractions of Cr(VI) exposed *T. pallida* parts: (a) leaves (b) roots (exposure period = 30 days).

Plant Tissue	Cr Conc. (mg/L)	Cr content (µg/g FW)				
		Cell wall (Fw)	Organelles (Fo)	Soluble fraction (Fv)	Total	Recovery (%)
Leaves	10	142.53 ±6.45 (39.5%)	1.44 ±0.06 (0.4%)	186.27±8.36 (51.6%)	368.11 ±18.9	91.5
	20	66.06 ±4.27 (39.8%)	1.62 ±0.09 (0.98%)	82.53 ±4.9 (49.6%)	166.41±11.74	90.38
	30	40.32 ±1.40 (32.0%)	2.00 ± 0.25 (1.59%)	65.52 ±4.1 (52%)	126.28 ±8.7	85.59
Roots	10	236.64 ±17.8 (57.82%)	6.52 ± 0.47 (1.6%)	131.78 ±9.3 (32.3%)	408.51 ±21.64	91.72
	20	139.14 ±10.5 (63.2%)	4.84 ±0.27 (2.2%)	61.64 ±4.47 (28%)	220.16 ±16.32	93.4
	30	122.10 ±8.34 (59.35%)	11.72 ±0.71 (5.7%)	56.37 ±4.91 (27.4%)	205.74 ±14.21	92.45

Results presented are average of three samples analysed with standard deviation±SD (n=3). The figures in bracket indicate the percentages of Cr accumulation in the different fractions.
Recovery (%) = (Cell wall + Soluble fraction +Organelle) × 100/Total.
F_w cell wall fraction, F_o organelle fraction, F_v cytoplasm and vacuoles fraction

Further, storage of these toxic Cr ions inside these sites, diverted Cr ions from metabolically active compartments, such as (chloroplast, mitochondria), resulting in a reduction of Cr toxicity in the plant cell. This is consistent with the literature reports on subcellular distribution of Cr by other hyperaccumulators which suggest vacuole as the major site for metal accumulation by hyperaccumulator plants (Zeng et al., 2011; Liu et al., 2009). It has been reported that plants detoxify Cr and other heavy metals by sequestration inside their vacuole, thereby keeping the heavy metal concentration low in the plants cytoplasm.

Figure 4.4 shows energy dispersive X-ray spectra of the Cr treated *T. pallida* leaves biomass at an initial concentration of 10 mg/L Cr(VI). The spectra showed that the electron dense areas contained high Cr. The spectrum further confirmed the significant amount of Cr inside *T. pallida* leaf cells.

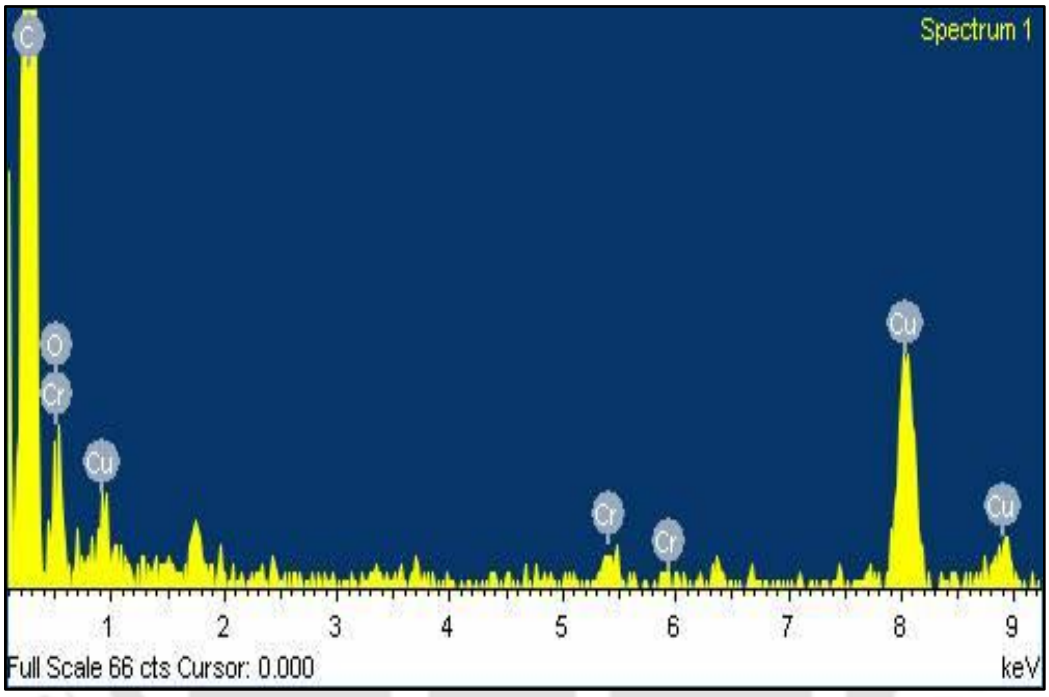

Figure 4.4: Energy Dispersive X-ray Spectroscopy (EDX) of Cr(VI)-treated *T. pallida* leaves at initial concentration of 10 mg/L (exposure period = 30 days).

All these results provide direct evidence on the role of vacuoles in the subcellular localization of Cr by *T. pallida*. The ability of *T. pallida* plants to tolerate and detoxify high Cr accumulation in its biomass, in part, due to its immobilization in the to different cell compartments (cell wall + vacoules) outside the key metabolic sites.

4.2. Effect of Chromium (VI) on *T. pallida* antioxidant enzyme systems and biochemical parameters

Following Cr bioaccumulation studies, using *T. pallida* Cr(VI)-treated and control *T. pallida* plants were analyzed for its antioxidative enzyme system and biochemical parameters to understand tolerance mechanism under Cr(VI) stressed condition. Besides analysing the activity of antioxidant enzymes, viz. catalase, peroxidase and ascorbate peroxidase, malondialdehyde (MDA) content (end product of lipid peroxidation) was measured to determine the oxidative stress on *T. pallida* due to Cr(VI) exposure. Biochemical changes in the plant were analysed in terms of carbohydrate content, protein content, proline content and chlorophyll content.

4.2.1. Effect of Chromium on antioxidant enzymes

Activity of antioxidant enzymes in Cr(VI)- treated and control plant were determined to understand the cellular defense machinery of *T. pallida* plants against Cr. Antioxidant enzymes are important components in preventing oxidative stress in plants by scavenging free radicals and peroxides (Gill and Tuteja, 2010). The roles of antioxidative enzymes are very important in plants while coping with ROS damaging effects.

Table 4.3 presents biochemical and antioxidant enzyme activity in *T. pallida* due to Cr(VI) exposure at an initial Cr(VI) concentration of 10 mg/L. These results show that Cr(VI)-treated plant has a high activity of lipid peroxidation, catalase, peroxidase and ascorbate peroxidase (APX) enzymes as compared with the control *T. pallida* plant.

Table 4.3a shows lipid peroxidation activity measured in terms of MDA, which increased in all Cr(VI)-treated plants compared with control. In addition, the Cr(VI)-treated plants showed a high activity of lipid peroxidation in its roots than in the other plant parts following the 90-day experimental period. Membrane destabilisation is generally attributed to lipid peroxidation due to an increased production of ROS (Suzuki et al.,

2011). Thus, in the present study, enhancement in lipid peroxidation due to Cr(VI) exposure suggests that Cr caused oxidative damage to the cell, particularly the membrane lipids due to ROS generation. Moreover, the increase in MDA content in Cr(VI)-treated plant parts can be interrelated with an increase in concentrations of Cr(VI) and duration of treatment.

Maximum catalase activity was observed in the Cr(VI)-treated plants leaves (Table 4.3b). Cr(VI)-treated plants shoots showed significantly lower H_2O_2 concentrations than that in the leaves and roots. Catalase efficiently scavenges the free radicals particularly H_2O_2 produced during stress, thereby preventing membrane lipids, proteins and nucleic acids against damage (Tamás et al., 2008). Significantly high values of the APX activity were observed in Cr(VI)-treated leaves, shoots and roots. High increase in APX activity (Table 4.3d) thus, suggests that APX is up-regulated under Cr(VI)-induced oxidative stress and plays a major role in the scavenging of H_2O_2 (Diwan et al., 2007).These results also indicate that APX was more efficient in destroying H_2O_2 than the other antioxidant enzymes.

Table 4.3: Changes in biochemical and antioxidant enzyme activity in *T. pallida* due to Cr(VI) exposure: (a) lipid peroxidation, (b) catalase activity, (c) peroxidase activity and (d) ascorbate peroxidase (APX) activity.

(a)	**Malondialdehyde (µM/g fresh weight)**					
Experimental period (days)	**Roots**		**Shoots**		**Leaves**	
	Control	Cr(VI) treated	Control	Cr(VI) treated	Control	Cr(VI) treated
30	8.3 ±0.4	39.7 ±0.3	7.6 ±0.2	28.6 ±0.3	9.5 ±0.2	26.3 ±0.2
60	20.2 ±0.6	54.1 ±0.2	16.9 ±0.5	31.3 ±0.3	15.4 ±0.4	37.2 ±0.3
90	22.8 ±0.4	57.5 ±0.4	14.2 ±0.3	28.9 ±0.3	18.6 ±0.4	40.1 ±0.3

(b)	**Catalase (µM H_2O_2 destroyed/mg protein/min)**					
Experimental period (days)	**Roots**		**Shoots**		**Leaves**	
	Control	Cr(VI) treated	Control	Cr(VI) treated	Control	Cr(VI) treated
30	5342 ±10.9	5923 ±15.4	4198 ±18.2	5605±21.2	5853±12.1	6854±21.2
60	5809 ±11.1	6107 ±13.0	4634 ±14.3	5490±17.4	6352±10.8	6912±21.2
90	6566 ±21.0	7650 ±11.3	4823 ±19.2	5499±12.2	6031±22.4	7696±19.0

(c)	**Peroxidase (U/mg protein)**					
Experimental period (days)	**Roots**		**Shoots**		**Leaves**	
	Control	Cr(VI) treated	Control	Cr(VI) treated	Control	Cr(VI) treated
30	5267 ±21.3	8952 ±17.3	4339 ±16.2	6971±21.3	4108±19.4	5877±18.4
60	6209 ±16.6	9977 ±21.6	4987 ±12.7	7085±27.7	5977±14.3	8568±13.5
90	6744 ±14.5	9806 ±18.4	4768 ±19.8	7100±11.6	6132±12.8	8892±15.4

(d)	**Ascorbate peroxidase (U/mg protein)**					
Experimental period (days)	**Roots**		**Shoots**		**Leaves**	
	Control	Cr(VI) treated	Control	Cr(VI) treated	Control	Cr(VI) treated
30	2006 ±26	3500 ±14	1407 ±11	3148 ±15	2210 ±18	3865 ±19
60	2783 ±18	9800 ±13	1922 ±18	8554 ±23	2630 ±21	8317 ±22
90	2976 ±13	9823 ±25	2314 ±17	9439 ±16	2965 ±14	9709 ±12

Results presented are average of three sample analyses with standard deviation (n=3)

Recently, several studies have demonstrated the dual role of reactive oxygen species (ROS) in plants subjected to abiotic stress (Suzuki et al., 2011). The ROS generation further helps in signalling activation of defence responses. For instance, various antioxidant enzymes viz., catalase, peroxidase and APX act synchronously to quench the free radicals produced. In chloroplasts, ascorbate–glutathione cycle is one of the major defence pathway by which H_2O_2 is scavenged and converted to H_2O and O_2 (Asada, 2006). The main enzymes in this pathway are APX and glutathione reductase enzymes; the pathway uses ascorbate and glutathione as oxidoreductants, H_2O_2 as an electron acceptor and nicotinamide adenine dinucleotide phosphate (NADP+) as an electron donor. These enzymes are also strictly compartmentalised and act in a highly coordinated manner.

Chromium (VI) is a known oxidising agent which after reduction to Cr(III) inside the plant cell produces free radicals, such as singlet oxygen, hydroxyl ions and hydrogen peroxide, thereby inducing damage at the cellular level (Ali et al., 2015). In this study, *T. pallida* plants treated with Cr(VI) showed a high oxidative damage and increased peroxidation of membrane lipids compared with those in the control plants. All these effects could be attributed to free radicals produced under Cr-stressed conditions, which induce oxidative damage to the plant. Also, the increased activities of the antioxidant enzymes in the present study is attributed to the plants defence mechanism to counteract the toxicity induced by Cr(VI).

4.2.2. Effect of Chromium on *T. Pallida* biochemical parameters

Chromium (VI) is known to interfere with several physiological and biochemical processes of plants resulting in changes in these processes (Oliveira, 2012). In the present study, total carbohydrates, protein, chlorophyll and proline content in *T. pallida* plants were examined at the end of experimental period to gain insight into the physiological

and biochemical mechanisms of Cr accumulation and detoxification. Table 4.4 presents the carbohydrate and protein content of *T. pallida* plant parts following treatment with Cr(VI).

Table 4.4: Change in (a) carbohydrate and (b) protein content of *T. pallida* plant parts following treatment with Cr(VI) (initial Cr(VI) conc. = 10 mg/L and exposure period = 90 days).

(a)	**Carbohydrate content (mg/100 mg of dry weight of plant part)**				
Roots		**Shoots**		**Leaves**	
Control	Cr(VI) treated	Control	Cr(VI) treated	Control	Cr(VI) treated
1.8783 ±0.1604	2.1058 ±.1681	1.7549 ±0.0921	1.8139 ±.1541	2.4814 ±0.2357	3.4231 ±0.1284

(b)	**Protein content (mg/g of fresh weight of plant part)**				
Roots		**Shoots**		**Leaves**	
Control	Cr(VI) treated	Control	Cr(VI) treated	Control	Cr(VI) treated
13.519 ±0.4541	12.961 ±0.9983	7.937 ±0.4230	7.233 ±0.1069	19.805 ±0.5512	15.533 ±0.5910

Results presented are average of three sample analyses with standard deviation S.D (n=3)

Results of carbohydrate analysis revealed that carbohydrate content was higher in roots and leaves of Cr-treated plants compared with that in the control plants (Table 4.4a). The soluble sugar content revealed that low concentration of Cr(VI) increased the soluble sugar content in all the plant parts. This is mainly due to a high starch accumulation in Cr(VI)-exposed leaves as it improves the resistance of the plant photosynthetic apparatus and lowers the starch export from the mesophyll (Ali et al., 2013). In addition, an enhancement in the carbohydrate supplements helps in protecting the plant biomolecules and membranes under stress conditions (Dubey et al., 1999). Conversely, protein content was lower in the Cr-treated plants as compared with that in the control plant parts (Table

4.4b). Moreover, the protein content was significantly reduced in the leaves as compared with that in the stem and roots of the Cr(VI)-treated plants. The decrease in the protein content of Cr(VI)-exposed plant suggests that proteins are easily susceptible to oxidative stress damage induced by Cr(VI) in these plants. This is in agreement with Costa et al. (1997), who observed a decrease in soluble protein content of *Lupinus albus* in the presence of heavy metals such as Cd.

The results pertaining to the effect of different initial Cr(VI) concentration on proline content is presented in Fig 4.5. Proline is one of the most significant metabolites produced in plant tissues under stress conditions. Treatment of *T. pallida* plants with different initial Cr(VI) concentration resulted in a significant Cr(VI) dose dependent accumulation of proline as compared to thar in the control. The maximum increase in proline content was observed in *T. pallida* leaves at a high initial Cr(VI) concentration of 30 mg/L. This has been attributed to the protective role of proline that acts as a ROS scavenger (Matysik et al., 2002), osmoprotectant (Vendruscolo et al., 2007) and membrane stabilizer (Agami et al., 2014). Moreover, proline is known to play an important role in enhancing metal tolerance and plays a crucial role in overcoming metal induced stress in plant cells (Huang and Wang, 2010). Figure 4.6 shows the effect of different initial Cr(VI) concentration on chlorophyll-a and chlorophyll-b content in *T. pallida* leaves. Treatment of *T. pallida* plants with different initial Cr(VI) concentration resulted in a decrease in both the chl a and b content as compared to that in the control.

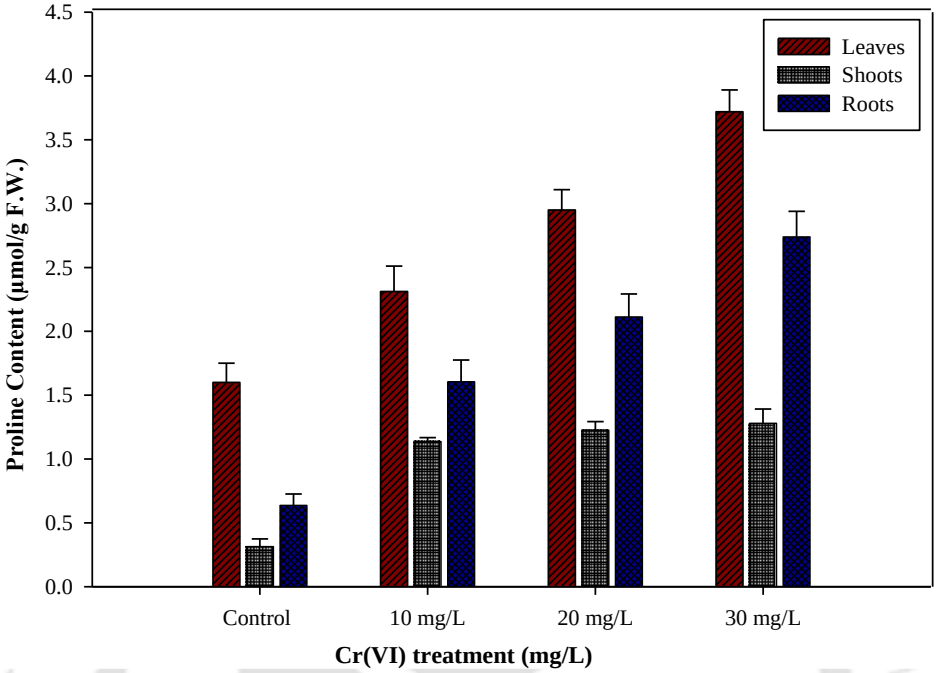

Figure 4.5: Effect of different initial Cr(VI) concentration on proline content in *T. pallida*: (a) leaves, (b) shoots and (c) roots.

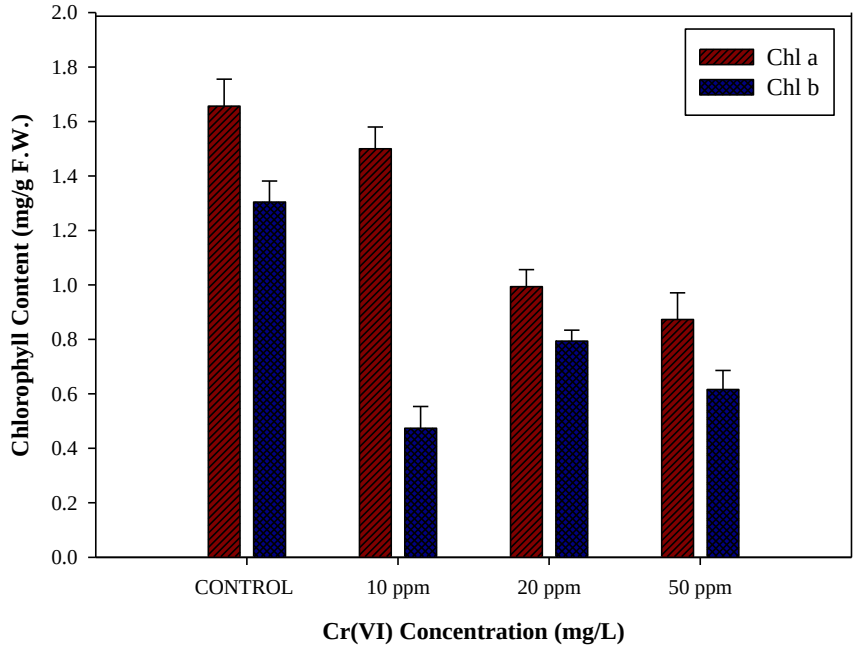

Figure 4.6: Effect of different initial Cr(VI) concentration on chlorophyll content in *T. pallida* leaves.

The chlorophyll degradations in Cr(VI)-treated leaves could be a result of the cytotoxic effect of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation (Upadhyaya et al., 2007). It is one of the major chloroplast components for photosynthesis, and relative chlorophyll content has a positive relationship with photosynthetic rate. Figure 4.7 shows the Cr(VI) tolerance and uptake uptake mechanism in *T. pallida.*

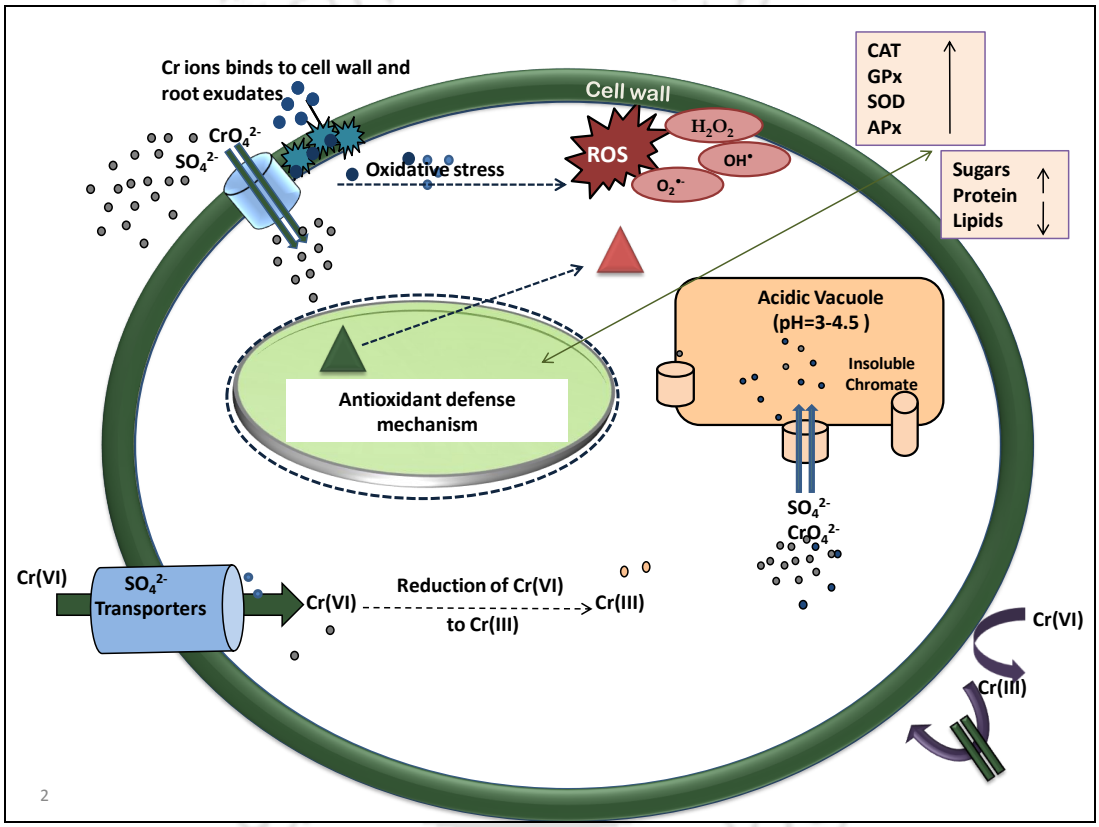

Figure 4.7: Cr(VI) tolerance and uptake mechanism in *T. pallida*

. Overall, this study showed excellent tolerance of *T. pallida* towards Cr(VI). The plant showed significant Cr accumulation, along with changes in the biochemical constituents. Up-regulation of antioxidative enzyme system played a key role in overcoming oxidative stress induced due to Cr(VI). *T. pallida* was found to be a potential plant for Cr remediation.

4.3. Simultaneous Removal of Chromium (VI) and Co-ions from Multi-ion System by *T. pallida*

Cr(VI) is mainly released in the environment by leather tanning, textile processing, electroplating, mining operations and pigment manufacturing industries which discharge wastewater heavily contaminated with Cr(VI), sulphate, nitrate, phosphate, etc., (Huang et al., 2009, Chen et al., 2010). Under this investigation, the potential of *T. pallida* for the simultaneous removal of Cr(VI), SO_4^{2-}, NO_3^- and PO_4^{3-} and the effect of these co-ions on Cr(VI) uptake by *T. pallida* were carried out. Co-presence and interaction between these co-ions may often exert a different effect on Cr uptake and its translocation in *T. pallida*. Therefore, the present study was carried out to evaluate the effect of SO_4^{2-}, NO_3^- and PO_4^{3-} on Cr uptake. Cr(VI) uptake kinetics mechanisms was determined using suitable kinetic models reported in the literature. Finally, analyses of the biochemical and enzymatic changes in *T. pallida* were carried out for a better understanding of the oxidative stress and detoxification responses adopted by *T. pallida* due to its exposure to Cr(VI) and co-ions.

4.3.1. Effect of Co-ions on Chromium Uptake by *T. pallida*

Table 4.5 presents the removal of Cr(VI), SO_4^{2-}, NO_3^- and PO_4^{3-} by *T. pallida* in the multicomponent system, which reveals that their removal efficiency varied depending upon their combination level in the respective experimental runs. In this study, a maximum removal of Cr(VI) (84%), SO_4^{2-} (87%), NO_3^- (94%) and PO_4^{3-} (100%) was achieved depending upon the initial concentration of Cr(VI) and the co-ions. The removal of Cr(VI), SO_4^{2-}, NO_3^- was maximum at a high initial concentration of 20, 150 and 20 mg/L, respectively. PO_4^{3-} removal, on the contrary, was high in all the experimental runs, irrespective of its initial concentration (high or low). Further, these results clearly indicated that the removal of Cr(VI) and co-ions depended on their initial concentration in

the mixture. Thus, an overall removal efficiency of more than 70% for Cr(VI) and the co-ions was achieved in the multicomponent system.

Table 4.5: Plackett-Burman experimental design matrix showing combination of levels of Cr(VI) and co-ions along with their removal by *T. pallida* in the different experimental runs.

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Exp.run no.	Initial concentration(mg/L)				Removal %			
	Cr(VI)	SO_4^{2-}	NO_3^-	PO_4^{3-}	Cr(VI)	SO_4^{2-}	NO_3^-	PO_4^{3-}
1	5	50	20.0	10.0	77.00	80.29	93.53	99.97
2	20	150	20.0	0.5	80.31	87.14	93.66	100.00
3	5	150	0.5	10.0	84.10	86.97	94.88	93.97
4	20	50	20.0	10.0	73.33	78.60	92.55	99.83
5	20	150	20.0	0.5	78.33	84.41	89.73	99.85
6	5	150	20.0	10.0	79.74	84.27	92.00	98.27
7	20	150	0.5	10.0	84.27	83.27	93.37	94.14
8	20	50	0.5	10.0	74.70	72.71	91.75	94.86
9	20	50	0.5	0.5	75.25	84.54	93.78	96.55
10	5	50	20.0	0.5	77.74	81.76	96.82	98.99
11	5	150	0.5	0.5	81.74	76.53	91.80	94.14
12	5	50	0.5	0.5	76.74	80.99	93.45	95.07

Figure 4.8 shows Pareto chart on the effect of Cr(VI) and co-ions on each other removal by *T. pallida* in the multicomponent system. In these Pareto charts, horizontal bars indicate the effects due to the individual factors and those exending past the vertical reference line were considered the significant ones ($\alpha = 0.05$). The Pareto chart has been described as a useful tool for identifying the most important effects, display the magnitude of each variables and is a convenient way to view the results of a Plackett–Burman design (Haaland, 1989; Strobel and Sullivan, 1999).

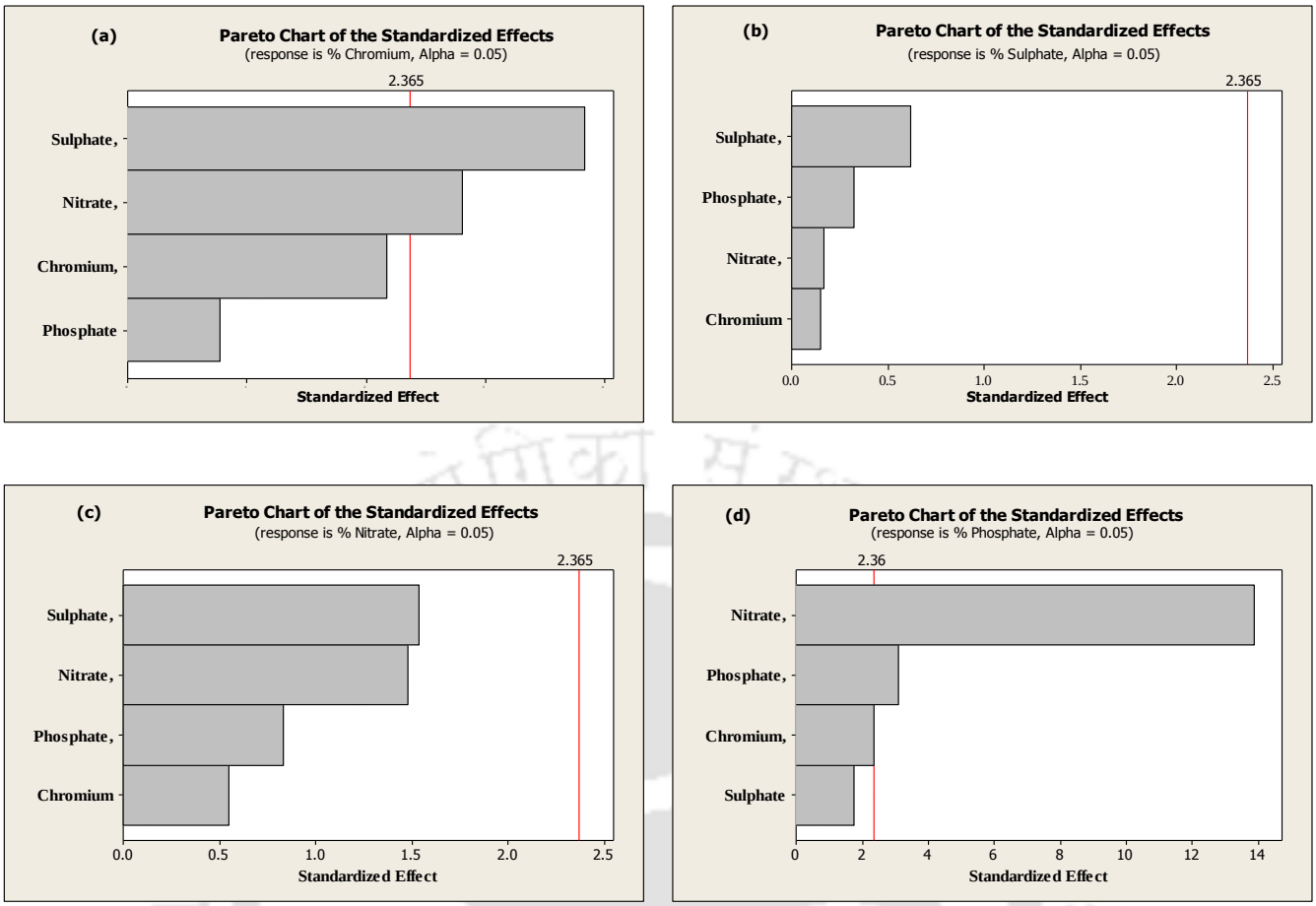


Figure 4.8: Pareto chart showing the effect of Cr(VI) and co-ions on each other removal by *T. pallida* in the different experimental runs: (a) Cr(VI) removal, (b) SO_4^{2-} removal, (c) NO_3^- removal and (d) PO_4^{3-} removal (vertical line shows significance cutoff at P value less than 0.05).

In this chart, the length of each bar on a standardized Pareto chart is proportional to the absolute value of its associated regression coefficient or estimated effect. The effects of all parameters, interactions as well as quadratic terms, are standardised (each effect is divided by its standard error). The order in which the bars are displayed corresponds to the order of the size of the effect of co-ions. The chart includes a vertical line that corresponds to the 95% limit indicating statistical significance. An effect is, therefore, significant if its corresponding bar crosses this vertical line (Rezzoug and Capart, 2003).

All these results show that Cr(VI) removal efficiency varied in the presence of different concentration of co-ions (SO_4^{2-}, PO_4^{3-} and NO_3^-). As shown in Fig. 4.8a, the most significant parameters for Cr(VI) removal is SO_4^{2-}. The co ions SO_4^{2-} and NO_3^- significantly affected the Cr(VI) uptake by *T. pallida*. Among the different co-ions, SO_4^{2-} enhanced Cr(VI) removal the most. It has been reported that most plants do not have specific transporters for Cr(VI) uptake mainly because it is not an essential nutrient (Singh et al., 2013). In this study, a high initial SO_4^{2-} concentration increased the accumulation of Cr(VI) by enhancing the transport rate of Cr(VI) into the cells, thus, suggesting that more number of SO_4^{2-} transporters were engaged at an elevated SO_4^{2-} concentration and through which concurrent uptake of SO_4^{2-} and Cr(VI) occurred (Schiavon et al., 2012). Figure 4.9 shows the proposed mechanism of Cr transport in *T. pallida*, where, Cr(VI) ions gains entry into *T. pallida* cells via sulfate transporters due to its structural homology with SO_4^{2-} ions. In a variety of plant species, Cr(VI) entry takes place through the sulphate ABC transporter, which is up-regulated following CrO_4^{2-} exposure (Vita et al., 2014). In a previous experiment (section 4.1.2), a substantial amount of Cr(VI) was observed to bioaccumulate in *T. pallida* shoots as well as in roots. Hence, low as well as high affinity sulphate transporters can be attributed to the simultaneous uptake of SO_4^{2-} and Cr(VI) observed in this study (Kaszycki et al., 2005).

At an initial Cr(VI) concentration of 20 mg/L, the effect of PO_4^{3-} on the Cr(VI) removal was negligible (Fig. 4.7a, Table 4.5), which is due to the fact that PO_4^{3-} ions act as an essential nutrient for plants, and, hence, it is assimilated over chromate by the plant (López-Bucio et al., 2014).

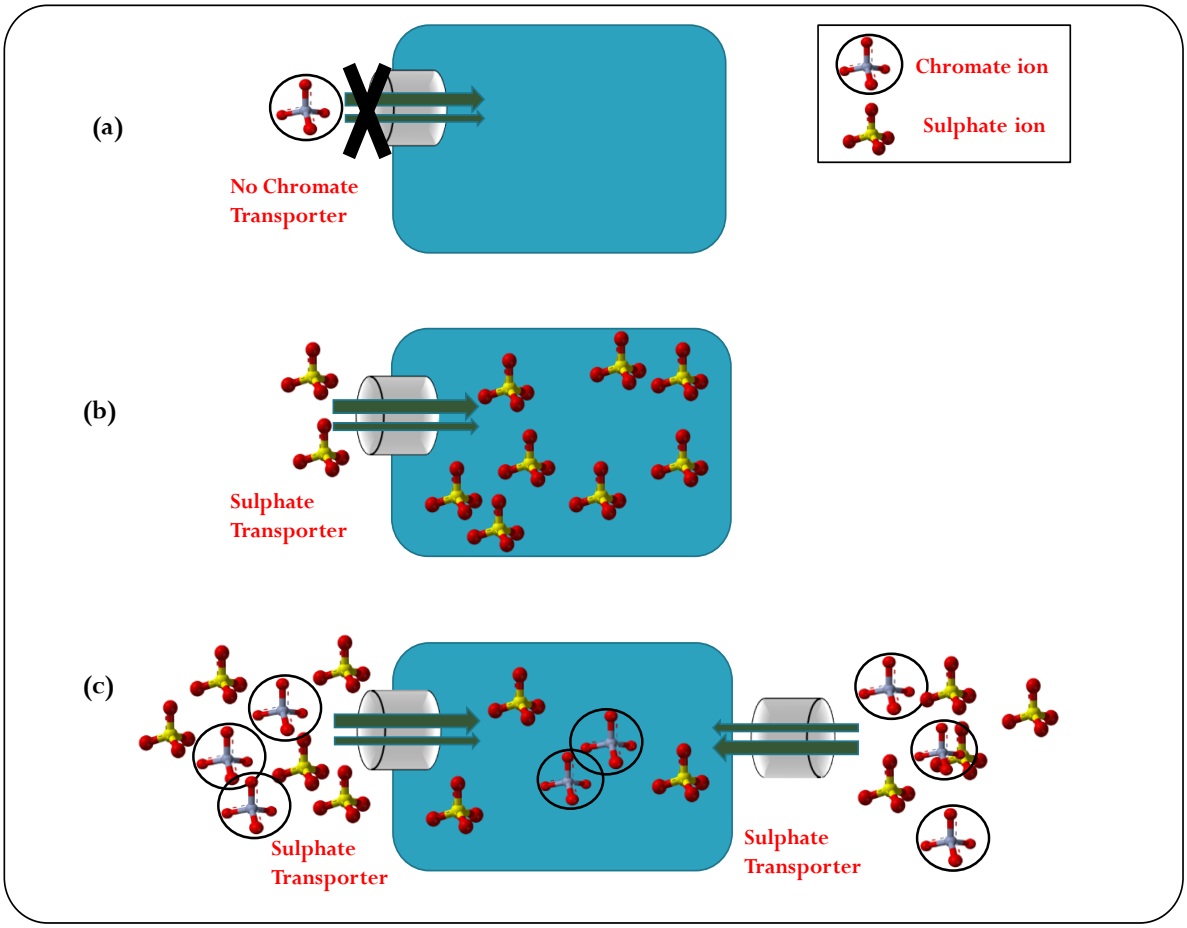


Figure 4.9: Schematic of Cr(VI) transport in *T. pallida* via sulphate transporters

In the present study, Cr(VI) uptake increased along with an increase in the initial PO_4^{3-} concentration which may be attributed to the plant's mechanism to tolerate Cr toxicity and to maintain the plant's nutrient homeostasis (Qian et al., 2013). A similar inference was drawn by López-Bucio et al. (2014), who reported that Cr induced the expression of PO_4^{3-} transporters in *Arabidopsis thaliana*. Several other reports have also shown that mineral nutrients, e.g. PO_4^{3-} attenuates Cr(VI) toxicity by decreasing Cr absorption and increasing the absorption of beneficial ions in plants such as *Raphanus sativus* L. (Sayantan, 2013) and *Pteris vittata* (de Oliveira et al., 2015). These authors also confirmed that a reduced Cr toxicity in plants is due to an increase in PO_4^{3-} uptake at a high initial Cr(VI) concentration in medium. Further, SO_4^{2-} showed negative effect on PO_4^{3-} removal. This may be attributed to the alteration in the expression of *SULTR2:1*

transporter, which increases SO_4^{2-} translocation at a low concentration of PO_4^{3-} (Rouached, 2011). The ion, PO_4^{3-} uptake increased along with an increase in the initial Cr(VI) concentration which may be attributed to tolerate Cr toxicity and to maintain nutrient homeostasis of the plants (Alam, 1999).

Figure 4.10 shows the proposed mechanism of SO_4^{2-}, NO_3^- and PO_4^{3-} transport in *T. pallida*, where each of these ions gains entry inside *T. pallida* cells through its own specific transporters. Since all these ions are plant's macronutrients, plant cell posesses large number of transporters for their uptake. Further, results show that PO_4^{3-} uptake was dependent on its own initial concentration (Fig. 4.8d).

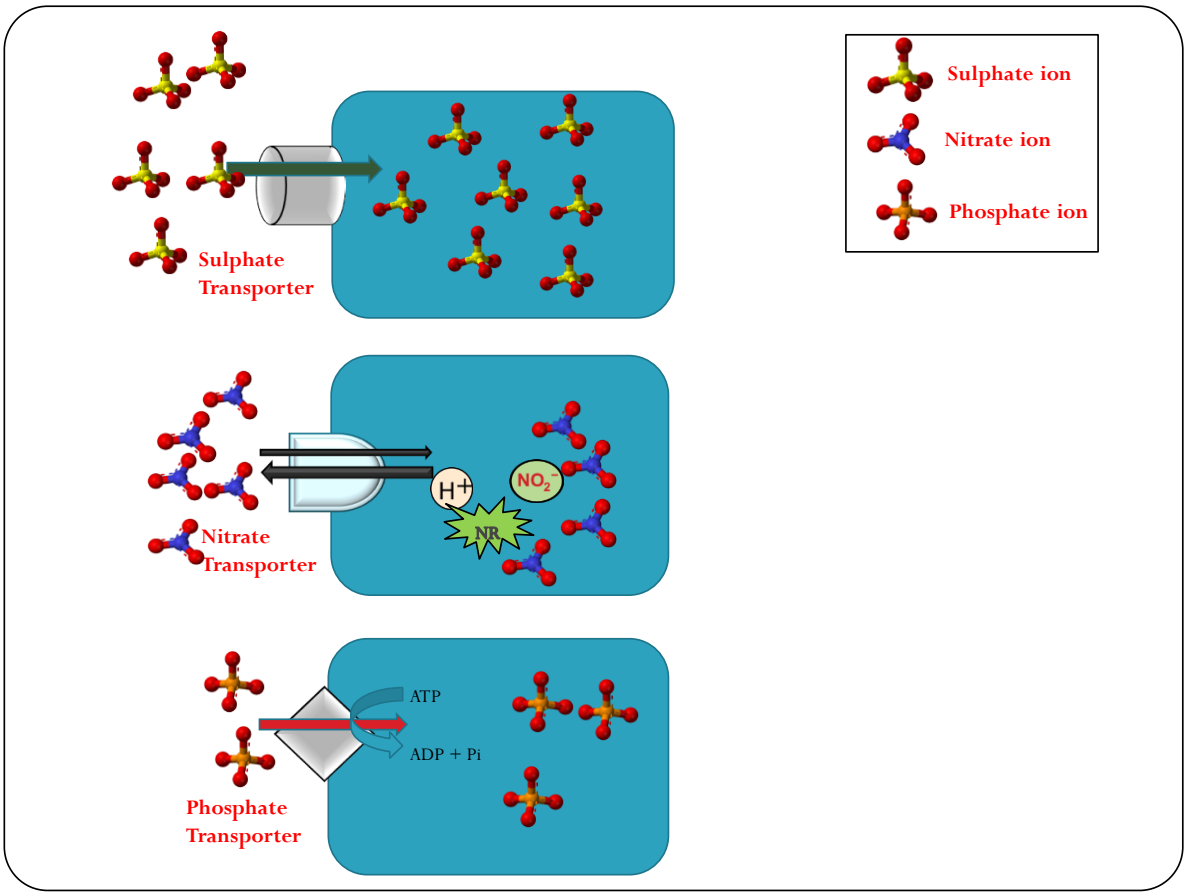
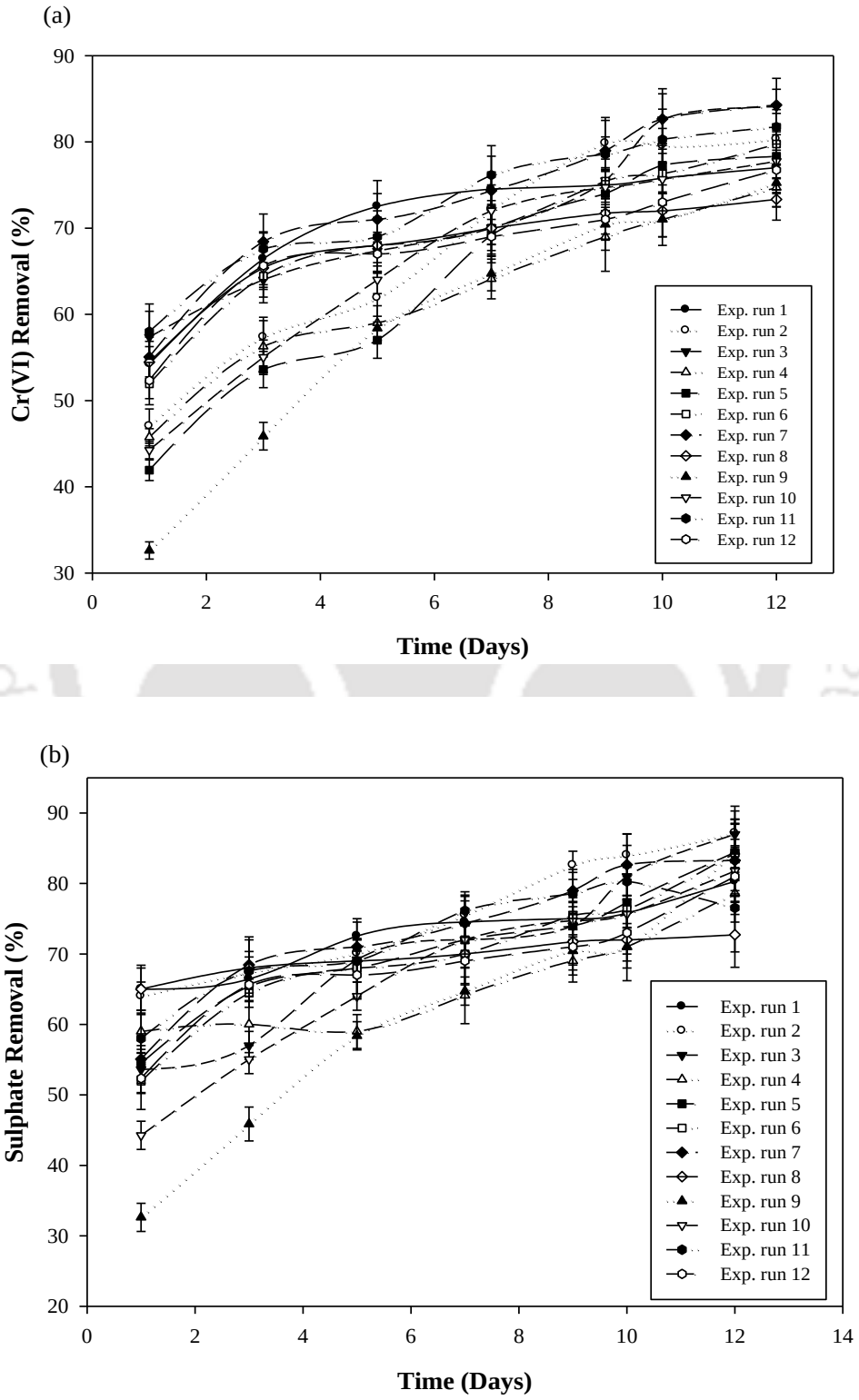


Figure 4.10: Schematic of SO_4^{2-}, NO_3^- and PO_4^{3-} transport in *T. pallida.*

4.3.2. Chromium (VI) removal kinetics by *T. pallida*

Time profile of Cr(VI), SO_4^{2-}, NO_3^-, PO_4^{3-} removal in the different experimental runs are depicted in Fig. 4.11.



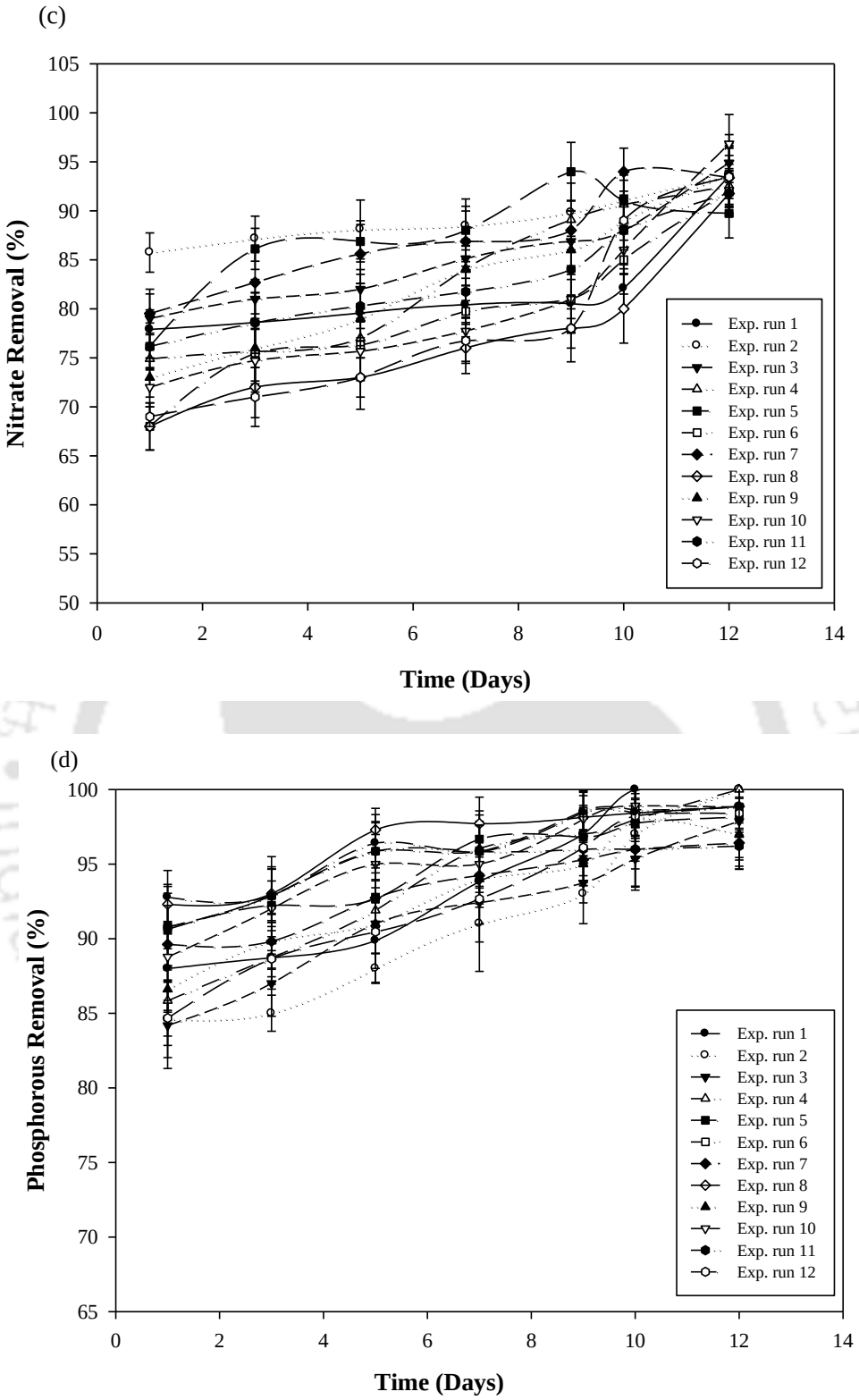


Figure 4.11: Time profile of removal of Cr(VI) and co-ions by *T. pallida* in the different experimental runs: (a) Cr(VI), (b) SO_4^{2-}, (c) NO_3^- and (d) PO_4^{3-}

These figures show a gradual uptake of Cr(VI) and the co-ions by *T. pallida* throughout the experimental period. Although some variation in the percentage removal of Cr(VI) and the co-ions was observed in each of the experimental runs, the removal, in general, was slow and steady for Cr(VI) and the co-ions. For a better understanding of the kinetics of Cr(VI) removal by *T. pallida*, the experimental results were fitted to three well-known kinetic models found in the literature, i.e irreversible, Lagergen's first-order and Ho's second-order kinetic models. The values of the estimated kinetic model parameters and the coefficient of determination (R^2) due to these models are presented in Table 4.6.

Table 4.6: Estimated kinetic model parameters of Cr(VI) removal by *T. pallida* in the different experimental runs.

			Irreversible		**Pseudo first order**			**Pseudo second order**	
Exp. run no.	q_{max} (mg/g)	Rate constant (k_1) (g/mg/ min)	R^2	q_{max} (mg/g)	Rate constant (k_2) (min^{-1})	R^2	q_{max} (mg/g)	Rate constant(k_3) (g/mg/min)	R^2
1	0.2136	0.2936	0.8656	0.2237	0.4467	0.7689	0.1887	4.7769	0.9955
2	1.1659	0.0536	0.8221	0.7650	0.3286	0.7367	2.86345	0.7654	0.9778
3	0.1896	0.3291	0.8873	0.1048	0.8768	0.8560	1.2244	1.1568	0.9732
4	1.0360	0.0603	0.8145	0.8669	0.4386	0.8348	1.1237	1.1679	0.9568
5	0.9854	0.0634	0.8097	0.9786	0.2478	0.7932	2.9756	0.7986	0.9324
6	0.2586	0.2412	0.8965	0.2678	0.5863	0.7332	1.1854	0.9864	0.9560
7	0.8678	0.0720	0.7898	0.8670	0.3258	0.7187	3.0478	0.7789	0.9588
8	0.6472	0.0965	0.8123	0.6890	0.2896	0.6987	1.4327	1.0964	0.9568
9	0.7896	0.0821	0.8026	0.656	0.3103	0.7860	1.4795	0.9446	0.9455
10	0.3210	0.1948	0.8676	0.1540	0.7689	0.7684	0.1789	4.8970	0.9899
11	0.2285	0.2735	0.8432	0.1865	0.7423	0.7457	1.3842	0.9875	0.9776
12	0.1180	0.5274	0.8321	0.2011	0.4867	0.7556	0.2012	5.237	0.9876

Table 4.6 clearly reveals that Cr(VI) removal kinetics followed the pseudo second-order kinetic model more accurately than the irreversible or pseudo first-order kinetics with a correlation coefficient (R^2) greater than 0.93. These results suggested that the initial Cr(VI) sorption by *T. pallida* is reaction controlled, involving chemisorption, i.e., the Cr(VI) was initially bound to the surface of *T. pallida* for its subsequent uptake (Sinha et al., 2015), which is in agreement with the literature (Espinoza et al., 2009, Kavita and Keharia, 2012). The maximum estimated biosorption capacity (3.04 mg/g) (Table 4.6) obtained using this model also matched well with the maximum Cr(VI) removal efficiency (84.27%) obtained in the experimental run 7 (Tables 4.5). Further, the effect of sulphate on Cr(VI) removal was observed both on the experimental Cr(VI) removal (Fig. 4.8a) and the estimated pseudo second-order biosorption capacity value (q_{max}) (Fig. 4.12).

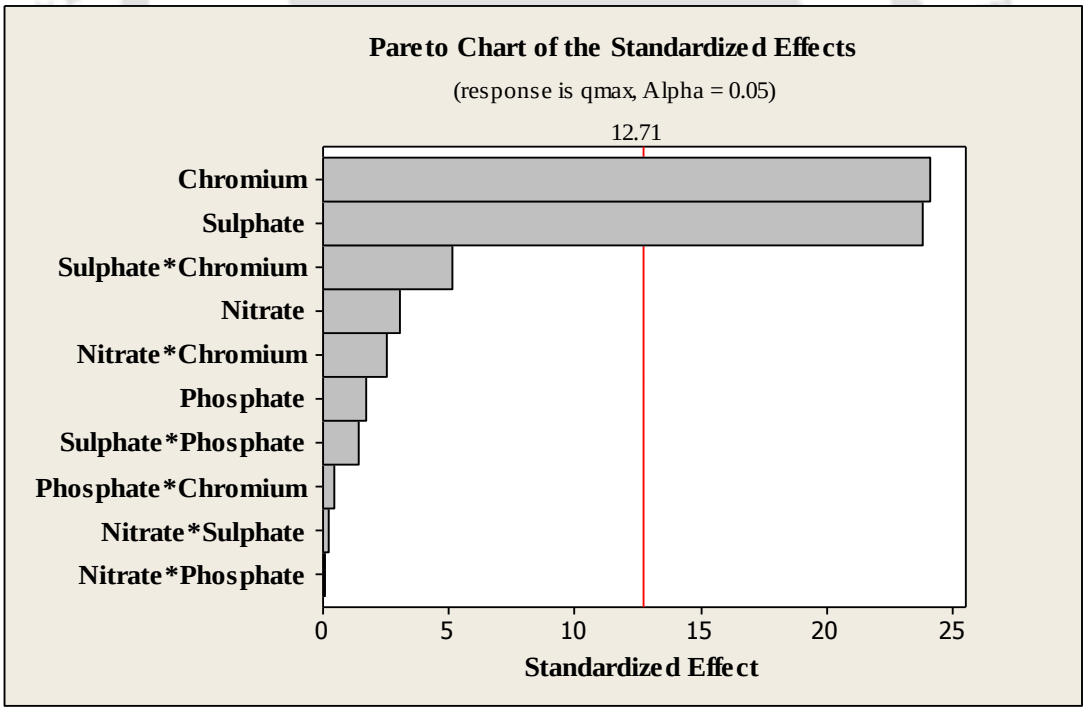


Figure 4.12: Pareto chart showing the effect of Cr(VI) and co-ions on the estimated maximum Cr(VI) uptake capacity (q_{max}) of *T. pallida* in the multicomponent system (vertical line shows significance cutoff at P value less than 0.05).

4.3.3. Factorial Analysis (ANOVA and Student's t test)

For a better understanding and evaluation of the relative significance of Cr(VI) and the co-ions on each other removal in the multicomponent system, the metal removal results were analysed statistically in the form of analysis of variance (ANOVA) and student's t test. The ANOVA result with a high Fischer's 'F' value and a low probability 'P' value of the regression model indicates its accuracy in explaining the variations in the results (Table 4.7).

Table 4.7: Analysis of variance (ANOVA) of removal of Cr(VI) and the co-ions by *T. pallida* in the multicomponent system.

Term	**Cr(VI) Removal [a]**		**SO_4^{2-} Removal [b]**		**NO_3^- Removal [c]**		**PO_4^{3-} Removal [d]**	
Values	P	F	P	F	P	F	P	F
Main effect	0.01	6.98	0.0	0.13	0.00	1.39	0.0	52.8
Cr(VI)	0.01	11.6	0.88	10.02	0.6	0.3	0.05	5.61
SO_4^{2-}	0.00	14.74	0.55	0.38	0.16	2.37	0.01	9.54
NO_3-	0.02	7.89	0.87	0.03	0.18	2.18	0.0	192.99
PO_4^{3-}	0.06	4.71	0.75	0.11	0.43	0.69	0.12	3.05

	S	PRESS	R-Sq	R-Sq (pred)	R-Sq (adj)
a	0.74	8.4918	97.96	94.12	91.52
b	0.40	4.45	97.14	92.0	88.0
c	0.85	5.93	94.19	93.0	92.3
d	0.58	7.04	96.79	90.57	94.96

The values of the statistical parameters, namely Fischer's F, Probability P, Standard error S, coefficient of determination (R^2) and adjusted R^2, collectively describe if the level means are significantly different from each other or not. Standard error is expressed in the same units as that of the response variable and it represents the standard measure between the experimental and the model predicted values. The parameter value also indicates goodness of fit of the regression model used to describe the experimental results. Thus, a

low value of S obtained in this study indicates a very good accuracy of the regression model in predicting the experimental data. On the other hand, R^2 and adjusted R^2 values describe the amount of variation in the observed response values that is explained by the predictors. Thus, minimum S value and maximum R^2 value indicate an accurate prediction ability of the model in this study (Roy et al., 2015). Moreover, values of the predicted residual error sum of squares (PRESS) presented confirmed that the regression based model were highly accurate in predicting the experimental results.

To further understand which of the individual factors (Cr(VI) and the co- ions) in the multicomponent system played a significant role on each other removal, student's t -test was performed, which is used as a tool to check the significance of the regression coefficient of the parameters. The estimated co-efficients of individual effect of Cr(VI) and the co-ions are presented in Table 4.8, in which the associated t and P values were used to check their significance. Any variable with a P-value less than 0.05 were considered statistically significant on the removal of Cr(VI) and co-ions. Positive or negative effect of the factors on each other removal was determined by their respective estimated t-values.

The following are the regression model equations (4.1-4.4) of the experimental design:

$$Y_1 = 79.38 - 0.46(X_1) + 2.29\,(X_2) - 1.68\,(X_3) + 1.3\,(X_4) \quad (4.1)$$

$$Y_2 = 82.39 + 0.17(X_1) + 0.73\,(X_2) - 0.2\,(X_3) + 0.38\,(X_4) \quad (4.2)$$

$$Y_3 = 93.08 + 0.74\,(X_1) - 0.38\,(X_2) - 0.36\,(X_3) + 0.20\,(X_4) \quad (4.3)$$

$$Y_4 = 97.01 + 0.40\,(X_1) - 0.38\,(X_2) - 0.36\,(X_3) + 0.20\,(X_4) \quad (4.4)$$

Where, Y_1, Y_2, Y_3 and Y_4 are Cr(VI), SO_4^{2-}, NO_3^- and PO_4^{3-} removal (%) and X_1, X_2 , X_3 and X_4 are Cr(VI), SO_4^{2-}, NO_3^- and PO_4^{3-} concentration (in mg/L) respectively.

Table 4.8: Student's t test of the model coefficient for Cr(VI) and co-ions removal by *T. pallida* in the multicomponent system: (a) Cr(VI), (b) SO_4^{2-}, (c) NO_3^- and (d) PO_4^{3-}.

(a)						**(b)**					
Term	**Effect**	**Coef**	**SE Coef**	**T**	**P**	**Term**	**Effect**	**Coef**	**SE Coef**	**T**	**P**
Constant		79.38	0.59	132.8	0.0	Constant		82.39	1.18	69.75	0.0
Cr(VI)	-0.92	-0.46	0.59	7.74	0.46	Cr(VI)	0.35	0.17	1.18	0.15	0.88
SO_4^{2-}	4.59	2.29	0.59	3.84	0.00	SO_4^{2-}	1.46	0.73	1.18	0.62	0.55
NO_3^-	3.36	-1.68	0.59	2.81	0.02	NO_3^-	-0.4	-0.2	1.18	-0.17	0.87
PO_4^{3-}	2.6	1.3	0.59	2.17	0.06	PO_4^{3-}	0.76	0.38	1.18	0.32	0.75
(c)						**(d)**					
Term	**Effect**	**Coef**	**SE Coef**	**T**	**P**	**Term**	**Effect**	**Coef**	**SE Coef**	**T**	**P**
Constant		93.08	0.24	378.41	0.0	Constant		97.01	0.16	574.33	0.0
Cr(VI)	0.27	0.14	0.24	0.55	0.6	Cr(VI)	0.8	0.4	0.16	2.37	0.05
SO_4^{2-}	-0.76	-0.38	0.24	-1.54	0.16	SO_4^{2-}	-1.04	-0.38	0.24	-1.54	0.016
NO_3^-	-0.73	-0.36	0.24	-1.48	0.18	NO_3^-	4.69	-0.36	0.24	-1.48	0.18
PO_4^{3-}	0.41	0.20	0.24	0.83	0.43	PO_4^{3-}	-0.59	0.20	0.24	0.83	0.43

Effect of co-ions on Cr(VI) removal by *T. pallida* was significant as revealed by their respective t and P values (Table 4.8). SO_4^{2-} exhibited a highly significant positive effect with a t value of 3.84 and a P value of less than 0.05 (Table 4.8a, Fig. 4.9a). NO_3^- also positively affected Cr(VI) uptake by *T. pallida* with a t value of 2.81 and P value of 0.02. On the other hand, PO_4^{3-} showed no significant effect on Cr uptake (t-value = 2.17 and P-value = 0.06). SO_4^{2-} and NO_3^- removals were rather found to be independent of the presence of the other co-ions as indicated by their respective insignificant P-values (P-value > 0.05). PO_4^{3-} removal was found to be positively affected by Cr(VI) (t- value = 2.37 and P-value = 0.05); however, it was negatively affected due to the presence of SO_4^{2-} (t- value = -1.54 and P-value = 0.016). Overall, it can be inferred that an increase in SO_4^{2-} and NO_3^- concentration affected Cr(VI) removal. On the other hand, the PO_4^{3-} removal was positively affected due to the presence of Cr(VI).

4.3.4. Effect of Multi-ions on *T. pallida* Antioxidant Defence Systems and Biochemical Parameters

In this multicomponent study, effect of Cr(VI) along with the co-ions on *T. pallida* antioxidant defence system and biochemical parameter was examined. Comparison of the effect was studied in presence and in absence of the ions, but in presence of Cr(VI) at 5 mg/L (low initial level) and 20 mg/L (high initial level). Plants with media containing Cr(VI) at low initial level (5 mg/L) or at a high initial level (20 mg/L) in absence of the co-ions, served as control A and control B respectively. Plants taken with media in the absence of both Cr(VI) and co-ions served as control C.

Plant biomass subjected to the experimental conditions 2, 3, 7 and 11(Table 4.5) showed maximum Cr(VI) removal. Therefore, plant biomass taken from these respective

experimental runs, was analysed for the effect of co-ions in the presence of Cr(VI) with respect to biochemical and antioxidant enzymes levels.

Fig. 4.13a shows the catalase activity in *T. pallida* leaves in the different experimental runs at different time intervals. Catalase activity was maximum at a high initial Cr(VI) concentration of 20 mg/L, revealing a maximum stressed condition of the plant due to the elevated level of Cr(VI). High catalase activity was also observed in the exp run nos. 7 and 2. A relatively low activity of the enzyme was observed in the exp run nos. 3 and 11. The least activity was also observed in the control (C) and control (A) i.e. in the absence of co-ions. Lipid peroxidation increased with a high initial Cr(VI) concentration in the absence of co-ions. The value was lower in case of the control (C) plants (Fig. 4.13b). Furthermore, the increased MDA content in presence of 20 mg/L Cr(VI) clearly indicated oxidative stress in *T. pallida*. Fig. 4.14a shows the carbohydrates content in *T. pallida* leaves in the different experimental runs at different time intervals. The total sugars content was high in all the experimental runs and a maximum value was observed at a low initial Cr(VI) concentration. The sugar content was also the least in the plant exposed to only 20 mg/L Cr(VI) (in the absence of co-ions) and in the control (C) plant (Fig. 4.14a). Increase in the sugar content due to the presence of Cr(VI) and co-ions shows that it helps the plant to regulate osmotic stress, thereby preventing damage to its biomolecules and membrane (Najafian et al., 2012). It has been reported that exposure to chromate results in overexpression of genes related to carbohydrate metabolism (glyoxylate metabolism, citrate cycle, pyruvate metabolism, glycolysis, gluconeogenesis) (Couée et al., 2006).

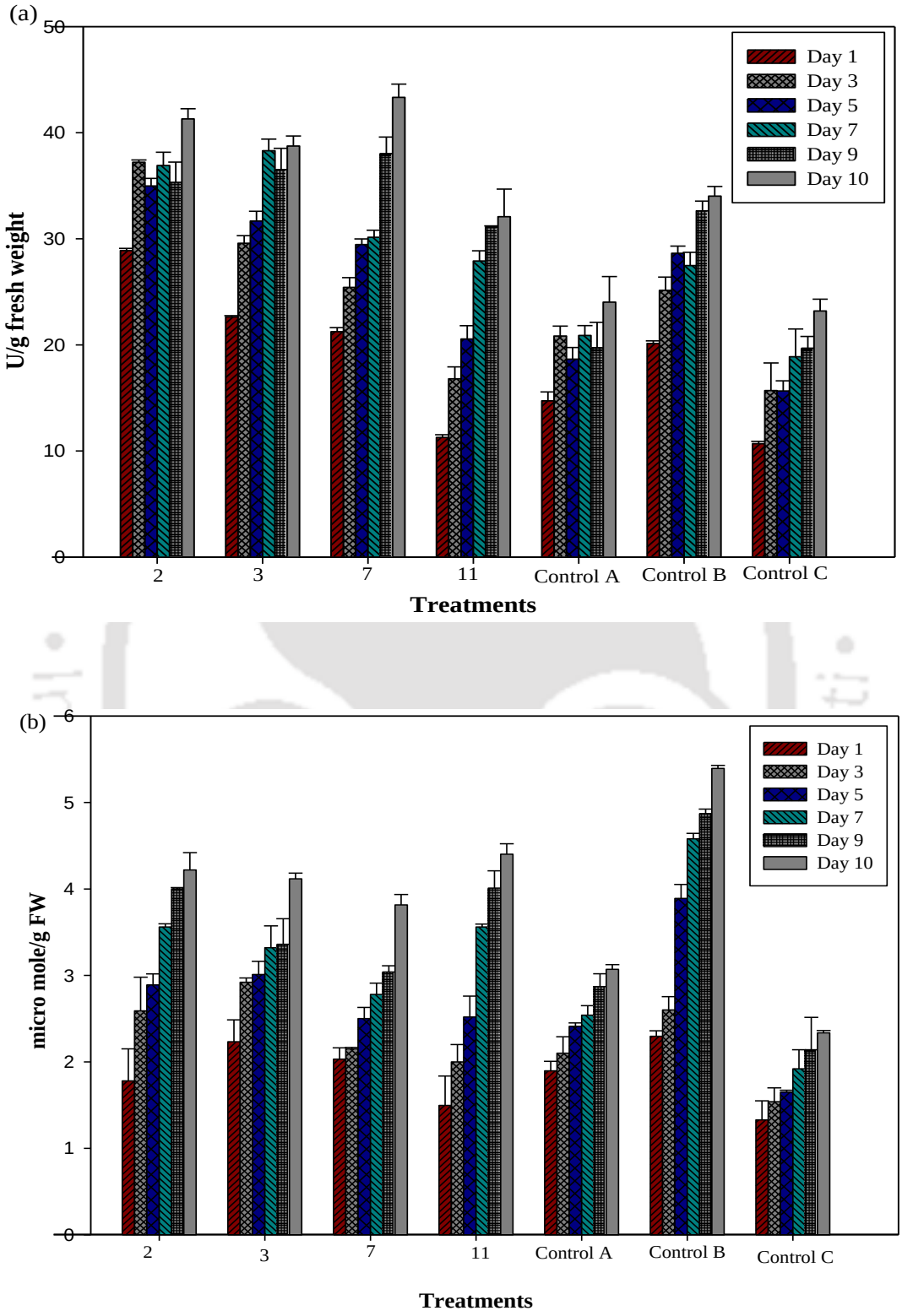


Figure 4.13: Effect of co-ions on enzyme activity of *T. pallida* (a) catalase and (b) MDA

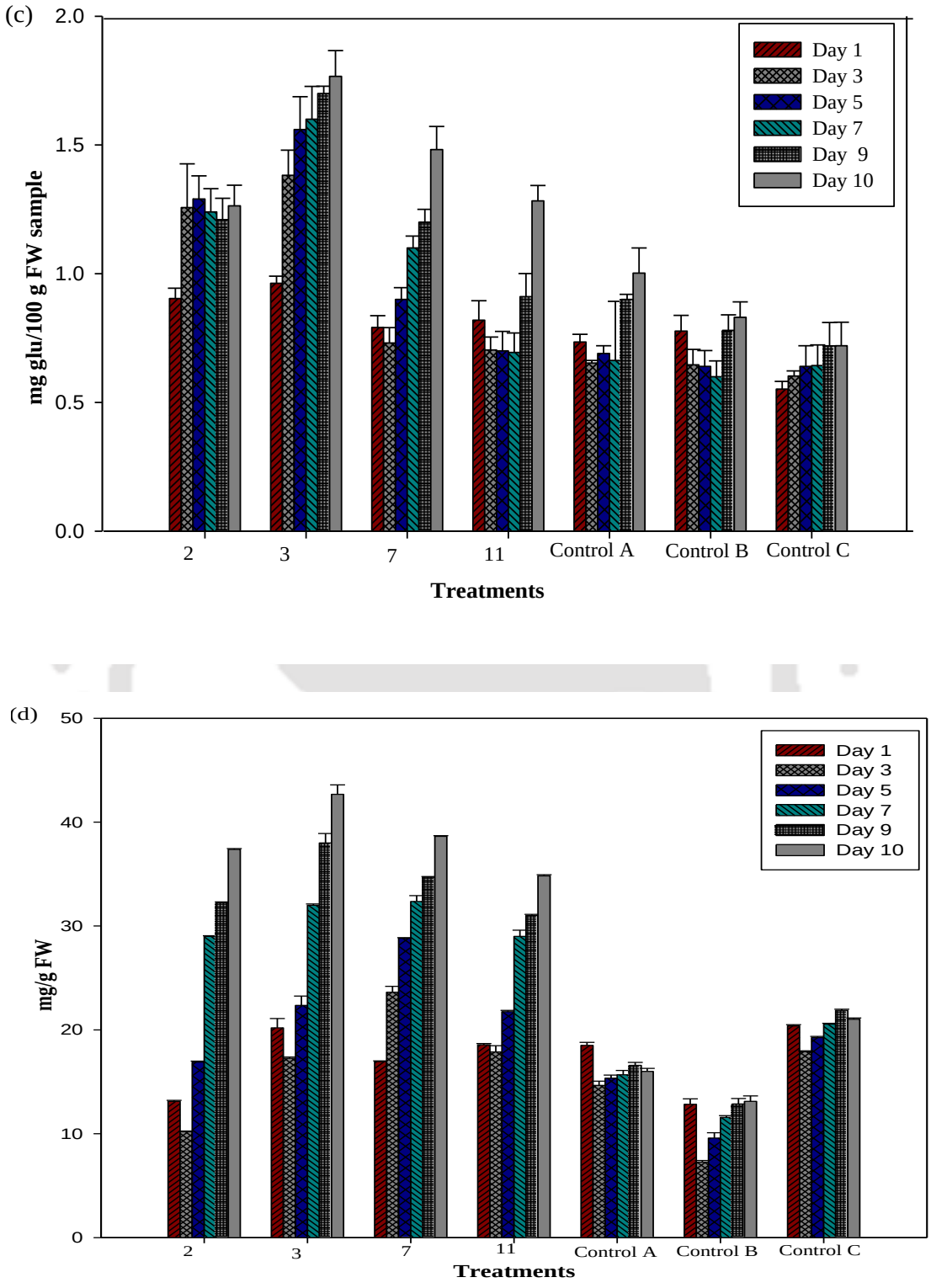


Figure 4.14: Effect of co-ions on biochemical parameters of *T. pallida* (a) carbohydrate and (b) protein content.

Total protein content was low in the first 2-3 days of experiments in all the experimental runs. Following this experimental time period, the protein content significantly increased in exp run nos. 2, 3, 7 and 11. The protein content was also low with control (A) and control (B) plant treated with 5 and 20 mg/L Cr(VI) respectively, in the absence of the co-ions (Fig. 4.14b). Negative correlation between initial Cr(VI) concentration and protein content could be observed in the absence of the co-ions. Whereas in the presence of co-ions, protein content gradually increased after day 3, suggesting that SO_4^{2-}, NO_3^- and PO_4^{3-} helped the plant to overcome the initial stress condition. This could be explained based on the fact that supplementation with NO_3^- helps the plant to synthesize more protein, which is a nitrogenous biomolecule. Similarly, SO_4^{2-} is incorporated with cysteine and other organic molecules, which play a key role in the generation of antioxidants molecules like glutathione and phytochelatin. These compounds get complexed and/ or sequestrated into vacuoles that further help the plant towards Cr tolerance (Brychkova et al., 2012).

Thus, the tolerance results obtained in this multi-component study (in presence of Cr(VI), SO_4^{2-}, NO_3^- and PO_4^{3-}) when compared with single component (in presence of only Cr(VI)) (section 4.2) revealed that the presence of co-ions SO_4^{2-}, NO_3^- and PO_4^{3-} in the tested range not only enhances Cr(VI) uptake but also helps in relieving Cr(VI) toxicity in *T. pallida* plants.

4.4. Continuous Removal of Chromium (VI) from Wastewater in a VSS-CW System

Previous experiments including screening of plants, Cr(VI) accumulation, tolerance and uptake mechanisms were carried out under batch hydroponics systems. These studies gave an insight into the mechanism adopted by *Tradescantia pallida* in response to Cr(VI) exposure. Cr(VI) removal efficiency and tolerance of *T. pallida* in batch studies were further verified in a laboratory scale vertical subsurface flow (VSSF) constructed wetland (CW) system with varying parameters under continuous mode. Long term wetland studies have been proposed as a means to achieve more realistic results that can be successfully used in wastewater treatment sites.

Thus, in this study the performance of *T. pallida* was evaluated to continuously remove Cr(VI) from contaminated water using a laboratory scale VSSF-CW system at different hydraulic retention time (HRT) and influent pH. Effect of metal loading rate and influent pH was studied on Cr(VI) removal in the CW units. Further, Cr(VI) concentration in CWs microcosms comprising soil, sand and *T. pallida* plants was analysed to understand the mechanism and distribution of Cr removal in the *T. pallida* based CW unit.

4.4.1. Effect of HRT on Chromium (VI) Removal

Figure 4.15 presents influent and effluent concentrations of Cr(VI) for control and planted CW units operated at three different HRTs and at an influent concentration of 20mg/L Cr(VI). During the initial phase of the experiments, the units were operated continuously at three-day HRT. Thereafter, the HRT was reduced to two-day and then to one-day. At three-day HRT, the mean effluent value for Cr(VI) in the planted and control CW units were found to be 0.27 and 0.34 mg/L, respectively, which are well below the

recommended Cr discharge limit of 0.5 mg/L. At two-day HRT, the mean Cr(VI) effluent value was found to be 0.337 mg/L in the planted unit, but in case of the control unit, it was high at 1.23 mg/L. The Cr(VI) removal efficiency further decreased at one-day HRT for both the units with a mean effluent concentrations of 2.96 mg/L and 4.29 mg/L, in the planted and control CW units, respectively.

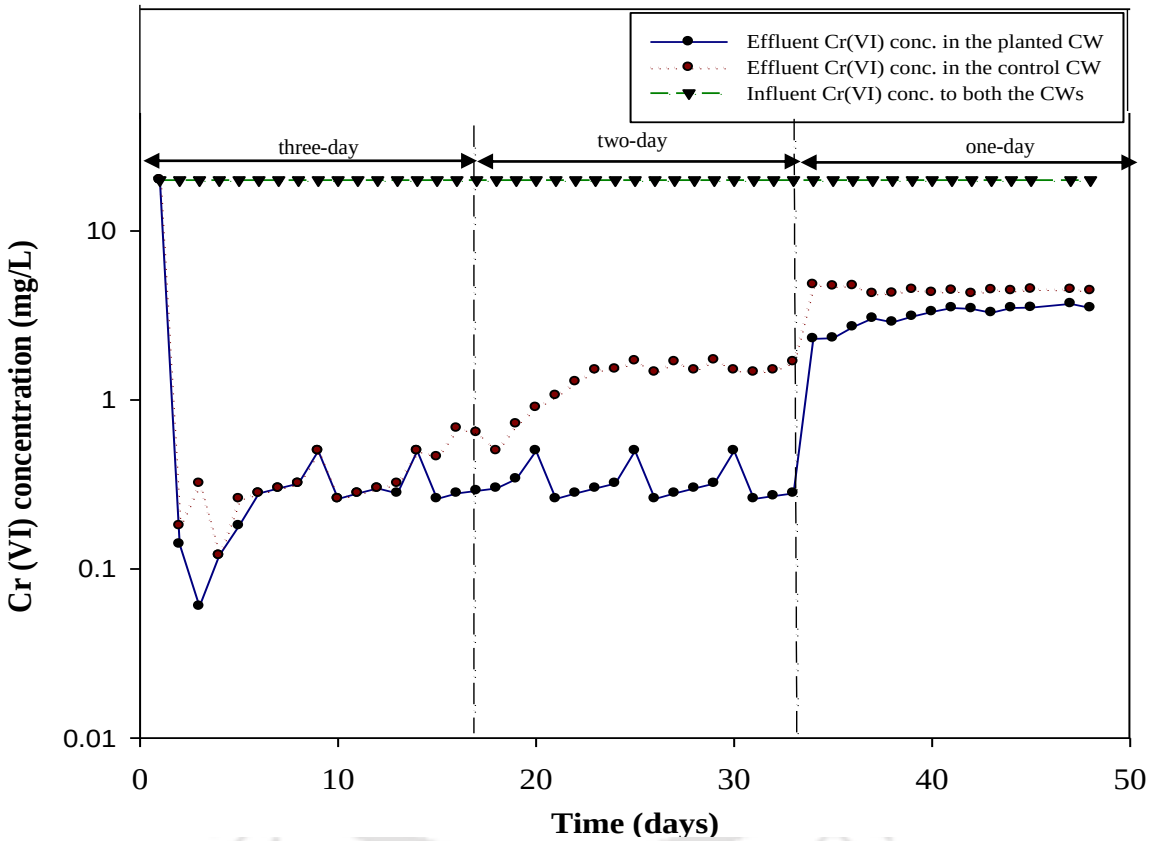


Figure 4.15: Effect of different HRT on the Cr(VI) removal by *T. pallida* in the control and planted CW units.

Thus, the planted CW unit showed a 3 fold increase in Cr(VI) removal as compared to the control unit. These results also revealed that Cr(VI) removal efficiencies were very high and stable for two-day and three-day HRTs. Maximum 97.2-98.3% Cr(VI) removal was achieved in the planted unit for two and three-day HRT. A very low Cr(VI) removal in the range 73.24-77.9% was observed in the control unit for one-day HRT.

As reported by many authors, plants are known to reduce Cr(VI) to Cr(III), which is a thermodynamically stable form of Cr in soil (Duarte et al., 2012; Rascio and Navari-Izzo, 2011). In a recent study, more than 90% of Cr(VI) was found to be accumulated in Cr(III) state in substrates and plants in CWs planted with *Leersia hexandra* Swartz (Liu et al., 2014). This Cr(VI) reduction is attributed to the plant roots which enhance aerobic condition in the soil, produces organic matter (El Zahar et al., 2014; DalCorso et al., 2013) and secretes root exudates containing carboxylic acids, amino acids, carbohydrates and proteins necessary for Cr(VI) reduction and sequestration in the soil (Ud Din et al., 2015). In addition to these mechanisms, precipitation of the less soluble $Cr(OH)_3$ occurs, thereby aiding in its retention within the soil. Thus, in the control CW without the *T. pallida* plant biomass, adsorption, reduction and precipitation seem to be the major mechanisms for Cr removal from the influent. Whereas, in case of *T. pallida* plant based CW unit, not only these three processes, but also bioaccumulation of Cr by *T. pallida* played a major role in continuous Cr removal from wastewater.

A direct comparision of results obtained in this study with those reported in the literature is difficult as the wetland operating conditions particularly HRT, influent Cr(VI) concentration followed in the literature are different compared with the same followed in the present study. In this study, 97.2-98.3% Cr(VI) and 86-88.2% total Cr removal efficiency was achieved for an influent concentration of 20 mg/L and two-HRT which is significant and comparable with Cr removal efficiency reported in the literature (Fig. 4.16). For instance, CWs planted with *Leersia hexandra* Swartz showed a maximum removal efficiency of 99% for a low influent Cr(VI) concentration of 2.5 mg/L (Liu et al., 2014). In another study, 72% Cr removal was achieved with *Phragmites australis* planted CW, in which the bed consisted of gravel of different sizes (Fibbi et al., 2012).

Fig. 4.16 further reveals that Cr removal increased with an increase in the HRT. It is evident from the results that even at a low HRT of two days, Cr removal from 20 mg/L influent Cr(VI) concentration, was always below the permissible discharge limit for total Cr and Cr(VI) (0.05 mg/L) (Förstner et al., 1979). Further, at two-day HRT, least variation in Cr removal was observed in the planted CW as compared with that in the control CW. Hence, it could be well said that the *T. pallida* plant based CW system is highly efficient for treating Cr(VI) containing wastewaters even at a low HRT.

4.4.2. Effect of Metal Loading Rate on Chromium (VI) Removal

Figure 4.16 shows the effect of Cr(VI) loading rate on Cr(VI) removal in the control and planted CW units. For a better understanding of the efficiency of the *T. pallida* based CW system in removing Cr(VI) from synthetic wastewater, the estimated values of Cr(VI) removal rate and Cr(VI) removal efficiency was re-plotted versus Cr(VI) loading rate.

Almost 98% of Cr(VI) removal efficiency is observed for both 6.67 and 10 mg/L/day Cr(VI) loading rate. A further increase in the Cr(VI) loading rate (20 mg/L/day) greatly reduced the Cr(VI) removal efficiency to 88% in case of the planted CW and to 77.8% in case of the control system. On the other hand, the Cr(VI) removal rate steadily increased with an increase in the Cr(VI) loading rate. In case of planted CW, a maximum Cr(VI) removal rate of 16.5 mg/L/day was observed for a maximum Cr(VI) loading rate of 20 mg/L/day. For the same Cr(VI) loading rate, the control CW showed a removal rate of only 15.5 mg/L/day.

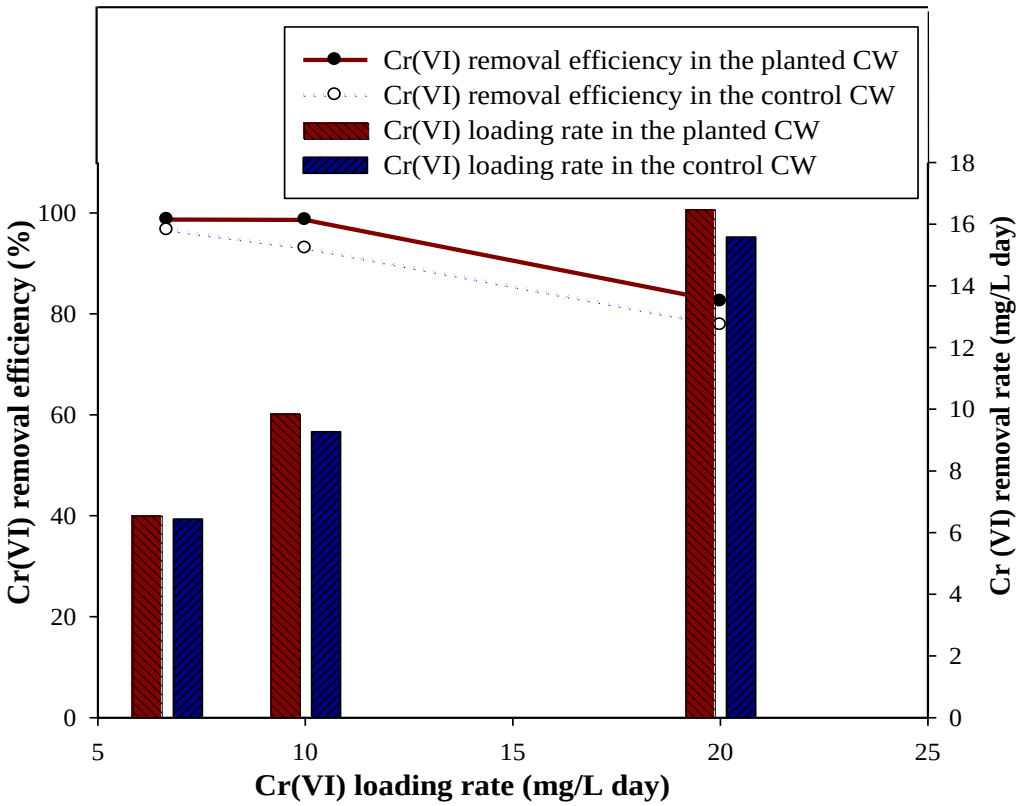


Figure 4.16: Effect of Cr(VI) loading rate on Cr(VI) removal in the control and planted CW units

Thus, in the planted system, Cr(VI) removal rate was higher than the control system when operated at same Cr(VI) loading rate. The significant Cr(VI) removal efficiency and rate of Cr(VI) removal achieved in the *T. pallida* planted CW unit shows the potential of this system in treating Cr(VI) laden wastewaters.

4.4.3. Effect of pH on Chromium (VI) and Total Chromium Removal

Among the different parameters, besides Cr(VI) concentration and HRT, wastewater pH is known to significantly affect Cr(VI) removal by plants due to its effect on Cr chemistry and speciation. Figure 4.17 shows the result of Cr(VI) and total Cr removal for two-day HRT in the CW units operated at pH 4 and 7, respectively. During the first 45 days of the experiments both the units were operated at an influent concentration of 20 mg/L each.

Thereafter, the influent concentration was increased to 30 mg/L and operated continuously for the next seven days.

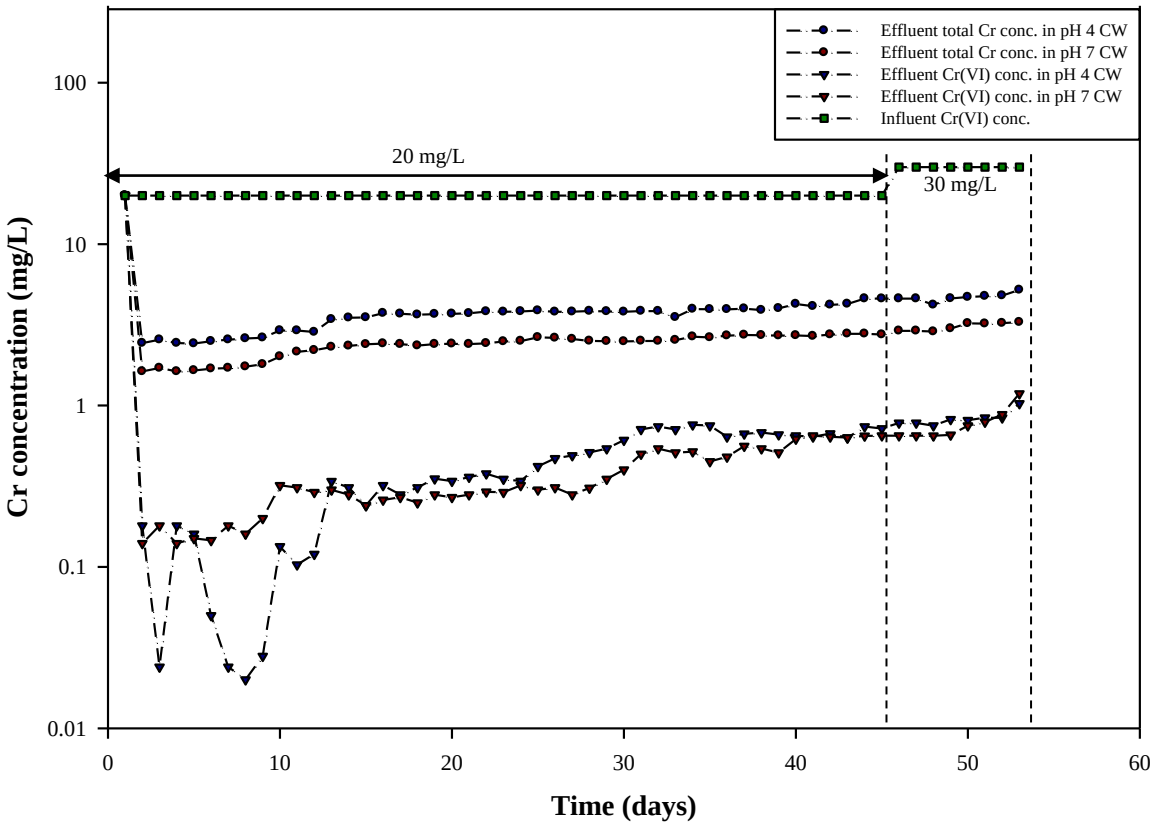


Figure 4.17: Effect of influent pH on Cr(VI) and total Cr removal by *T. pallida* in the respective CW units.

For an influent pH of 4 and 7, the mean effluent Cr(VI) value was found to be 0.441 mg/L and 0.379 mg/L, respectively, at an influent concentration of 20 mg/L. For the same influent concentration, mean effluent value of total Cr was found to be 3.59 mg/L and 2.352 mg/L at pH 4 and 7, respectively. At an influent concentration of 30 mg/L, the mean effluent Cr(VI) value was found to be 0.907 mg/L and 0.896 mg/L at pH 4 and 7, respectively. For the same influent concentration, the mean effluent value of total Cr was 4.86 mg/L and 3.173 mg/L at pH 4 and 7, respectively. These results reveal that the maximum Cr(VI) removal efficiency is observed in the planted CW operated at an

influent pH of 7, in which case maximum 97.2-98.3% Cr(VI) and 86.0-88.2% total Cr removal was achieved.

To determine the Cr(VI) reduction rate under pH 4 and pH 7 of the influent, amount of Cr(VI) and Cr(III) was determined at regular intervals. Figure 4.18 shows percentage amount of Cr species vs. treatment time in the respective CWs.

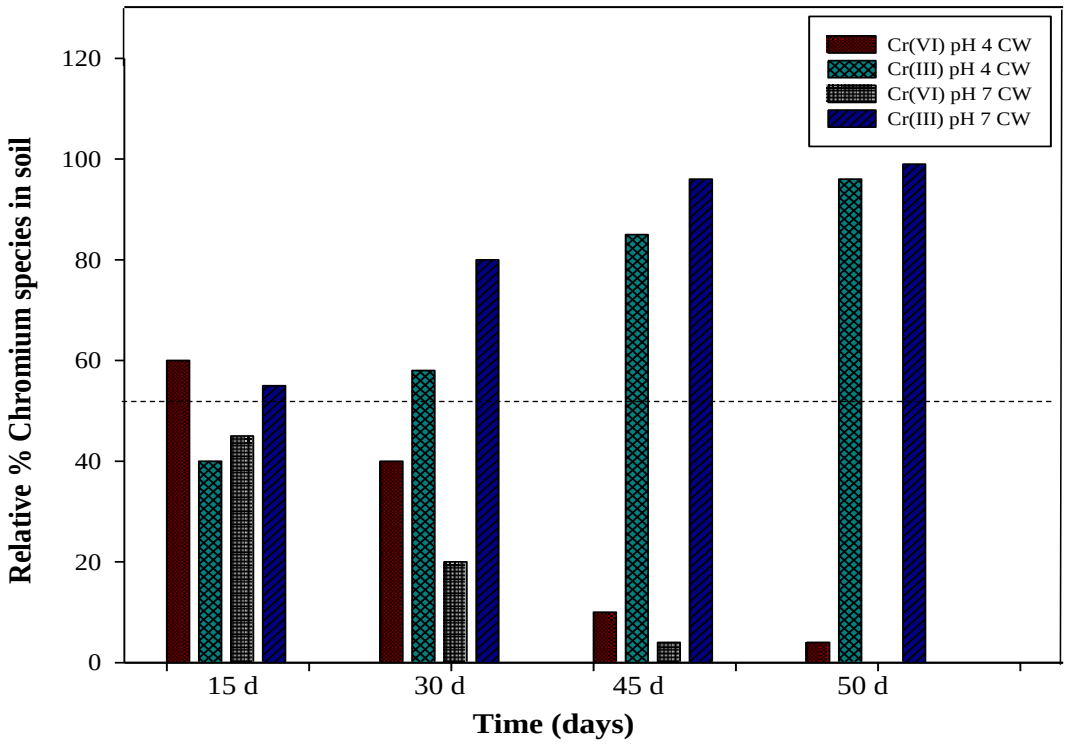


Figure 4.18: Effect of influent pH on Cr(VI) reduction rate in the respective CW units

These results show that initial pH of the influent had important affect the Cr(VI) reduction rate as the time required to achieve 50% reduction of Cr(VI) at pH 7 was very less as compared to that shown by the pH 4 CW unit. After the first 30 day of the experimental period, percentage of Cr(III) in total Cr was significantly higher for the pH 7 CW (81.24%) as compared to that in the pH 4 CW (58.7%). Hence, Cr(VI) reduction rate is found to be faster in the pH 7 CW as compared to pH 4 CW.

After 50 days of treatment time, complete Cr(VI) reduction occurred in both pH 7 and pH 4 CWs.

Both, pH 4 and pH 7 CW units, showed significantly high removal of Cr(VI) from the influent. Whereas, total Cr removed was high in case of pH 7 CW unit, indicating an increased Cr(VI) reduction efficiency and retention capacity than the pH 4 CW unit. This is mainly because of the fact that at neutral pH value (pH 7), Cr(III) is immobilized more effectively in the soil matrix as insoluble complexes than at an acidic pH (pH 4). Cr(III) is reported to form insoluble $Cr(OH)_3$ in the pH range 6-9 (*K*sp, 6.7×10^{-31}) that remains tightly adsorbed to the soil. Further, Cr(III) remains relatively insoluble at the pH values (pH 6.95–7.59) and, hence, gets retained in the soil (Barrera-Díaz et al., 2012).

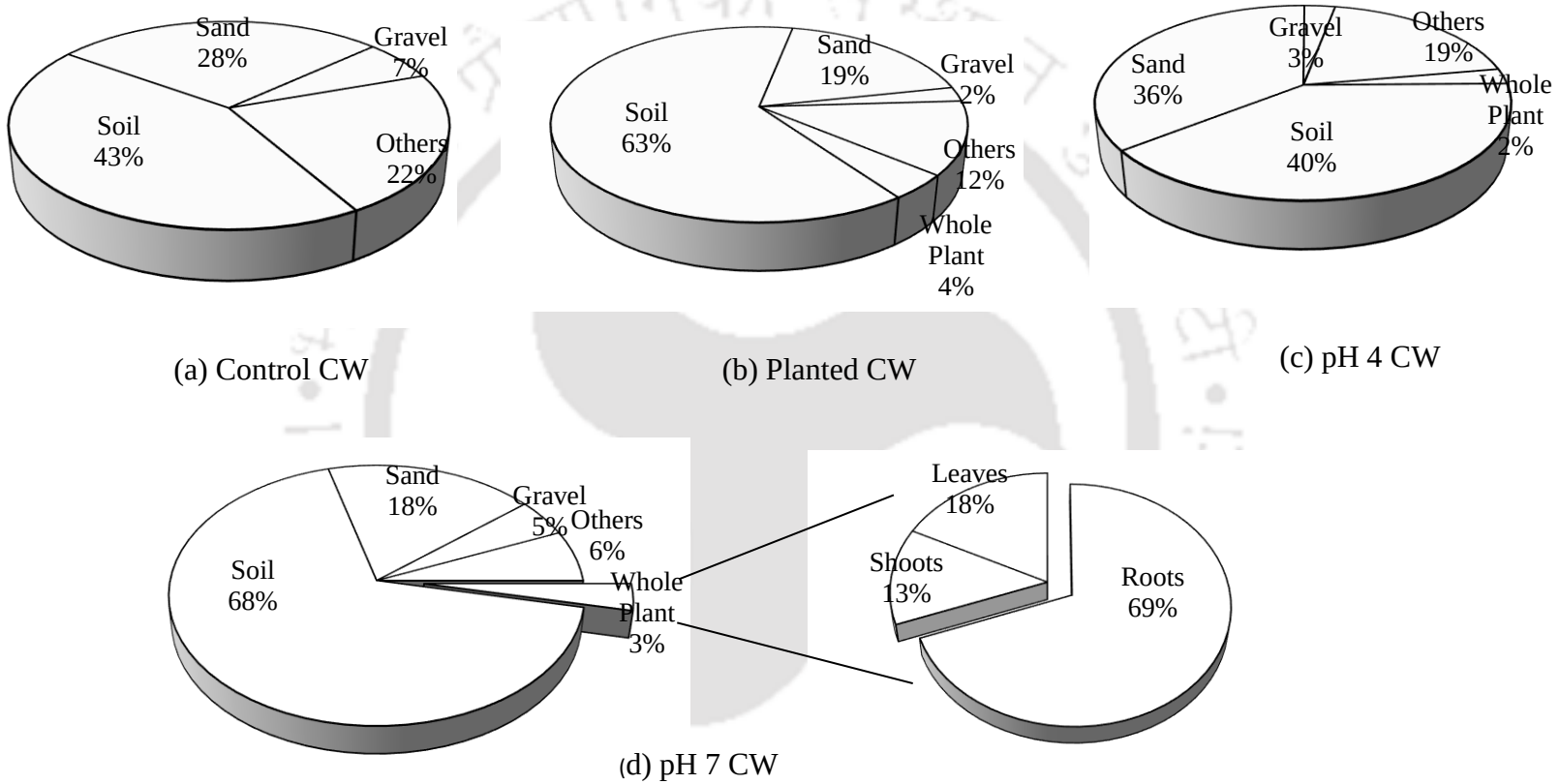
In the pH 4 CW unit, the primary Cr(VI) removal mechanism is attributed to the adsorption of Cr(VI) ions. It is already reported that Cr(VI) adsorption increases at a faster rate as pH decreases. At low pH values, the hydronium ions surround the surface of soil matrix that strongly bind with the anionic Cr(VI) species (CrO_4^{2-}, $Cr_2O_7^{2-}$). Under acidic soil conditions, Cr(VI) reduction to Cr(III) is reported to occur in lesser proportion as compared with that under neutral or alkaline soil conditions (Gupta and Bhattacharyya., 2011). Further, it is reported that low pH soil result in higher K_d values for Cr (Cainelli and Cardillo, 2012). Hence, at acidic pH, Cr(VI) along with other metals such as Si, Al undergo leaching from soil and gets deposited in the sand forming a thick orange colored complex, as also observed in this study using the pH 4 CW unit.

Overall, these studies reveal that the reduction and retention of Cr in the planted CWs were strongly pH dependent, i.e. an acidic pH value of the influent favoured Cr(VI) adsorption and neutral pH of the influent enhanced both reduction and total Cr removal in the respective CWs.

4.4.4. Chromium Accumulation and Distribution in the CW

During the experiments carried out to study the effect of different HRT on Cr(VI) removal by the CW units (planted/Control), the total Cr(VI) mass loading into the system is estimated to be 962.49 mg each. Fig. 4.19 presents the distribution of total Cr in each fraction shown as a percentage of total Cr loaded into the CWs over the entire experimental period.

In the present investigation, a different distribution pattern of Cr was observed in the planted CW as compared with that in the control CW (Fig. 4.19; Table 4.9). These results revealed that the soil in these CW acts as the main sink for Cr, sequestering up to 40-68.41% of the total Cr supplied to the system. On the basis of the plant biomass produced and the bed constituent weight, the amount of total Cr accumulated in the whole plant (dw basis), soil, sand and gravel in the planted CW is estimated to be 55.68, 552.575, 242.73 and 46.7 mg, respectively. Whereas in the control CW, the Cr accumulated in the soil, sand and gravel is estimated to be 471, 194 and 48.23 mg, respectively. This can be explained due to the fact that plants not only help in Cr uptake but also play a major role in its reduction and retention within the soil on which they grow (Prado et al., 2010).



The mass of Cr in each fraction is presented as percentage of the total amount of Cr loaded into the wetland over the experimental period.

Figure 4.19: Distribution of total Cr in the wetland systems (a) Control CW (b) Planted CW (c) pH 4 CW (d) pH 7 CW.

Table 4.9: Cr distribution in each of the CW units at the end of the study.

	Control CW	Planted CW	pH 4 CW	pH 7 CW
Whole Plant (mg/kg dw)	-	3%	2%	4%
Soil (mg/kg)	43%	68%	40%	63%
Sand (mg/kg)	28%	18%	36%	19%
Gravel (mg/kg)	7%	5%	3%	2%
Unaccountable(others)	22%	6%	19%	12%

During the experiments to study the effect of influent pH on Cr(VI) removal by the CW units (pH 4/pH 7), Cr(VI) mass loading in the system was 1785 mg. For the pH 4 CW unit, amount of total Cr accumulated by the whole plant (dw basis), soil, sand and gravel is estimated to be 35.69, 408.57, 292.73 and 46.7 mg respectively. Whereas in the case of pH 7 CW unit, Cr accumulated by the plant, soil, sand and gravel is found to be 61.32, 618, 264 and 45.23 mg, respectively. All these results clearly reveal that the *T. pallida* plant biomass played a highly significant role in Cr retention capacity in the soil fraction (62.7–68.41 % of the total Cr inflow). In the same unit, a high Cr accumulation was observed in the plant roots (754 µg Cr/g dry wt.) compared to that in the shoots and leaves (318 and 321 µg Cr/g dry wt.) of the plant. Although Cr accumulation by the plant is less (2–4%), Cr removal in terms of µg removed per g of biomass is very high, i.e., 464 µg/g in comparision with the Cr uptake values reported in the literature. In this study, results of Cr distribution in the constructed wetland revealed a high retention of Cr in the soil as compared with that in the control unit without any plants. Thus, Cr mainly got retained in the bed of the wetland media (soil and sand). Soil bed was the most important fraction that acted as filter media in CWs followed by sand. Theses bed fractions provided

surfaces to allow direct sorption of Cr ions under different soil pH conditions. Gravel was found to have a least Cr removal capability among the bed fractions, as also reported in by Singhakant et al. (2009). The results obtained are consistant with the other similar studies which confirm that the major metal removal mechanism in such CWs was adsorption and thus trapping of pollutants within the CW bed (Kosolapov et al., 2004, Sheoran and Sheoran, 2006).

4.4.5. Effect of Continuous Chromium (VI) Removal by *T. pallida* on its Growth

Table 4.10 shows *T. pallida* growth parameters in the CW operated at different conditions. To analyse the effect of influent pH on the plant growth and Cr uptake, *T. pallida* weight, height, chlorophyll content and bioremoval paremeters were determined at the end of the experimental period.

Table 4.10: Growth and bioremoval parameters of *T. pallida* plants from the different CW units at the end of the study.

CW	Fresh. Wt (g)	Dry. Wt (g)	RWC (%)	Height (cms)	Total chl (mg/g)	BCF	TFs/r	TFr/soil
Control CW	24.6±3.25	8.2±0.91	65.83	35±3.5	1.532	-	-	-
Planted CW	22.8±2.74	6.1±0.89	72.21	34±4.30	1.484	20.92	0.425	1.3
pH 4 CW	23.0±2.83	6±0.75	73.9	32±4.8	1.481	16.45	0.377	0.93
pH 7 CW	22.6±2.30	5.1±0.67	77.44	33±6.3	1.4909	23.21	0.90	2.504

Results presented are average of three samples analysed with standard deviation±SD (n=3)

*RWC = relative water content, BCF = bioconcentration factor, TFs/r = translocation factor from root to shoot, TFr/soil = translocation factor from soil to root.

As control for this study, a planted CW unit that received only tap water (pH 5.6) and no input Cr(VI) was used as the influent. The values of BCF in the different CW units

ranged from 16.45 to 23.21. Maximum Cr(VI) uptake was observed with the unit supplied with Cr(VI) containing influent at pH 7 and BCF value was 23.21. In plants, Cr uptake is directly related to Cr availability and mobility that depends on the soil pH conditions. Maximum BCF $_{soil/root}$ value was found at pH 7 as, Cr(VI) remains in soluble form at near neutral pH values and, thus, it is more bioavailable than at low pH values. The fact that accumulation was maximum in *T. pallida* roots could be attributed to this adsorption from the soil. In pH 7 CW, Cr(VI) reduction increased with time, also suggesting the role of the plant roots in the CW (Zazo et al., 2008). Whereas in the pH 4 CW, adsorption and leaching of Cr occur at a fast rate due to which Cr remains less available for plants and its microcosm to act upon (Banks et al., 2006). TFs/r ranged from 0.37 to 0.90 indicating that maximum Cr accumulated by *T. pallida* was mostly retained in the plant roots.

The Relative Water Content (RWC) of plants was determined and found to be significantly high in both the CW units receiving influent at pH 7 (77.44%) and pH 4 (73.9) as compared with the RWC of the plants in the control CW (65.54). Total chlorophyll amount in the plants from the different CW units revealed that pH had no significant effect on this parameter. Furthermore, no toxicity in terms of growth rate and chlorophyll content was observed in the plants grown in the different CW units, suggesting a high tolerance of *T. pallida* for the phytoremediation of Cr(VI) containing wastewater over a wide pH range.

This continuous Cr(VI) removal study demonstrated that wastewater containing Cr(VI) can be efficiently treated using *T. pallida* plants based subsurface CWs under continuous operation mode. The planted CW unit showed better removal in terms of both Cr(VI) and total Cr removal as compared with the control CW unit. Further, this investigation showed the importance of pH of the influent which controls the Cr removal mechanism in the CW. These results clearly revealed that *T. pallida* plants can effectively enhance the

Cr(VI) reduction and retention in the soil, thereby serving as an efficient system for treating Cr contaminated water. The high Cr removal efficiency combined with the extremely low construction and operation cost of *T. pallida* based CW suggest that it could be an attractive treatment system for Cr(VI) removal from wastewater.

4.5. Feasibility Study on the Disposal of *T. pallida* Plant Biomass Following Chromium Accumulation in Constructed Wetland

One of the major concerns for commercial implementation of the phytoextraction has been the disposal of the hazardous plant biomass. After successful phytoextraction of Cr using a constructed wetland, it is important to find an economical and feasible method for the reuse or disposal of Cr accumulated plant biomass. Thus, after Cr accumulation, large quantities of Cr loaded plant wastes are generated that needs to be stored or disposed appropriately so that it does not pose any risk to the environment. Recently, a sufficient number of studies have been carried out by different researchers for the safe disposal of metal contaminated plants. Several methods of disposal of contaminated plant biomass are described such as pyrolysis (Sas-Nowosielska et al., 2004) that reduces the crop volume. Furthermore, volume reduction processes, such as composting and compaction of Cr accumulated biomass have been proposed as a post harvest biomass treatment (Shukla et al., 2009). But the end-product of these processes is still a hazardous waste. Other proposed methods are liquid extraction, incineration and ashing of harvested biomass, but the cost of these operations is prohibitely high (Ghosh et al., 2005). Although several methods are proposed, a suitable technology for metal recovery and recycling has yet to be demonstrated (Mulligan et al., 2001). Thus more research is needed in this direction in order to make phytoextraction a commercially applicable and an environmentally sound process.

An efficient solution to this problem could be reuse of the plant biomass as biosorbent for removal of the same metal from constituent wastewater. Hence, Cr(VI) loaded *T. pallida* plant parts were investigated for its reuse as a biosorbent for the removal of Cr(VI) ions under batch mode. Reuse of such waste plant biomass would reduce the costs for waste disposal and at the same time serve as a valuable resource.

4.5.1. Characterisation of *T. pallida* Biomass for Chromium Biosorption

Structural characterisation of the *T. pallida* was done by using Fourier transform infra red (FTIR) and Scanning Electron Microscopy (SEM). FTIR relies on the fact that most molecules in a given sample absorb light in the infra-red region (0.8–25μm) of the electromagnetic spectrum; this absorption corresponds specifically to the bonds present in the molecule (Table 4.11). Figure 4.20 shows the FT-IR spectra of *T. pallida* leaf biomass before (control) and after sorption (Cr(VI)-loaded).

Table 4.11: IR absorption bands and corresponding possible functional groups.

Frequency (cm^{-1})	**Functional group**	***T. pallida* leaf biomass (control)**	**Cr(VI) loaded *T. pallida* leaf biomass**
3300-3450	-OH, -NH stretching	3372	3415
2910-2920	-CH stretching	2918,2849	2917,2649
1730-1740	C ═O stretching	1735	1736
1600-1640	-COO, C ═O stretching	1598	1639
1300-1450	Amide or sulfamide bond	1393	1320
1270	-C –O stretching	1153	1159
1030-1070	S=O stretching	1035,666	1035,614

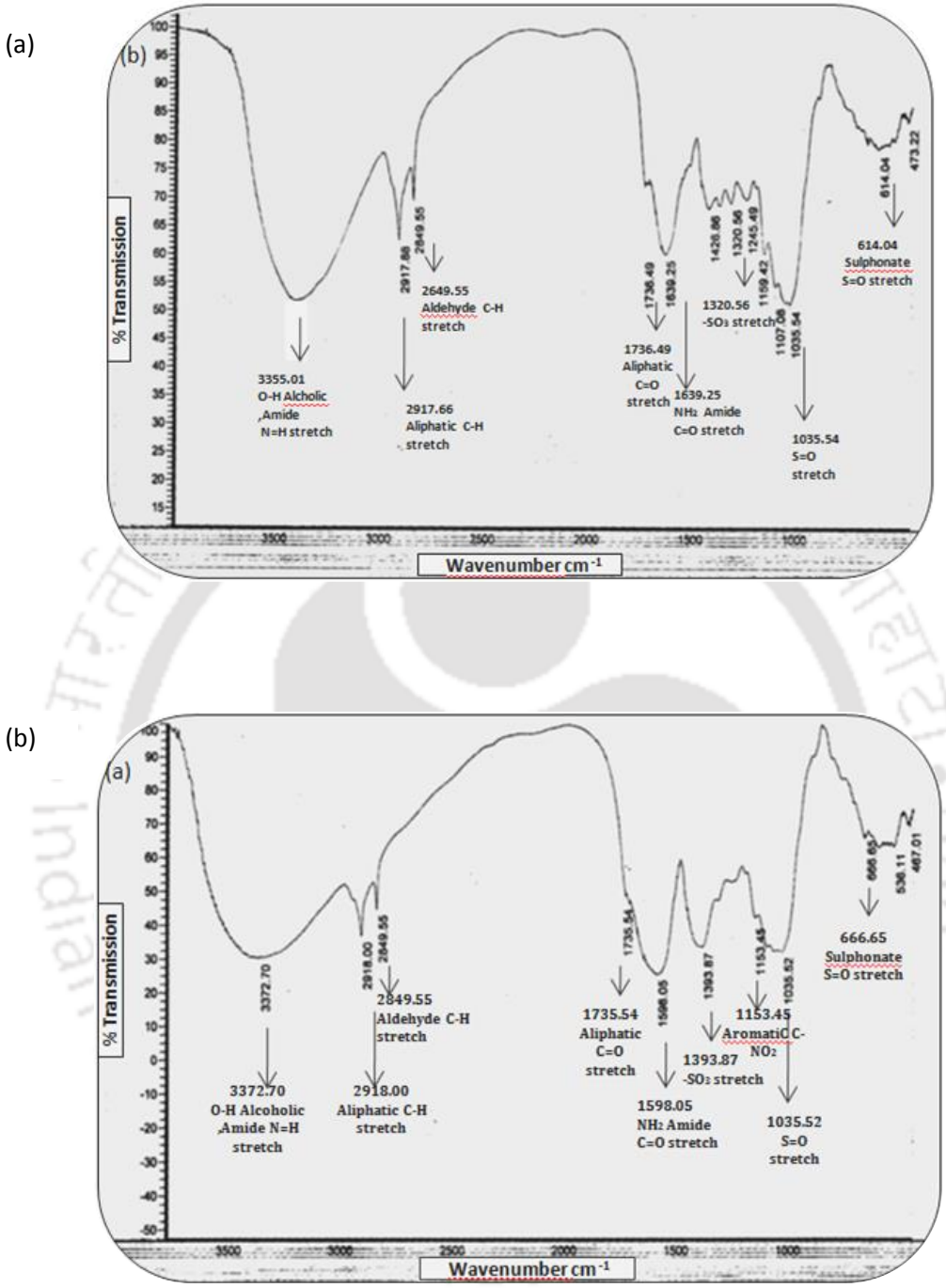


Figure 4.20: FTIR spectra of Cr(VI) exposed *T.pallida* leaf biomass : (a) control and (b) Cr(VI)-loaded

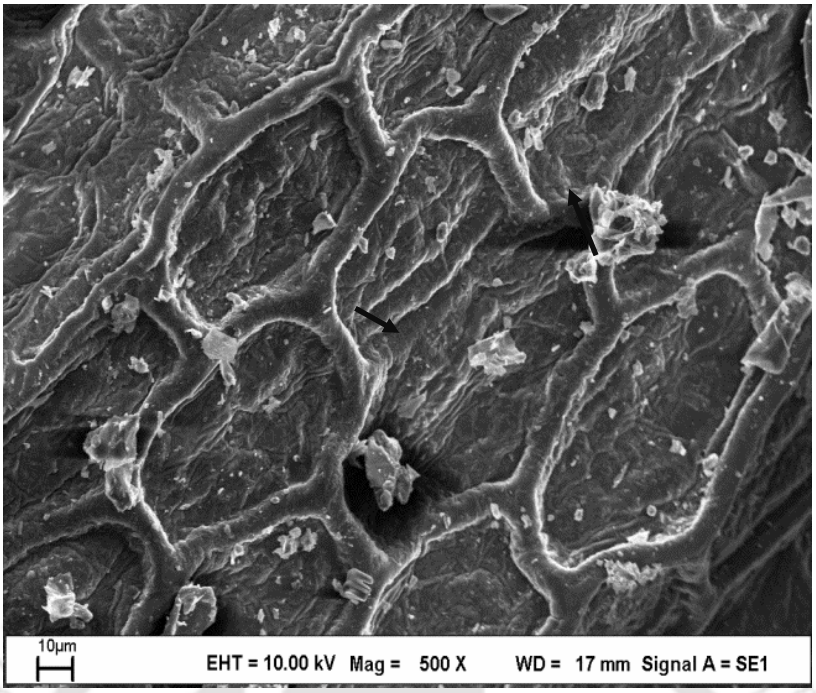
It revealed the vibrational frequency changes in the functional groups in the leaf biomass, which showed better Cr(VI) removal than the other plant parts. Cr(VI) sorption capacity

of the *T. pallida* depended upon its porosity and chemical reactivity of the functional groups present. In the spectra obtained using the Cr(VI)-loaded biomass (Fig. 4.20b), a band shift to 3355 cm^{-1} from 3372 cm^{-1} along with a substantial decrease in its intensity represented the –OH groups of cellulose and $–NH_2$ asymmetric stretching due to amines of proteins present in the biomass (Shroff and Vaidya, 2012). This broad shift indicates overlapped stretch of hydroxyl and amines groups on the leaf surface. These functional groups are mainly part of the protein and sugar content of leaf biomass, which strongly interacted with Cr(VI) ions for binding (Poorter and Jong, 1999). The major peak shift from 2849 cm^{-1} to 2649 cm^{-1} observed in the Cr(VI)-loaded biomass can be attributed to stretching vibration of the C–H group (Barbu et al., 2010). The band ranging from 1500 cm^{-1} to 1650 cm^{-1} represents amide I and amide II groups of peptide and ammonium groups of carboxylic acid compounds (Li et al., 2011). A clear shift in the band position to 1320 cm^{-1} from 1393 cm^{-1} represents $–SO_3$ stretching, which indicates binding of Cr(VI) mostly with $–SO_3$ groups in the leaf biomass (Murphy et al., 2009). The additional peak shift at 666 cm^{-1} can be assigned to the bending due to aromatic compounds present in the biosorbent (Bansal et al., 2009). These results thus indicated that –NH, amide, hydroxyl and sulphonate groups were mainly involved in Cr(VI) binding to the *T.pallida* leaf biomass.

FTIR analysis results of the Cr(VI) loaded leaf biomass revealed the involvement of –NH, amide, hydroxyl and sulphonate as the major functional groups for Cr binding by the plant biomass. This can be attributed mainly to the plant leaves which posses photosynthetic function with a rich content of proteins, lipids, nitrogen, organic acids and sugars (non-structural carbohydrates as well as structural carbohydrates) than woody stem tissues (Poorter and Jong, 1999). These components thus provide a large number of functional groups which could bind with Cr(VI).

Surface morphology of the biosorbent was studied using a scanning electron microscope. Figure 4.21 shows the scanning electron micrographs of Cr(VI) biosorbed *T. pallida* leaf biomass which reveals irregular and porous surface along with the complexed Cr(VI) ions. These SEM images provided direct evidence of Cr(VI) binding to the pores of the leaf biomass. Based on the morphology, as well as on the fact that high amounts of Cr are concentrated on the external epidermis of *T. pallida* leaves, it can be concluded that the plant leaf presents an adequate morphological profile to retain Cr(VI) ions.

(a)



(b)

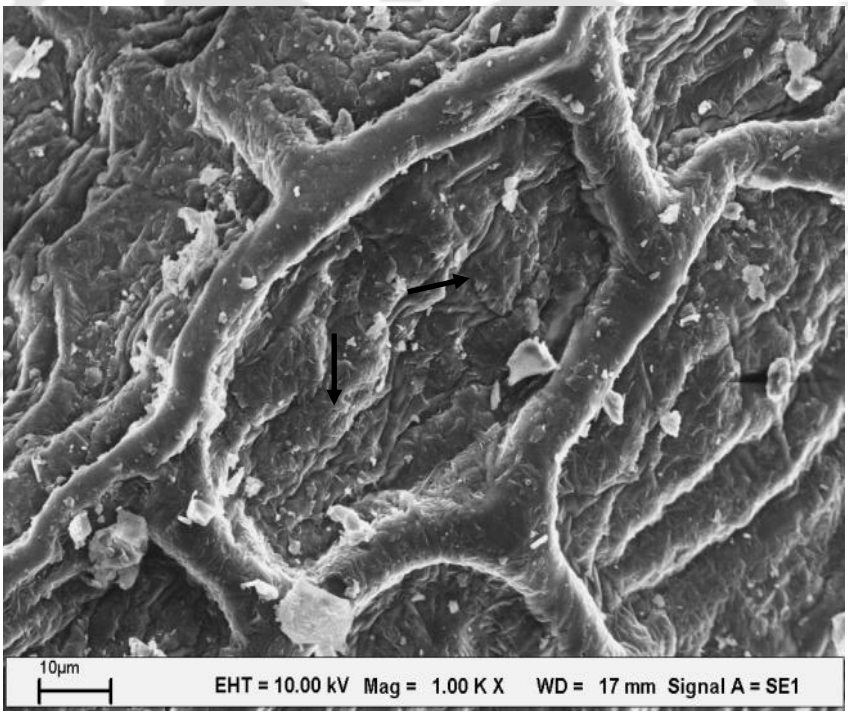


Figure 4.21: SEM images of Cr(VI) biosorbed *T.pallida* leaf biomass showing multiple well developed pores (a) 500x magnification and (b) 1000X magnification (Arrows inside these two figures indicate sorbed Cr(VI) species).

4.5.2. Effect of Process Parameters on Chromium (VI) Biosorption

This study was carried out to examine Cr(VI) sorption capacity of the Cr accumulated *T. pallida* plant and its efficiency was compared with that of the Cr(VI) unexposed plants. Comparison was also made among the different plant parts (roots, shoots and leaves) in order to define the metal binding potential of each.

Figure 4.22 and Figure 4.23 show the effect of solution pH, contact time, temperature and sorbent dose on Cr(VI) sorption by Cr(VI) exposed/unexposed *T. pallida* plant parts. The optimum pH for the Cr(VI) biosorption is thus found to be 2, and was fixed to analyse the effect of the other parameters on Cr(VI) biosorption (Fig. 4.22a and Fig. 4.23a). This is mainly because not only the metal speciation depends on solution pH, but also the dissociation of active functional sites on the biosorbent (Yu et al., 2003). $HCrO^{4-}$ and $Cr_2O_7^{2-}$ are the predominant species in the pH range 0 - 6.5 and CrO_4^{2-} ions dominates at a pH above 6.5 (Levankumar et al., 2009; Blázquez et al., 2009). With a decrease in the solution pH, functional groups such as carboxyl (-COOH) and amino ($-NH_2$) gets protonated. Due to the positively charged biomass surface at a low solution pH, its coordination with the anionic Cr(VI) species is highly enhanced. Thus, a lower pH in the present study favoured Cr(VI) biosorption by the plant biomass than did the higher pH (Moussavi and Barikbin, 2010). Among the different process parameters examined for their effect on Cr(VI) biosorption by *T. pallida* leaf biomass (Cr(VI) exposed/ unexposed), pH was found to be the most significant.

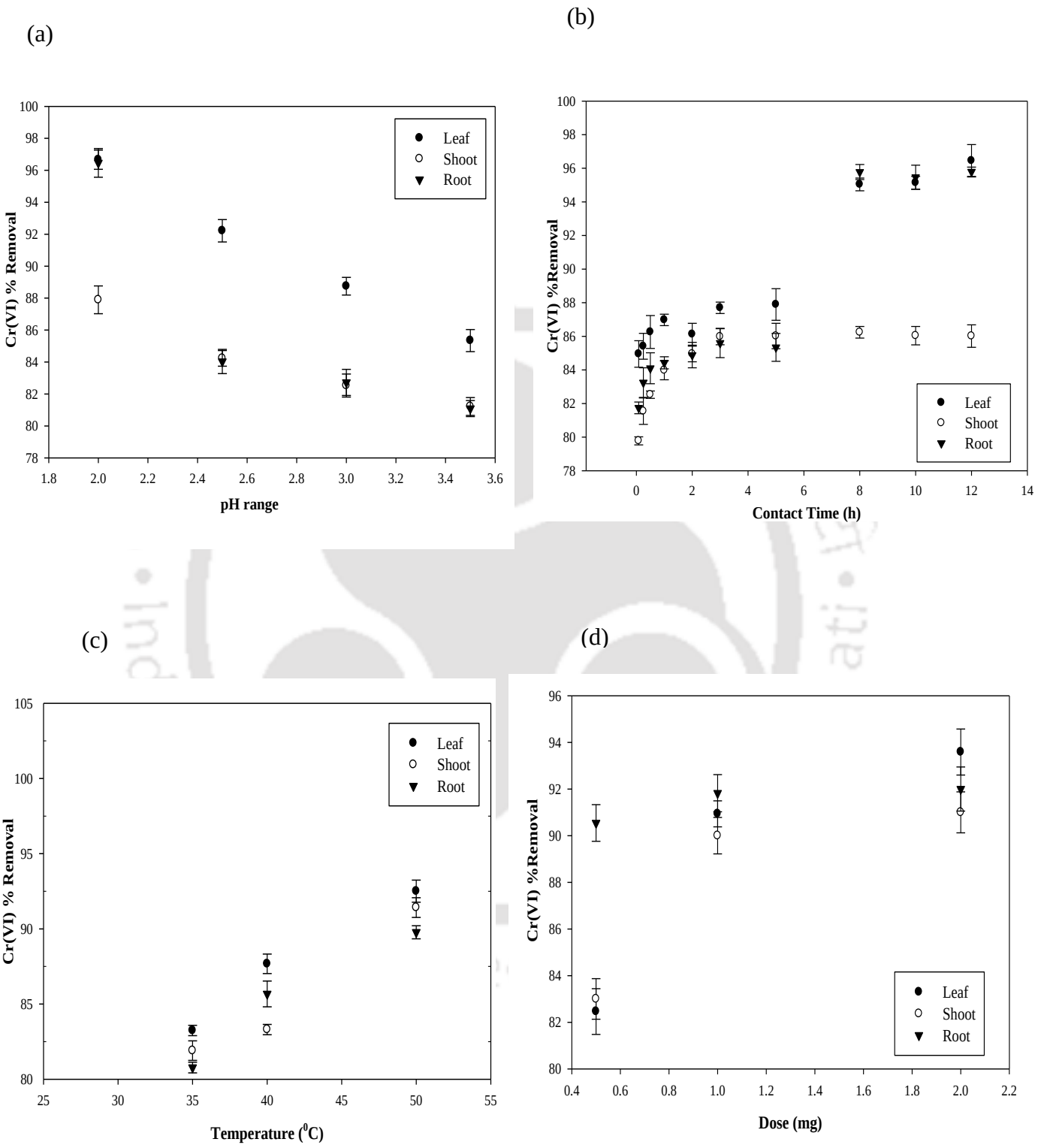


Figure 4.22: Effect of different parameters on Cr(VI) biosorption by the different plant parts of unexposed *T.pallida* (a) pH (b) contact time (c) temperature (d) dose.

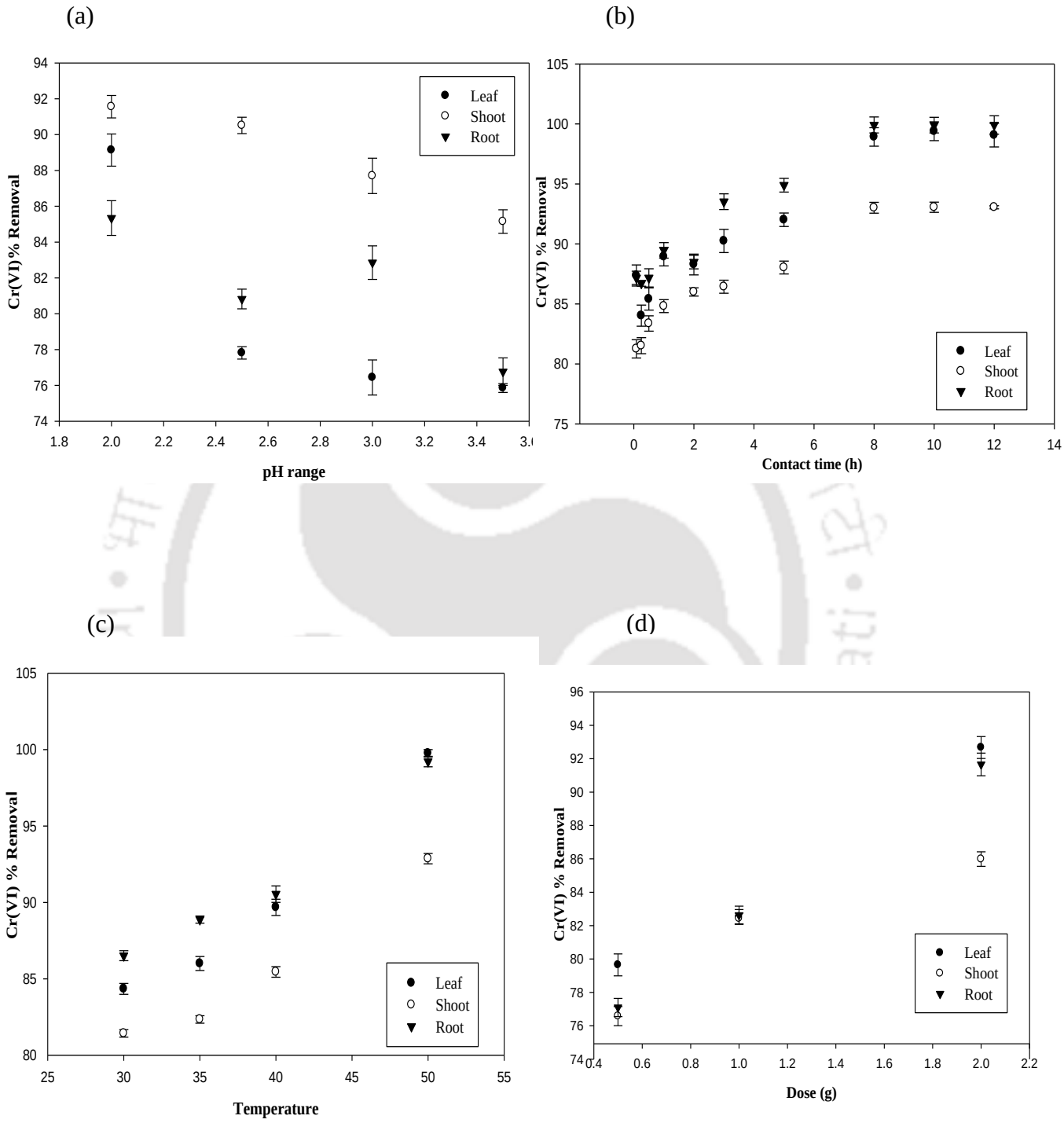


Figure 4.23: Effect of different parameters on Cr(VI) biosorption by the different plant parts of Cr(VI) exposed *T.pallida* (a) pH (b) contact time (c) temperature (d) dose.

Fig. 4.22b and Fig. 4.23b shows the effect of contact time on Cr(VI) removal. It shows an initial curved portion followed by a linear profile and a plateau depicting that the equilibrium was achieved within the initial 5 hours. After this time period the removal remained nearly the same. Thus, biosorption increased with an increase in the contact time within the initial 5h. These results are in accordance with other biosorption studies using various groups of microorganisms and plants, which reported fast initial rates of metal binding, followed by slower onset of equilibria (Wu et al., 2008).

To examine the temperature effect on Cr(VI) biosorption, experiments were performed at four different temperatures (30, 35, 40, 50°C). The results indicate an increase in Cr(VI) sorption with a raise in the temperature (Fig. 4.22c and 4.23c).

Fig. 4.22d and 4.23d shows the effect of biosorbent dose on the Cr(VI) removal. A series of biosorption experiments were carried out with different sorbent dosages (0.5-2 g/50ml). The results show that the percentage of Cr(VI) removal efficiency increased with an increase in the biosorbent dose. This was attributed to the increase in concentration of biosorbent, resulting in the increase of adsorption surface area. However at 2g biosorbent dose, the maximum removal was 94% as compared with 92% at 1g, biomass dosage, which indicates saturation of available sites in the biomass for Cr(VI) binding above 1g. Thus, a maximal removal efficiency of 94% was obtained using *T. pallida* leaf biomass at a Cr(VI) initial concentration of 100 mg/L and at a 50°C temperature. This is in agreement with the literature reports indicating possible increase in surface area and availability of more adsorption sites (Kannan, 1991, Namasivayam et al., 1996).

In all these batch experiments, no significant difference in Cr(VI) removal efficiency was observed between the treated and control *T. pallida* leaf biomass. Thus, the results

showed that the Cr(VI) removal due to either Cr(VI) exposed or unexposed plant parts was the same. However, among the different plant parts, *T. pallida* plant leaf biomass exhibited a higher Cr(VI) removal than *T. pallida* roots or shoots (exposed or unexposed).

4.5.3. Kinetics of Cr(VI) Sorption and Estimation of Kinetic Paremeters

Cr(VI)-treated plant biomass was further examined for its Cr(VI) sorption kinetics, isotherm and thermodynamics. In order to describe the Cr(VI) adsorption kinetics the data was fitted to kinetic models most commonly reported in literature, which are pseudo-first-order and pseudo-second-order. The pseudo-first order equation of Lagergren's is described earlier in Table 3.4. The equation applicable to experimental results generally differs from a true first order equation in two ways:the parameter $k_1(qe - qt)$ does not represent the number of available sites; and the parameter log *qe*is an adjustable parameter which is often not found equal to the intercept of a plot of log (qe –qt) against *t*, whereas in a true first order sorption reactionlog qe should be equal to the intercept of log(*qe* – *qt*) against *t*.In order to fit equation to experimental data, the equilibrium sorption capacity, *qe* must be known. In many cases is unknown and as chemisorption tends to become unmeasurably slow, the amount sorbed is still significantly smaller than the equilibrium amount. The pseudo first order rate constant can be obtained from the slope of plot between log (*qe* – *qt*) against time, t. Table 4.12 presents the results of fitting the Cr(VI) sorption kinetics to pseudo first and second-order kinetic models. The plot of t/q_t versus t showed that Cr(VI) sorption kinetics in the study followed the pseudo second-order rate expression more accurately than the pseudo first-order kinetics in which case the R^2 value was 0.999. The higher values confirm that the adsorption data are well represented by pseudo-second order kinetics. Using this model, the amount of Cr(VI) sorbed at equilibrium (q_e) was estimated to be 2.299 g/mg/min. Pseudo second-order

kinetic model fitting of the data revealed intraparticle diffusion of Cr(VI) ion as the rate controlling step.

Table 4.12: Estimated kinetic parameters of Cr(VI) biosorption obtained using the pseudo first-order and second-order kinetic models.

(a) Pseudo first-order kinetics

	Cr(VI) exposed *T. pallida* biomass			***T. pallida* biomasss (unexposed)**		
	Leaf	**Shoot**	**Root**	**Leaf**	**Shoot**	**Root**
Coefficient of determination (R^2)	0.886	0.485	0.841	0.842	0.754	0.831
Rate Constant (k_1)(min^{-1})	0.001	0.001	0.005	0.001	0.001	0.001
Equilibrium amount of metal ion (qe) (mg/g)	0.908	0.606	1.195	0.913	0.597	0.983

(b) Pseudo second-order kinetics

Coefficient of determination (R^2)	0.998	1	0.981	0.999	0.999	0.991
Rate Constant (k_2) (g/mg/min)	0.207	0.232	0.207	0.199	0.213	0.198
Equilibrium amount of metal ion (qe)(mg/g)	2.299	0.245	1.859	1.913	1.619	1.513

4.5.4. Chromium (VI) Sorption Isotherm Parameters

The equilibrium sorption isotherms are important for understanding the mechanism of the biosorption process. It also provides sufficient information for defining the chemical equilibrium between Cr(VI) ions and the biosorbent.

Table 4.12 presents the Langmuir and Freundlich isotherm model parameters for Cr(VI) sorption applied in the study (Pehlivan et al., 2008). These isotherms models are used to represent adsorption of components from liquid phase on to a solid phase. The Freundlich isotherm model is the well-known earliest relationship describing the adsorption process. This model applies to adsorption on heterogeneous surfaces with the interaction between

adsorbed molecules and the application of the Freundlich equation also suggests that sorption energy exponentially decreases on completion of the sorption centers of an adsorbent. This isotherm is an empirical equation and can be employed to describe heterogeneous systems and is expressed as follows in linear form as described earlier in Table 3.6. Freundlich equilibrium constants were determined from the plot of log Q_e versus log *Ce*. The *n* value indicates the degree of non-linearity between solution concentration and adsorption as follows: if $n = 1$, then adsorption is linear; if $n < 1$, then adsorption is a chemical process; if $n > 1$, then adsorption is a physical process. The values of regression coefficients R^2 are regarded as a measure of goodness of fit of the experimental data to the isotherm models.

The Langmuir isotherm assumes monolayer adsorption on a uniform surface with a finite number of adsorption sites (Awwad and Farhan, 2012). Once a site is filled, no further sorption can take place at that site. As such the surface will eventually reach a saturation point where the maximum adsorption of the surface will be achieved. The linear form of the Langmuir isotherm model is described earlier in Table 3.6. Values of Q_o, *b* and regression coefficient R^2 are listed in Table 4.13.

Results of the equilibrium adsorption data shows that, the data fitted well with the Langmuir sorption isotherm with a Q_o value 64.672 mg/g, and equilibrium constant, b, value of 0.328 mg/L. The model yielded significant details of the biosorbent properties and its affinity to Cr(VI). A better fit of the isotherm data to this model further suggested that Cr(VI) sorption was restricted to monolayer on the biomass surface with a finite number of identical sites (homogenous). A large q_{max} value along with a high b value for Cr(VI) binding further indicated, respectively, a very high binding capacity and good affinity of the leaf biomass for Cr(VI).

Table 4.13: Langmuir and Freundlich isotherm parameters estimated for Cr(VI) sorption on Cr(VI) exposed *T. pallida* leaf biomass.

	Langmuir constants			Freundlich constants		
Temperature (K)	Q_o (mg/g)	b (L/mg)	R^2	n	K_f (L/g)	R^2
303	53.968	0.208	0.997	1.0	1.648	0.935
308	54.032	0.334	0.997	1.2	1.613	0.934
313	59.098	0.330	0.998	1.0	1.656	0.954
323	64.672	0.328	0.999	1.4	1.572	0.854

4.5.6. Thermodynamics of Chromium (VI) Biosorption onto *T. pallida* Leaf Biomass

In order to describe thermodynamic behaviour of the biosorption of Cr(VI) ions onto *T. pallida* plant powder, thermodynamic parameters including the change in free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) were calculated using Van't Hoff equation as described earlier in Table 3.7.

The Van't Hoff plots for Cr(VI) is presented in Figure 4.5. It shows a highly linear Van't Hoff plot obtained between ln b and 1/T for Cr(VI) sorption in this study. From the slope and intercept of the Van't Hoff plot, shown in Fig. 4.24, the values of change in enthalpy (ΔH°), and entropy (ΔS°), for Cr(VI) sorption process were estimated and are presented in Table 4.14. The values of these parameters were found to be 11.346 kJ/mol and 0.391 kJ/mol K, respectively. The negative value of ΔG° at different temperatures shows that the Cr(VI) biosorption process is spontaneous and that the *T. pallida* leaf biomass has a high affinity for the metal ions at high temperatures. The maximum sorption capacity, Q_o, and the sorption equilibrium constant, b, increased from 53.968 to 64.678 mg/g and 0.208 to 0.328 L/mg, respectively, as the temperature was raised from 303 to 323 K.

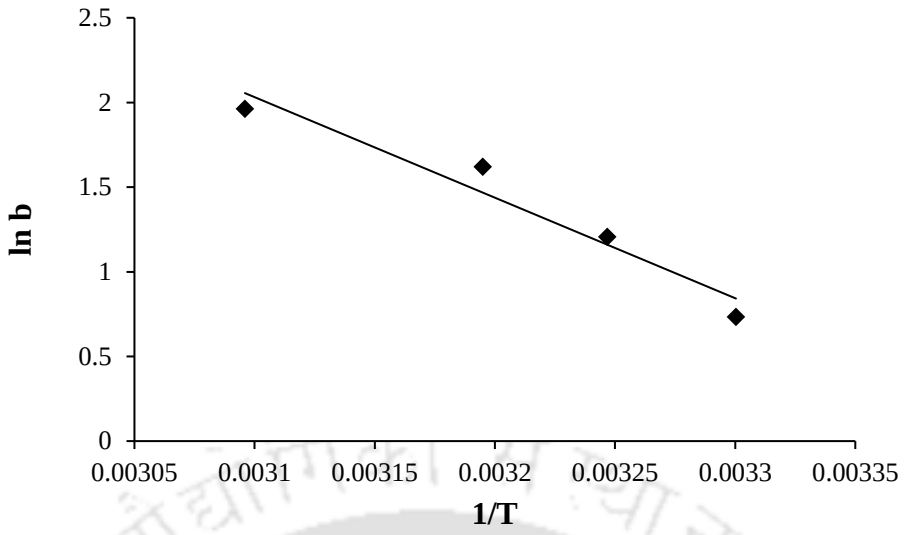


Figure 4.24: Van't Hoff plot (temperature = 35–50 °C, pH = 2, Cr(VI) conc. = 50 mg/L, biomass dose 0.5 g)

The increase in the Cr(VI) sorption equilibrium constant value with temperature reveals an increase in heat of sorption with a raise in the temperature, which clearly demonstrates that Cr(VI) sorption on to *T. pallida* biomass is an endothermic process. The positive value of ΔH° further indicates that Cr(VI) biosorption onto the biosorbent is endothermic. The positive values of ΔS° reveal that randomness of the Cr(VI) sorption process increases at the solid–liquid interface. It also reflects the affinity of the biosorbent for Cr ions and structural changes in the biosorbent following Cr(VI) sorption. All these values of the respective thermodynamic parameters obtained in this study revealed a feasible and endothermic nature of the Cr(VI) sorption process by *T. pallida* plant biomass.

Table 4.14: Thermodynamic parameters of Cr(VI) sorption obtained using the Cr(VI) exposed *T. pallida* leaf biomass.

Temperature (K)	Equilibrium constant K	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (kJ/mol K)
303	16.39774	-1.84814	11.346	0.391
308	28.07096	-3.09003		
313	42.85714	-4.21988		
323	77.88162	-5.27626		

The high Cr(VI) removal efficiency obtained using either treated or untreated *T.pallida* plant biomass demonstrates its potential for use in treating Cr(VI) contaminated wastewaters by both hyperaccumulation and by biosorption even after it has ceased to grow. Cr(VI) can further be recovered from the loaded biomass by desorption using suitable agents, which however needs to be investigated further.

In this study, Cr accumulated *T. pallida* plant biomass showed high biosorption capacity that can further be effectively utilised in treatment of Cr(VI) contaminated wastewater. This would be economical as well as advantageous to minimize the disposal of Cr accumulated *T. pallida* in constructed wetland.

CHAPTER 5 SUMMARY AND CONCLUSION

The present research work successfully investigated the potential of an indigenous plant species, *Tradescantia pallida*, for Cr(VI) removal. Owing to its low maintenance, fast growth and rich biomass content, *T. pallida* is well suited for Cr phytoremediation. The BCF and TF index, which indicates the translocation from media to plant parts and further translocation to aerial parts, revealed a very good potential of the plant for Cr bioremediation. The uptake of Cr ranged from 80 to 536 μg/g dw, with the highest Cr accumulated in the *T. pallida* root system. A good correlation between Cr accumulation and the plant's biochemical and antioxidant enzyme system was observed. Among the different antioxidant enzymes, ascorbate peroxidase activity increased maximally indicating its active role in the plants defence system against Cr(VI) induced oxidative stress. Subcellular fractionation of Cr-containing *T. pallida* plant tissues showed that most of the accumulated Cr was found in the root vacuoles and cell wall fractions.

Multicomponent experiments for simultaneous removal of Cr(VI), SO_4^{2-}, NO_3^- and PO_4^{3-} and the effect of these co-ions on Cr(VI) uptake by *T. pallida* was studied. The bioremoval of Cr(VI) by *T. pallida* was enhanced due to the presence the co-ions viz. SO_4^{2-}, and NO_3^- and PO_4^{3-}. Among the bioremoval of Cr(VI) and co-ions in this multi-ion study, PO_4^{2-} was removed with a high efficiency followed by NO_3^-, SO_4^{2-} and Cr(VI). The results showed that Cr(VI) tolerance by *T. pallida* increased with an increase in the initial concentration of NO_3^- and PO_4^{3-}, which was attributed to this protective role against Cr(VI) induced toxicity in *T. pallida*. Biochemical analyses of the plant indicated that its tolerance towards Cr(VI) may be related to high constitutive levels of carbohydrates and catalase activity. The Cr(VI) removal kinetics by *T. pallida* followed the chemisorption based pseudo second-order kinetics which revealed that both biosorption and bioaccumulation of the metal played an important role in Cr(VI) removal by *T. pallida*.

Overall, this study showed not only very good Cr(VI) removal by the *T. pallida* but also an excellent tolerance towards the metal even in presence of the co-ions from contaminated water.

Continuous removal of Cr(VI) by *T. pallida* using a laboratory scale vertical subsurface flow (VSSF) constructed wetland system was studied. The planted CW unit showed better removal in terms of both Cr(VI) and total Cr removal as compared to the control CW unit. The importance of pH of the influent which controls the Cr removal mechanism in the CW was further examined for continuous Cr(VI) removal. The pH 7 CW removed Cr(VI) ions with 95–97% Cr(VI) removal efficiency at two-days HRT. In case of total Cr, high removal efficiency of 86–88.2% was achieved in pH 7 CW as compared to 78-80% removal in pH 4 CW. Distribution of Cr in the CW revealed that the soil acts as the main sink for Cr, sequestering up to 40-68.41 % of the total Cr supplied to the system. These results clearly revealed that *T. pallida* plants can effectively enhance the Cr(VI) reduction and retention in the soil, thereby serving as an efficient system for treating Cr contaminated water. The high Cr removal efficiencies combined with the extremely low construction and operation cost, of *T. pallida* based CW posses an attractive treatment method for Cr(VI) removal.

A thorough investigation on the reusability of the Cr(VI) accumulated plant biomass from CW was carried out by studying its Cr(VI) biosorption potential under batch mode. as a biosorbent was done. The batch biosorption study showed that Cr(VI) exposed *T. pallida* leaf biomass can be used as an efficient biosorbent to remove Cr(VI) from aqueous solution. Among the different parameters examined for Cr(VI) biosorption, solution pH was the most significant. Sorption mechanism of Cr(VI) was investigated by applying suitable adsorption models, which explained the Cr(VI) sorption kinetics and equilibrium by *T. pallida* plant biomass. Biokinetic parameters in the study revealed that Cr(VI)

biosorption followed the pseudo second-order kinetics with intra particle diffusion of Cr(VI) as the rate controlling step. The structural details of the biosorbent further revealed that –NH, amide, hydroxyl and sulphonate groups were mainly involved in the binding of Cr(VI). An accurate evaluation of kinetics, isotherm and thermodynamics studies, helped to prove this adsorption system as a feasible process. Thermodynamics of Cr(VI) sorption in this study revealed that the process is highly spontaneous and therefore highly feasible for the removal of Cr(VI) from contaminated water.

Thus, the positive results obtained indicate very good potential of the *T. pallida* for treating Cr(VI) contaminated wastewater. The high Cr removal efficiencies combined with extremely low construction and operation cost, of *T. pallida* based CW system is an effective solution for the treatment of Cr laden wastewater discharged by tanneries, metallurgical, textile and other industrial setups. This approach can be used in developing countries where wastewater treatment is a major problem due to fast urbanization and industrialization.

Scope for Future Work

The present research work focused on the potential of an indigenous plant species, *T. pallida*, for Cr(VI) removal from industrial wastewater and its application using a VSSF CW system. The following are suggested as future work to continue in this area of research:

1) Development of transgenic *T. pallida* plants to enhance their tolerance and accumulation rate at environmentally relevant concentrations.

2) Understanding the inter-conversion of the Cr species within the plant system and its localization following uptake in order to unravel the complete metabolic machinery and to develop the transgenic.

3) Up-regulation of genes responsible for Cr uptake, transport and sequestration, or antioxidant enzymes involved in the detoxification mechanism.

4) Pilot plant scale set up and its evaluation for continuous Cr removal.

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1. Sinha, V., Pakshirajan, K., Arul Manikandan, N., & Chaturvedi, R. (2017) Continuous removal of Cr(VI) from wastewater by phytoextraction using *Tradescantia pallida* plant based vertical subsurface flow constructed wetland system. ***International Biodeterioration & Biodegradation***, *119*, 96-103.

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