ISOLATION AND IDENTIFICATION OF CHROMIUM TOLERANT BACTERIAL STRAINS AND THEIR POTENTIAL TO PROMOTE PLANT GROWTH

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2016

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In

Environmental Science

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

2016


بسم الله الرحمن الرحيم

CERTIFICATE

It is certified that the contents and form of the thesis entitled **"Isolation and identification of Chromium tolerant bacterial strains and their potential to promote plant growth,"** submitted by Ms. Anza Javaid has been found satisfactory for partial fulfilment of the requirements for the degree of Master of Science in Environmental Science

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DEDICATED TO…

MY BELOVED MOTHER AND TO THE LOVING MEMORY OF MY FATHER

My greatest source of inspiration and the reason behind all my achievements

ACKNOWLEDGEMENTS

I am grateful to Allah Almighty by whose Grace and Benevolence this dissertation has been made possible.

It is with great pleasure that I hereby acknowledge the valuable support, guidance and contributions made by several individuals throughout the period of my research work until the completion of this thesis.

First and foremost, I would like to express my sincere gratitude to my supervisor, ***Dr. Muhammad Arshad***, whose patience, motivation and knowledge enabled me to progress. I highly valued his ideas, suggestions and support through the course of my MS study and research. I also wish to express my earnest thanks to my GEC members, ***Dr. Imran Hashmi*** and ***Dr. Saud Ahmed Khan***, who provided insight and expertise that assisted my research work.

I express special appreciation to my lab fellows for being helpful mentors and to the lab staff of Environmental Biotechnology and Microbiology lab at IESE (SCEE) for their technical assistance and cooperation.

Last but not the least; I thank my family, for holding my back and believing in me, and my group of friends, especially ***Um-e-Habiba***, for their encouragement and unwavering personal and professional support during this tough but adventurous academic journey.

Anza Javaid

CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis Of Variance
APHA	American Public Health Association
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Unit
Cr (VI)	hexavalent Chromium
CRB	Chromium-Reducing Bacteria
DNA	Deoxyribonucleic Acid
EC	Electrical Conductivity
FASTA	Fast Alignment
HPC	Heterotopic Plate Count
IAA	Indole Acetic Acid
mg/kg	milligram per kilogram
MIC	Minimum Inhibitory Concentration
MSA	Mannitol Salt Agar
NCBI	National Center for Biotechnology Information
OD	Optical Density
PGP	Plant Growth Promoting
PGPB	Plant Growth-Promoting Bacteria
ROS	Reactive Oxygen Species
SE	Solubilization Efficiency
USEPA	United States Environmental Protection Agency
UV-Vis	Ultra Violet- Visible
WHO	World Health Organization
XRF	X-ray Fluorescence

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ABSTRACT

Chromium (Cr) presence in soil higher than the permissible limit and its uptake in plants results in reduced growth and yield. The current study was thus aimed at the isolation and identification of chromium-tolerant bacterial strains and to evaluate their potential in improving edible plant growth. Four soil samples from Gujrat District, Pakistan, were collected and analyzed for Cr content. Thirteen bacterial colonies were isolated from the sample with highest chromium content. Tests were performed with the isolates to check chromium tolerance and plant growth-promoting (PGP) abilities; indole acetic acid production and phosphate solubilization, in *in vitro*. The tolerant isolates which also exhibited PGP abilities were used as inoculants in germination and pot trial experiment with spinach plant. The isolates A5, identified as *Pseudomonas plecoglossicida*, and A6, identified as *Staphylococci saprophyticus* through 16S rRNA gene sequencing, were found to be tolerant up to 700 mg/L of Cr (VI). They were both effective in solubilizing phosphate but only A5 (*Pseudomonas plecoglossicida*) was able to produce indole acetic acid. A5 also increased the percentage of seed germination by 17 to 46% and also increased spinach plant's biomass by 44% with respect to the control. Keeping in view the effects on spinach germination and pot experiments, A5 (*Pseudomonas* sp.) can be developed as a bio-inoculant for agricultural applications and may potentially be useful for bioremediation of Cr using plants.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Industrialization, agricultural practices and various anthropogenic activities lead to the build-up of heavy metals in the environment. These heavy metals pose serious threats to terrestrial and aquatic ecosystems. The heavy metals which are generally regarded as pollutants are normally the ones with densities greater than 5 g cm^{-3} (Abdelatey *et al*., 2011). Chromium is one such toxic metal with regulatory limits set at 1.3 mg/kg in plants (WHO), 0.1 mg/L in water (WHO) and 100 mg/kg in soil (Dutch Standards) (Marsh and McInerney, 2001). Table 1.1 lists some common toxic metals and their permissible limits for soil.

Table 1.1: Toxic heavy metals and their regulatory limits in soil (Salt *et al*., 1995)

Metal	Concentration range (mg/kg)	Regulatory limit (mg/kg)
Lead	1 - 6900	600
Cadmium	0.1 - 345	100
Arsenic	0.1 - 102	20
Chromium	0.005 - 3950	100
Mercury	0.001 - 1800	270
Copper	0.03 – 1550	600
Zinc	0.15 - 5000	1500

Chromium is hazardous for humans and the ecosystem. It is one of the seventeen chemicals that pose the highest danger to human health according to the United States environmental protection agency (USEPA) (Kafilzadeh and Saberifard, 2016). As a consequence of industrial and manufacturing activities more than

170,000 tons of chromium waste is discharged into the environment annually (Kamaludeen *et al*., 2003). Once up taken by plants, chromium does not only enter our food chain but also damages plants and reduces a major food source. To curtail the detrimental effects of chromium, soil remediation is required. Remediation through microorganisms, like bacteria, may be cost effective and beneficial for the plants grown. Work needs to be done in the area in order to rehabilitate environment and address the food scarcity issue.

1.2 CHROMIUM IN PAKISTAN

In Pakistan, untreated sewage and industrial waste water is commonly used for irrigation, especially in peri-urban regions, where it is used for vegetable production. This practice has not only led to the build-up of heavy metals in a major food source but also contaminates the soil and groundwater in contact.

1.2.1 Chromium toxicity in soil

Concentrations of heavy metals, like chromium, are reported to be much higher in Pakistani soil. Soil around industries namely textile, tanning, cement, coal and oil powered plants and mines contains chromium at higher levels than those permissible. For example, the soil in Kasur has been recorded to contain 246 to 1980 mg/kg of chromium which is much higher than the permissible limit; 100 mg/kg (Iqbal *et al*., 2011). Table 1.2 lists a few other areas across Pakistan and the amount of chromium found in their native soil.

Table 1.2: Chromium concentrations in soil in various regions across Pakistan (Waseem *et al.*, 2014)

Sample type and location	Cr range (mg/kg) (mean)
Soil, Hattar Industrial Estate, KPK	0.24–34.06 (5.96)
Soil near tannery effluent, Peshawar, KPK	0.81–100.2 (29.9)
Contaminated Pazang site	42–756 (146)
Reference Pazang site	60–409 (252)
Contaminated Lahore site	92–850 (439)
Reference Lahore site	123–154 (139)
Coastal sediments of the Arabian sea along the urban Karachi, Sindh	2.95–180.90
Surface sediments in Karachi coastal sites, Sindh	12.0–319 (96.75)

1.2.2 Chromium toxicity in plants

Chromium toxicity for plants varies from 5 to 100 mg/kg of the chromium available in soil (Ghosh and Singh, 2005). Recent studies, on Pakistani soil and plants, have reported high content of chromium in leaves and edible portions of vegetables i.e. from an average of 3.74 mg/kg in leaves to an average of 7.56 mg/kg in edible parts (Perveen *et al*., 2012). Another study reported 3.93 mg/kg Cr in spinach which was irrigated with industrial effluent. Table 1.3 shows the amount of chromium found in vegetables grown in industrial wastewater.

Table 1.3: Amounts of Chromium found in vegetables grown in industrial effluent across Pakistan (Waseem *et al.*, 2014)

Vegetables (Punjab)	Chromium (mg/kg)
Cabbage (leaves)	11.0
Garlic (bulb)	14.6
Tomato (fruit)	16.2
Cauliflower	16.0
Spinach (leaves)	3.93
Okra (vegetable)	3.09
Onion (bulb)	7.2
Peas (seed)	6.2
Radish (root)	3.6
Squash (leaves)	3.32

1.3 OBJECTIVES

Based upon the information presented above, the objectives of the study conducted were:

1. Isolation and Identification of chromium tolerant bacterial strains in soil.
2. Evaluating the potential of Cr-tolerant bacterial strains in improving plant growth.

1.4 SCOPE OF THE STUDY

In the present study, bacteria were isolated from chromium-rich soil collected from Gujrat (32.57°N, 74.08°E), Pakistan. It is important to ascertain tolerance of microbes to chromium as it would serve as a basis for selecting the microbes that may help cleanse environment from chromium. Chromium tolerant species were

thus isolated and identified. Moreover their plant growth-promoting abilities were studied in context with edible plant growth. The study was conducted to support bioremediation as an efficient method, in increasing overall yield of edible plants and reduce food scarcity by reducing environmental stress caused by heavy metals like chromium and by studying if these bacteria improved growth and development of plants.

CHAPTER 2

LITERATURE REVIEW

2.1 HEAVY METALS IN THE ENVIRONMENT

2.1.1 Heavy metal contamination

Industrialization has led to environmental contamination by all sorts of substances. Industrial waste is a major source of heavy metals and toxins which pollute the environment. Many areas worldwide face the issue of abnormal levels of heavy metals in soil and water due to the unregulated discharge of industrial effluents. Heavy metals considered dangerous for biological systems include chromium (Cr), copper (Cu), lead (Pb), antimony (Sb), arsenic (As), beryllium (Be), cadmium(Cd), nickel (Ni), selenium (Se), silver (Ag), thallium (Tl), zinc (Zn) and mercury (Hg). Accumulation of such heavy metals in the environment above the critical levels disrupts the normal functioning and activities of microbes, biodiversity and soil fertility which in turn disturbs the natural balance of several ecosystems (Ahemad, 2015).

2.1.2 Effects of heavy metals

Heavy metal toxicity increases physiological health risks. These metals enter the human bodies through inhalation, ingestion and absorption and cause damage. Major health issues that arise from metal intake include cardiovascular diseases, chronic anemia, cognitive impairment, cancer, kidneys damage and damage to central nervous system, skin, teeth and bones (Wuana and Okieimen, 2011).

2.2 CHROMIUM AND ITS TOXICITY

Chromium is a transition element that is located in Group 6 of the periodic table. It is a lustrous and brittle metal that appears silver-grey in color and occurs naturally in rocks, plants, animals, soil, gases and in volcanic ash (Joutey *et al*., 2015). It exists

in the environment in several oxidation states of which two oxidation states are the most stable and common: hexavalent Cr (VI) and trivalent Cr (III). Both these oxidation states exhibit different properties. Cr (III) is considered to be a vital element, for glucose, lipid and protein metabolism in animals, in small amounts. It can however persist in the environment indefinitely and its build up can cause irreversible damage to the living bodies. Cr (VI), on the other hand, is extremely toxic for biological systems (Suresh *et al.,* 2011). It disseminates easily into soil and water as it is highly soluble, mobile and permeable through biological membranes (Smrithi and Usha, 2012). It also has an oxidizing ability which combines with the rest to make it hazardous.

2.2.1 Industrial applications of Chromium

Environmental pollution due to the presence of chromium is a prevalent problem today because of its widespread use in industrial applications. Though it is an essential micronutrient to living things but elevated levels of chromium lead to toxicity. Chromium presence in the soil is because of geochemical and various anthropogenic sources (Raicevic *et al.,* 2013). The chromium attaches strongly to the soil particles and does not get easily washed down into the groundwater, staying in soil to affect the soil vegetation. Chromium is resistant to corrosive reagents as it immediately reacts with oxygen producing an oxide layer that prevents further oxygen diffusion and protects the metal underneath the layer. It is hence used in electroplating, electro-painting and all sorts of alloy products like stainless steel to impart resistance to corrosion and to give a shiny finish to the alloy (Ahemad, 2015). It is discharged in the waste from tanneries, foundries, textile industries, cement industries, mines, from coal and oil combustion and from wood treatment etc. (Mohan *et al.,* 2014).

2.2.2 Effects of Chromium

2.2.2.1 Health effects of Chromium

Cr (III) is not easily disseminated by any exposure route unlike Cr (VI) so is not considered potentially dangerous. Its deficiency in the human body can cause disruptions in metabolism and diabetes whereas exposure to Cr (III) can cause skin irritation and rashes. On the other hand, Cr (VI) has become a subject of interest as its presence has a huge adverse impact on the environment. Many countries in the world classify hexavalent chromium under priority pollutants (Sayel *et al*., 2014). It is toxic, carcinogenic and mutagenic for humans and other living organisms (Shekhar *et al.*, 2014). Exposure to chromium has been recognized as an important risk factor for lung cancer. It is an element that can be absorbed by lungs, gastrointestinal tract and even skin to some extent. Cr (VI) causes irritation and ulcerations in the airways and on skin and causes allergic reactions, asthma, dermatitis, sinus cancer, chronic bronchitis, chronic rhinitis and other problems related to the respiratory system (Halasova *et al*., 2009). Absorption of chromium through the skin can cause allergic dermatitis leading to dryness, scaling and swelling of skin with other allergic responses. Chromium ingestion causes damage to the stomach and intestines that may lead to cancer. Tests conducted on lab animals have shown that Cr (VI) intake damages the sperm and reproductive systems in males (Kim *et al.,* 2012). Furthermore, it has also been known to damage the developing fetus in a few cases (Asmatullah and Shakoori, 1998).

Chromium affects the microbial population adversely and is known to decrease their cell metabolism at high concentrations (Shi *et al*., 2002). Cr (VI) is usually present in the form of chromate (CrO^{-4}) and dichromate (CrO^{-7}) (McLean and Beveridge, 2001). Chromate is a very strong oxidizing agent which gets reduced from Cr (VI)

to Cr (V) inside the cells. This unstable radical leads to the formation of reactive oxygen species (ROS) and together they react with nucleic acids and cell components (Ibrahim *et al.*, 2011). These cause cell DNA and protein damage thus producing mutagenic and carcinogenic effects in biological systems (McLean and Beveridge, 2001).

2.2.2.2 Effect of Chromium toxicity on plants

Plants growing on heavy metal contaminated soils show a reduction in growth and development due to the changes caused by metals in their physiological and biochemical activities (Chibuike and Obiora, 2014). Chromium compounds are also detrimental to the growth and development of plants and change their maturity patterns. The effects of chromium exposure in plants include reduction in seed germination, root length, plant biomass and in plant height (Sayel *et al.*, 2014). Toxic level of Cr (VI) in plants shows drastic effect on plant's dry matter and yield (Khan *et al*., 2008). Further effects of chromium on plants include:

i. Decreased nutrient uptake as well as reduced water potential,
ii. Alterations in the tissues and cell walls of plant cells,
iii. Chromosomal abnormalities causing addition, absence or transformation of chromosomal DNA,
iv. Inhibition of DNA replication and cell division by shutting down cell cycles,
v. Increase and decrease in the plants' transpiration making it lose and retain more water than normal,
vi. Changing the rate at which active enzymes work,
vii. Affecting the amount of active enzymes present in the plant,
viii. DNA damage and mutations,

ix. Changes in cell metabolism and structure due to the presence of reactive oxygen species (ROS) (Ahemad, 2015).

All the above mentioned effects and changes disrupt the normal functioning of plants leading to reduced growth and damage.

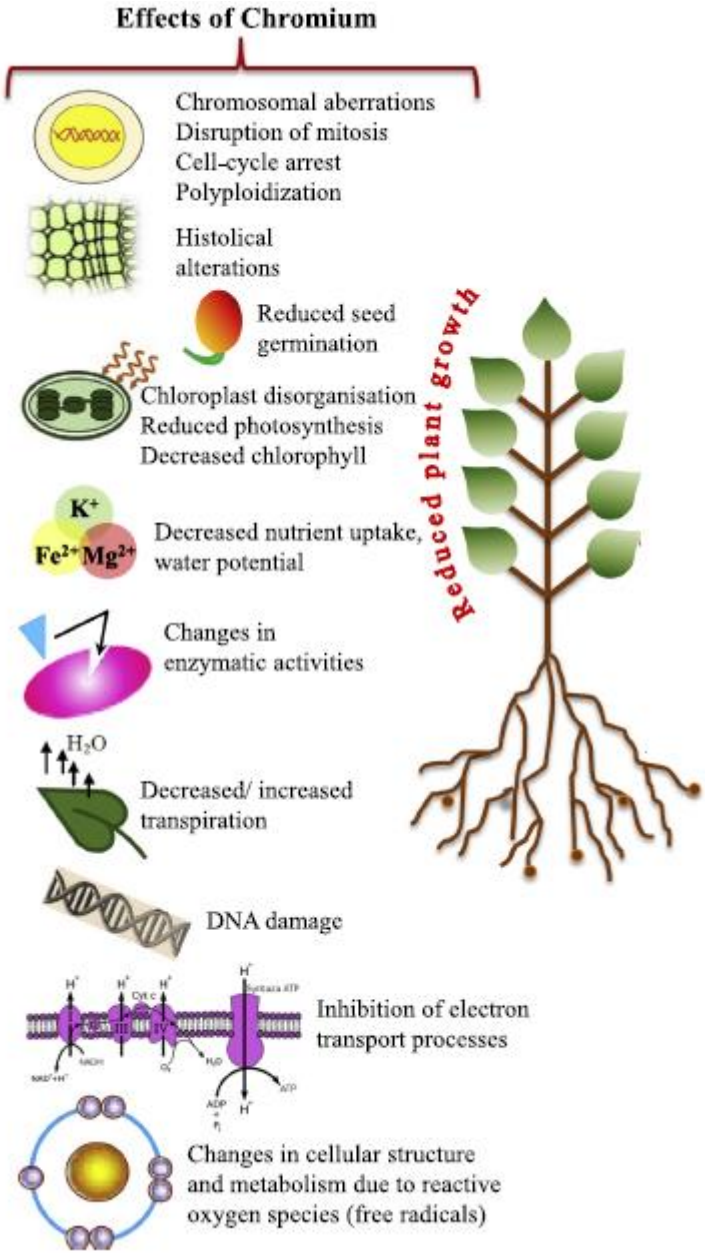

Fig 2.1 Chromium induced changes and effects on plants (Ahemad, 2015).

2.3 SOIL REMEDIATION

As of late soil remediation has attracted the attention of various factions because of the drastic effects these contaminated sites have on health and environment. Rehabilitation of heavy metal contaminated soils has gained importance and industries and companies have started allocating resources to resolve the soil pollution and rehabilitation issue. The environment needs to be conserved. There are a number of ways to remove heavy metals or reduce them in order to rehabilitate the environment as shown in Fig 2.2.

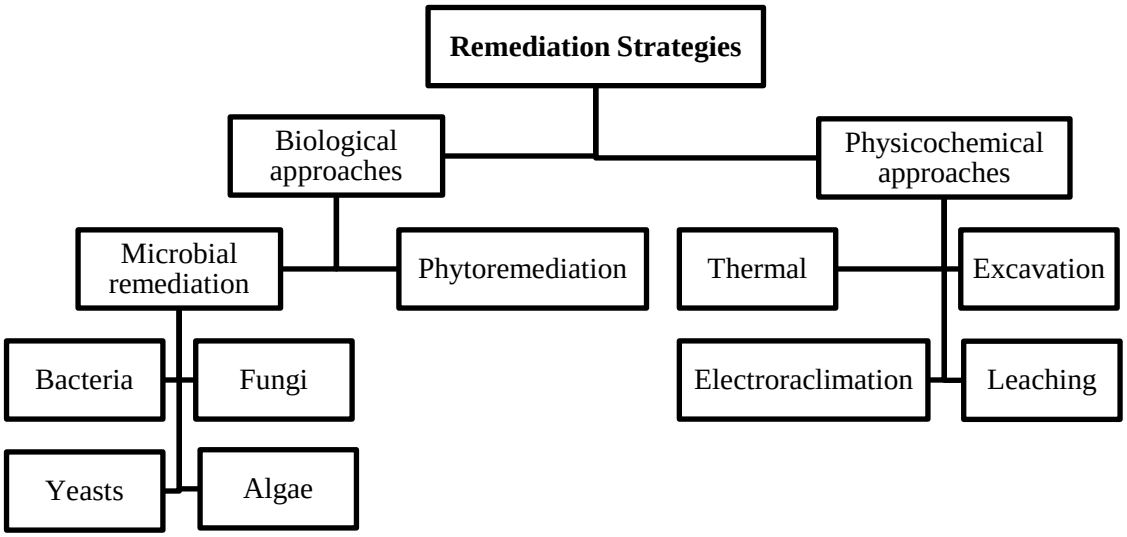

Fig 2.2 Remediation strategies for heavy metals (AbidUllah, 2015)

Cr (VI) can be removed by methods like membrane processing, ion exchange, chemical precipitation, electro dialysis and liquid extraction. However, all these methods at large scale are expensive, generate waste and are highly energy intensive (Kafilzadeh and Saberifard, 2016). Development needs to be done in a manner that does not cause destruction of nature or extinction of species but instead keeps the balance with nature undisturbed. Research has provided various solutions out of which bioremediation is fast gaining popularity in remediating the toxicity caused

by heavy metals in soil and water. The method does not involve the development of heavy or intensive methods and machinery and is found to be affordable, pollution free and an efficient means to tackle the problem.

2.3.1 Bioremediation

Bioremediation is one of the "Top 10 Biotechnologies to Improve Global Health" (Pieper and Reineke, 2000). It is an effective means to restore the environment by eliminating the contamination owing to the presence of heavy metals (Mohan *et al*., 2014). Bioremediation employs the characteristics of plants and microorganisms to transform toxic compounds in the soil or water to less harmful ones. Bioremediation which involves only microorganisms like bacteria, yeasts, fungi and algae etc. is known as microbial remediation (Pieper and Reineke, 2000).

2.3.2 Microbial remediation

Many microorganisms can accumulate or reduce certain heavy metals easily without considerable effort on our part (Ezaka and Anyanwu, 2011). Microbes have a high surface area to volume ratio hence they provide a large area of contact to interact with the metals in their surroundings (Zouboulis *et al.*, 2004). The polymers in these cells provide acidic functional groups, like carboxyl and amino groups, to react with dissolved metals (Chaturvedi, 2011). Research has also proved that some growing cells can remove metals through their internal detoxification mechanisms (Godlewska-Zylkiewicz, 2005).

Heavy metals do have detrimental effects on most microorganisms but many have evolved over time to curtail those effects (Suresh *et al.*, 2011). Many microorganisms also possess the abilities to not only survive heavy metal toxicity but also to use it or reduce it for their survival and proliferation. However, it is essential to identify and isolate heavy metal tolerant microorganisms which can be

used to decontaminate the environment from the metal toxicity. These microorganisms can further benefit the environment if they work simultaneously to improve other issues and aspects under concern. Thus, heavy metal-resistant plant growth-promoting bacteria are a novel and promising approach to solving soil toxicity and plant growth issues (Khan *et al.*, 2015).

2.3.3 Remediation of Chromium through microorganisms

Researchers Romanenko and Koren'Kov (1977) were the first ones to report the reduction of Cr (VI) by microorganisms. They observed Cr (VI) reduction capability in Pseudomonas species that was grown under anaerobic conditions. This bacterial strain had been isolated from sewage sludge and was identified as *Pseudomonas dechromaticans*. Since that discovery, many microorganisms that can reduce Cr (VI) to Cr (III) have been studied and isolated (Joutey *et al*., 2015).

Bacteria equipped with the ability to reduce chromium (VI) levels in the environment are called chromium-reducing bacteria or CRB (Somasundaram *et al*., 2009). These CRB, like *Bacillus* sp. (Chandhuru *et al*., 2012), *Acinetobacter*, and *Pseudomonas* sp. (Farag and Zaki, 2010), are isolated from areas which are known to possess chromium which usually include industrial effluents; from tanneries (Farag and Zaki, 2010; Chandhuru *et al*., 2012), textile industries (Cetin *et al*., 2008), electroplating manufacturing (Seema *et al*., 2012), and from soil contaminated with these effluents (Sharma and Adholeya, 2012).

2.4 PLANTS-MICROBE INTERACTIONS

2.4.1 Plant growth-promoting bacteria

Plant growth-promoting bacteria (PGPB) help improve plant growth and development even in contaminated soil due to their different growth-enhancing abilities, either directly or indirectly as illustrated by Fig 2.3. Usually the bacteria

found in the rhizosphere of a plant are plant growth-promoting and protect the plant from pathogens. These bacteria are provided with rich supplies of substrates by the roots, with the help of which they synthesize and release auxins which act as secondary metabolites and aid plant development (Strzelczyk and Pokojska, 1984). They also help in the degradation of contaminants which hinder plant growth (Glick, 2010). Various bacterial species like *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have been classified as PGPB as they have been reported to enhance plant growth (Lwin *et al*., 2012). Furthermore, PGP bacteria like *Pseudomonas putida*, *Pseudomonas aeruginosa* (Dogan *et al*., 2011), *Pseudomonas corrugate* (Christl *et al*., 2012) and *Bacillus* sp. (Wani and Khan, 2010) have been reported to be capable of restoring chromium contaminated sites.

2.4.2 Plant beneficial traits of PGPB

Studies are still being made in order to fully understand the exact mechanisms by which PGPB promote plant growth. As of now, the direct mechanisms are thought to include:

i. The ability to produce or alter the levels of plant growth regulating hormones like Indole Acetic Acid (IAA), ethylene, gibberellic acid etc. (Arshad and Frankenberger, 1993),
ii. Nitrogen fixation (Boddey and Dobereiner, 1995),
iii. Production of siderophores (Scher and Baker, 1982) and cyanide (Flaishman *et al*., 1996); to combat phyto-pathogenic microorganisms and,
iv. Phosphate solubilization (De Freitas *et al*., 1997),
v. Sequestering of iron by the production of siderophores and the production of phytohormones for synchronizing plant growth,

vi. Lowering of ethylene concentration in order to reduce wastage Gupta *et al*., 2014).

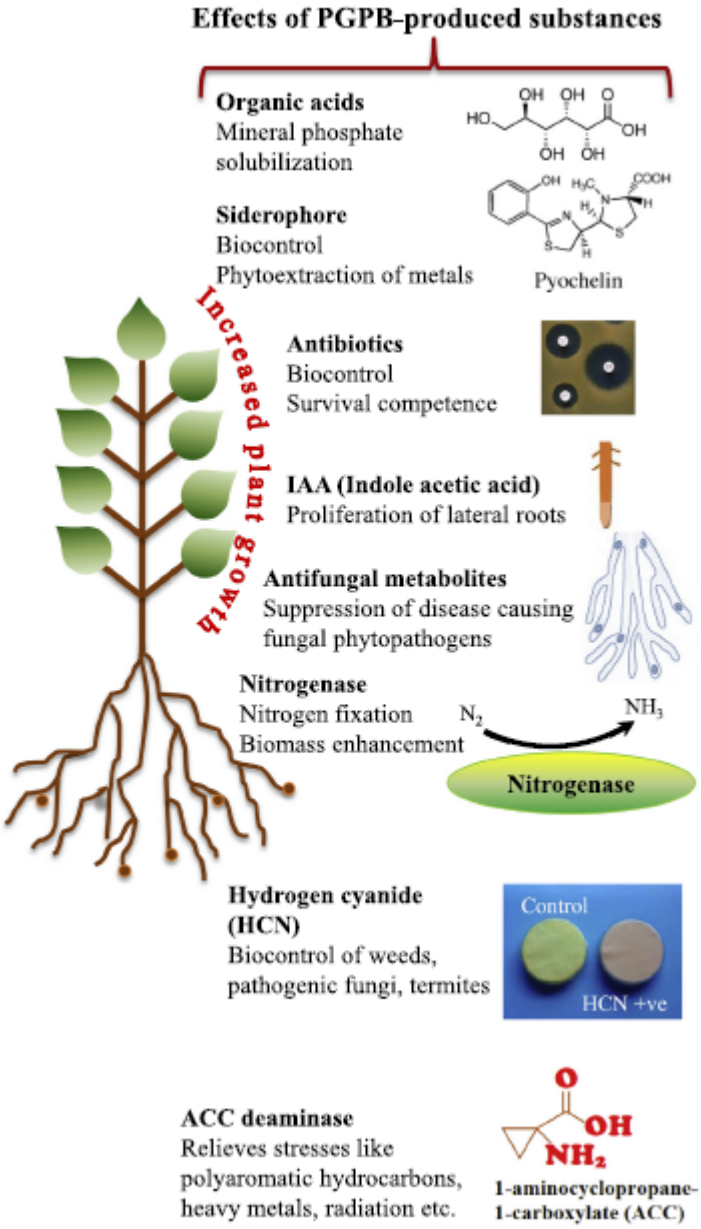

Fig 2.3 Effects of different plant growth-promoting bacteria on plants (Ahemad, 2015)

The indirect mechanisms for the promotion of plant growth by PGPB include:

i. Production of antibiotics (Shanahan *et al*., 1992)

ii. Depletion of iron from the rhizosphere,

iii. Synthesis of antifungal compounds to suppress disease,

iv. Production of fungal cell wall for reduction in food wastage Gupta *et al*., 2014).

2.5 PLANT GROWTH PROMOTING TRAITS

2.5.1 Indole Acetic Acid (IAA) production for plant growth

Auxins are hormones which stimulate lengthwise growth of plants and are distributed in the growth regions of the plant. The most important of the auxins is β-indolylacetic acid or indole acetic acid (IAA) which is formed from the breakdown of glycosides (type of carbohydrate) or from the amino acid tryptophan. IAA affects plants by acting on the chemical bonds of the carbohydrates with which the cell wall is made (Britannica, 2016). IAA also induces cell elongation, cell division, coordinates plant growth and serves as a signaling molecule for the development of plant organs (Zhao, 2010). IAA production by bacterial strains like *Klebsiella pneumoniae* (Sachdev et al., 2009), *Enterobacter cloacae, Klebsiella oxytoca* (Mirza *et al*., 2001), *Bacillus megaterium, Lactobacillus casei, Bacillus subtilis, Bacillus cereus* and *Lactobacillus acidophilus* (Mohite, 2013) has already been studied and reported earlier.

2.5.2 Solubilization of phosphates

Phosphorus (P) is an essential element for the sustenance and development of plants. It plays a vital role in key functions in a plant like energy transfer, photosynthesis, nutrient mobility within the plant, transformation of sugars and transfer of genetic traits (Theodorou and Plaxton, 1993). Absence or deficiency of Phosphorus in soil severely affects metabolism and the yield of plants. Phosphorus occurs naturally in organic and inorganic forms. It can remain unavailable to plants even if it is present in the soil due to several reasons. Solubilization of phosphates entails its conversion to a form which can be easily assimilated by the plants, hence, enhancing their

growth and yield (Ahemad and Zaidi, 2011). Many plant growth promoting bacteria have phosphate solubilizing efficacy. PGPB which have been studied as efficient solubilizers of phosphates include *Bacilllus thuringiensis* (Shahab *et al*., 2009) and *Pseudomonas* species identified as *Pseudomonas aeruginosa, Pseudomonas cepacia, Pseudomonas fluorescence and Pseudomonas putida* (Gupta *et al*., 2014). Further research is being conducted in this area.

It can be concluded from reviewing literature that chromium metal, in its hexavalent oxidation state, is a hazardous element which poses serious risks for animals, plants and microorganisms. Soil is a major sink of these heavy metals from which these enter plants and thus contaminate the food chain. Several soil remediation techniques are available of which remediation through microbes is an emerging fast, effective and environmentally friendly approach. Literature verifies the existence of heavy metal tolerant microorganisms which can be employed for bioremediation and also certain plant growth promoting bacteria that enhance yield. Exploiting the naturally occurring microorganisms found in chromium stressed soil to resist Cr (VI) and to promote plant growth in its presence has been explored in this study.

CHAPTER 3

MATERIALS AND METHODS

3.1 SOIL SAMPLING

Four soil samples were obtained from fields in Gujrat (32.57°N, 74.08°E), Pakistan. The upper surface, 0-15 cm (depth), and sub surface, 15-30 cm (depth), soil was extracted in bulk amounts. The collected samples were stored in air tight sampling bags and transported to laboratory for analysis.

3.2 SAMPLE ANALYSIS

3.2.1 Physicochemical analysis

The samples were air dried in lab. The dried soil was then ground using mortar and pestle and sieved with a 2mm-sieve. Powdered soil samples were then stored in air tight polyethylene bags at room temperature. The physicochemical properties like pH and Electrical Conductivity (EC) were measured using pH meter (HANNA HI 2211 USA) and an EC meter (WTW Germany Series 720).

3.2.2 Analysis via Elemental Analyzer

Elemental analysis was done for four soil samples using an x-ray fluorescence spectrometer elemental analyzer (JEOL JSX 3202 M) (Na→ U). The analyzer was used in order to ascertain the heavy metal composition which included the percentage of chromium in samples. The samples were prepared in the form of pellets, by applying a pressure of 30 Pascal inside an O-ring, and then placed inside machine. Data was recorded through associated computer software.

3.2.3 Analysis via UV-Vis Spectrophotometer

Soil sample with maximum chromium mass percentage, obtained using elemental analyzer, was selected. Further analysis was carried out in order to find concentration of hexavalent chromium (Cr (VI)) in sample. One gram of soil sample was first digested with addition of Nitric acid and Hydrochloric acid, in the ratio 3:1, and the mixture was brought to boil on a hotplate set at 250°C for one hour.

The preparation of Cr (VI) stock solution and standards was done as per standard methods (APHA, 2012):

i. 50 mg/L of Cr; dissolve 141.4 mg $K_2Cr_2O_7$ in 1 liter water
ii. 0.25, 0.5, 0.75, 1, 1.5 mg/L by dissolving 0.5, 1, 1.5, 2, 3 mL sub-stock in 100 mL water respectively
iii. Diphenylcarbazide solution: dissolve 250 mg 1,5-diphenylcarbazide in 50 mL acetone.
iv. Sulfuric Acid (0.2N): 0.56 mL of 96% pure H_2SO_4 in distilled water made up to 100 mL volume

Analysis method as per standard methods (APHA, 2012):

i. Use 0.2N H_2SO_4 + pH meter (bring pH to 1 ± 0.3 of all standards and sample)
ii. Transfer to 100 mL flask, dilute to 100 mL
iii. Add 2 mL diphenylcarbazide + acetone solution
iv. Allow color development (5 to 10 min)
v. Transfer to cuvette and measure absorbance at 540 nm using UV-Vis spectrophotometer (T-60U, PG INSTRUMENTS UK)

3.3 MICROBIOLOGICAL ANALYSIS

3.3.1 Serial Dilutions and Spreading

For isolation of chromium tolerant bacterial strains, plate count technique was adopted and selected soil sample was serially diluted (10^{-7}). One gram of soil was added to nine milliliter sterile distilled water in a test tube and vortexed (This denotes the 10^{-1} dilution). 1 mL of this dilution was transferred to another tube with 9 mL sterile water (10^{-2} dilution). The process was repeated and 7-fold sequential dilutions were made. Aliquots, of 1 mL, were drawn from all the dilutions and plated on sterile nutrient agar plates. These plates were then incubated at 37°C for 24 hours to allow bacterial growth.

3.3.2 Heterotopic plate count (HPC)

The plates were checked for bacterial colony growth after 24 hours. Bacterial colonies were enumerated by spread plate counting technique as Colony forming units (CFU) (APHA, 2012). Population density of bacterial colonies, counted using a colony counter (SUNTEX CC560, Taiwan), was expressed as CFU mL^{-1}.

$$\mathbf{CFU/mL} = \frac{\text{Number of colonies per mL plated}}{\text{Total dilution factor}}$$

3.3.3 Isolation and Purification

Thirteen bacterial colonies were picked from incubated plates and streaked on fresh nutrient agar plates separately. These were then streaked repeatedly on same medium plates in order to purify cultures. After three purifications, plates were stored in a refrigerator at 4°C. (The cultures were refreshed after every 8-11 days).

3.4 IDENTIFICATION

For identification of chromium tolerant bacterial strains the following morphological and biochemical tests were conducted (Table 3.1) using standard bacteriological techniques (Holt *et al*., 1994).

Table 3.1: Tests for the Identification of Cr tolerant bacterial strains

Morphological	Biochemical
Cell morphology (gram staining), colony morphology	Oxidase, Catalase, Motility, Simmons' citrate agar test, Mannitol salt agar test, MacConkey agar test

3.4.1 Morphological Tests

Cell and colony morphological characteristics were observed in order to identify and characterize isolated bacterial strains.

3.4.1.1 Colony Morphology

Colonies were observed on agar plates while performing heterotrophic plate count (HPC) and characterized with respect to characteristics mentioned in Table 3.2.

Table 3.2: Colony morphological characteristics

Characteristics	Description
Size	pinpoint, small, large
Configuration	round, filamentous, rhizoid, irregular
Margin (edges)	smooth, lobate, filamentous
Elevation (as seen from the side)	flat, raised, convex
Surface	smooth, rough
Color	cream, yellow, pink etc.

3.4.1.2 Cell morphology via Gram staining

Gram staining was done to observe cell morphology. The staining test helped identify shape of the cell i.e. either cocci, bacilli or cocci-bacilli. The test also helped classify strains as gram negative or gram positive as it can distinguish between differing membrane structure.

The bacteria were mounted on clean slides, air dried and heat fixed by passing the slide through a flame quickly. The area was covered with crystal violet solution and allowed to rest for 30 seconds for the bacteria to get stained. After washing with distilled water the area was again flooded with iodine solution and left for another 30 seconds. A decolorizing agent (ethanol) was then added drop wise to the stained area and made to run off until no color was observed. The last step involved the addition of Safranin solution to the slide to be washed off with distilled water after 30 seconds. The cover slip was then placed on the stained region, with a drop of immersion oil on top, and observed under light microscope (LEICA: 100x oil immersion). Gram positive bacteria appeared as blue-violet whereas gram negative bacteria appeared pink.

3.4.2 Biochemical Tests

A number of biochemical tests were also carried out in order to identify and characterize the isolated bacterial strains.

(Note: All the tests were conducted using inoculums from 24 hours fresh bacterial cultures.)

3.4.2.1 Catalase Test

Bacterial isolates were smeared on clean slides using a sterilized wire loop. Hydrogen peroxide (H_2O_2), 3%, was then added to the smear drop wise. The

immediate appearance of bubbles indicated a positive test and presence of catalase enzyme was confirmed. The enzyme broke down H_2O_2 into oxygen and water.

3.4.2.2 Oxidase Test

Loops full of isolates were smeared on strips of filter paper. A drop of the oxidase reagent i.e. N, N-dimethyl-p-phenylenediamine dihydrochloride solution was added to strip. Appearance of bluish or purple color, within the first 30 seconds, where bacteria were present denoted positive test. A positive test indicated presence of enzyme cytochrome oxidase.

3.4.2.3 Simmons' Citrate Agar test

Bacteria which can use citrate as their sole source of growth are able to grow on this media. The media contains a pH indicator, Bromothymol blue, which turns from green (acidic) to royal blue (alkaline) when citrate utilizing bacteria are present. The media was prepared according to manufacturer's instructions and strains were streaked on agar plates. The plates were left to incubate at 27°C for 24 hours and growth and color changes were observed.

3.4.2.4 Mannitol Salt Agar Test

Mannitol salt agar is a selective media for gram positive bacteria. It also favors growth of 'salt loving' bacteria as it contains high amount of Sodium Chloride (NaCl). It is also a differential media that contains a pH sensitive dye, phenol red, which changes color from red (alkaline) to yellow (acidic) if bacteria grown ferment the mannitol present. Growth but no color change indicates gram positive bacteria which do not ferment mannitol. The media was prepared according to manufacturer's instructions and strains were streaked on agar plates. The plates were left to incubate at 37°C for 24 hours and then growth observed.

3.4.2.5 MacConkey Agar Test

MacConkey is a selective media for the growth of gram negative bacteria and inhibits growth of gram positive strains. (The bacteria are 'lactose fermenters' if they turn pink after incubation). The media was prepared according to manufacturer's instructions and strains were streaked on agar plates. The plates were left to incubate at 37°C for 24 hours and then growth observed.

3.4.3 Molecular characterization

3.4.3.1 16Sr RNA gene sequencing

The 13 isolates were freshly cultured on nutrient agar plates. Once growth was obtained, bacteria were sprayed with 1 mL of autoclaved-distilled water and inoculums were collected in 2 mL Eppendorf tubes with the help of glass spreaders and a micro pipette. The tubes were then centrifuged at 2,000 rpm for 10 minutes to separate supernatant from bacterial culture pellet. The supernatant was then pipetted off. For preservation of bacteria, 1 mL of 50% glycerol solution and 1 mL of 30% nutrient broth were added to tubes containing pellet. These isolates were then sent to Genome Analysis Department Macrogen Inc., South Korea, for 16S rRNA gene sequencing.

3.4.3.2 Phylogenetic analysis

The sequences obtained after 16S rRNA gene sequencing were further processed to identify bacterial strains. Peak data of sequences was studied, using the software BioEdit sequence alignment editor, and ends of sequences were trimmed accordingly to remove any junk data. The final sequences obtained were analyzed using BLAST nucleotide search in the National Center for Biotechnology Information (NCBI) databases (genus names were verified using EzTaxon database). Sequences with highest matches to isolates were selected and their accession

numbers obtained. These FASTA sequences were then run in MEGA 7 software in order to construct the phylogenetic tree that showed linkage between isolated strains and those from the GenBank (NCBI).

3.5 CHROMIUM TOLERANCE ASSAYS

Two approaches were employed to check chromium tolerance of the bacterial isolates.

3.5.1 Growth response to Cr (VI) concentrations; qualitative analysis

For qualitative analysis nutrient agar plates, spiked with various concentrations of chromium (100, 150, 200, 250, 300, 350, 500, 600, 700, 800 mg/L) were prepared. Potassium dichromate ($K_2Cr_2O_7$) was used as Chromate (Cr VI) source and was added in quantities 42.4, 63.5, 84.7, 105.9, 127.1, 148.3, 211.8, 254.2, 296.5, 338.9 mg respectively to nutrient agar media prepared in 150 mL flasks. The media was then autoclaved and poured on to petri plates to solidify. Loops full of pure bacterial cultures were streaked on the prepared plates and were left to incubate at 37°C for 24 hours. Chromium tolerance was recorded based upon the growth of isolates.

3.5.2 Growth response to Cr (VI) concentrations; quantitative analysis

For quantitative analysis, the isolates were subjected to minimum inhibitory concentration (MIC) assay that tells the minimum concentration that inhibited the growth of all the isolates (Cappuccino and Sherman, 2013). Nutrient broth (10 mL solution in test tubes) was spiked with different concentrations (100, 200, 300, 400 mg/L) of chromium and autoclaved. The media was inoculated with loops full of bacterial isolates separately and incubated at 37°C. Inoculated broth tubes, without chromium, were used as control. The optical density (OD) was measured using a portable OD meter (HACH DR/2400) after 24 and 48 hour periods at 600 nm (Mohan *et al*., 2014; Nieto *et al*., 1989).

3.6 PLANT GROWTH PROMOTING ACTIVITY

3.6.1 Plant Growth Promoting (PGP) assays

3.6.1.1 Test for phosphate solubilizing activity

Chromium tolerant bacteria were tested for their phosphate solubilizing activity by plate assay described by Gupta *et al*. (2014). Inorganic phosphate, 5 g/L Calcium phosphate ($Ca_3(PO4)_2$), was added to nutrient agar medium. Each isolate was streaked on the center of the plates and left to incubate at 37°C for 5 days. The zone of clearing i.e. the formation of a halo around the colony confirmed phosphate solubilizing ability. The larger the diameter of the halo, the better was the phosphate solubilizing activity. The solubilization efficiency (SE) was calculated by the formula (Mohan *et al*., 2014):

$$\mathbf{SE} = \frac{(\text{halo zone} - \text{colony diameter})}{\text{growth diameter}} \times 100$$

3.6.1.2 Test for Indole Acetic Acid (IAA) production

Indole Acetic Acid is a plant growth stimulating hormone. To check IAA production by the bacterial isolates, they were first grown in Luria Broth (LB) medium that contained tryptophan as one of its constituents. The inoculated media was incubated at 37°C for 24 hours. After the incubation period, the culture broth tubes were centrifuged at 2,000 rpm for 20 min and 1 mL of the supernatants were transferred to empty sterile test tubes. 1 drop of Orthophosphoric acid and 2 mL of freshly prepared Salkowski's reagent (50mL; 49 mL, 35% $HClO_4$ + 1mL, 0.5M $FeCl_3$) was added to the tubes. The tubes were left to incubate for 30 minutes (Bent *et al*., 2001). The development of a pink color confirmed IAA production by the isolate.

3.7 PLANT GROWTH AND YIELD

3.7.1 Germination Test

A germination test is done to check the viability and growth potential of the seeds. Germination of spinach seeds bought from the farmer's market was tested using the paper towel method and the Tupperware germination test method (Yaklich, 1985) with all the reagents added.

3.7.1.1 Paper towel method

A paper towel was spread on a flat surface and moistened with tap water. 12 to 16 seeds were placed on the sheet in rows spaced evenly. Another moistened towel was laid on top so that the seeds were sandwiched between two wet sheets. The sheets were placed inside an air tight plastic bag, to retain moisture, and put in a relatively cool area with stable room temperature. The seeds were left to germinate for a period of 9 days. After the germination period the seeds were unwrapped carefully to avoid any damage to the seedlings. The number of seedlings was counted and the root and shoot lengths were recorded. Percentage of germination was calculated as:

$$\textbf{Percentage germination} = \frac{\text{Number of strongly germinating seeds}}{\text{Total number of seeds tested}} \times 100$$

3.7.1.2 Tupperware test method (with all the reagents added)

Another test was carried out to assess the effect of chromium and bacterial isolates on spinach seed germination. Moistened filter paper was placed inside petri plates and three to four seeds were added to every plate. Two milliliter of chromium solutions (100, 200, 300 and 400 mg/L) were added to the seeds followed by the addition of bacterial strains A5 and A6 with their optical density set at 0.5 (OD_{600}). The strains were cultured beforehand on nutrient agar plates and the inoculums were collected in vials (autoclaved water and spreaders were used to create bacterial suspensions). The optical density was checked using UV-Vis spectrophotometer

with wavelength set at 600 nm. Distilled water was added to the suspensions to decrease the OD down to 0.5. Once the process was completed, the plates were sealed with para films to retain moisture and placed in an area with stable, cool temperature. The root and shoot lengths were recorded after a germination period of 12 days.

3.7.2 Soil preparation

Soil in bulk quantity was spread outside in the sun for 2 to 3 days till it became completely dry. The dry soil was then added to a ball mill (soil crushing machine) to obtain crushed and powdered soil. The crushed soil was further passed through a 2 mm sieve in order to obtain very fine particles. Soil packets, 3 kg each, were made and spiked with chromium salt (1697, 3394, 5091, 6787 mg) to make varying concentrations (100, 200, 300, 400 mg/kg). Organic compost was also added to each of the five packets (one set as control) and given a contact time of two days. After two days 250 g of soil was transferred to each of the labelled pots.

3.7.3 Plant cultivation

Once the soil was ready and transferred to the pots, the pots were lightly watered. The next day 2 to 3 seedlings were sowed about half an inch deep in the moist soil and watered again. The seedlings transplanted were 3 weeks old. Seedlings were sown in a total of 50 pots; one set for both the bacterial strains (A5 and A6) to be added with 5 treatments and 5 replicates. Spinach required cool temperature, consistent watering and mulching to grow.

3.7.4 Plant harvest

Five week old spinach plants were harvested. The roots were carefully extracted from the soil and washed to remove any excess. Physical parameters like root, shoot

length and fresh weight of all the plants were measured and recorded. Dry weight was recorded the next day after leaving the plants in an oven at 65°C for 24 hours.

3.8 STATISTICAL ANALYSIS

Microsoft Excel data analysis tools were used to analyze data for germination, root and shoot lengths, fresh and dry weights. Data of replicates of each treatment, was subjected to single factor and two-way analysis of variance (ANOVA). The means and standard deviations for all parameters were also calculated.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 SOIL ANALYSIS

4.1.1 Physicochemical analyses of soil

The pH, electrical conductivity (EC) and percentage of chromium by mass in four soil samples and NUST soil tested are listed in Table 4.1. The pH of the soils fell in slightly acidic to slightly basic range, 6.5 to 7.5, whereas EC varied considerably. This was probably due to inherent factors like soil minerals, climate and soil texture. EC is a measure of amount of salts present; hence higher EC indicates more salinity. Analysis via XRF spectrometer elemental analyzer revealed that sample B2 had the highest chromium percentage by mass i.e. 1.0989% and was thus selected as chromium-rich soil for isolation of bacteria.

Table 4.1: Physicochemical parameters and chromium composition of soil samples collected from Gujrat and NUST

Sample ID	Physico-chemical		Elemental Analyzer	
	pH	EC µS/cm	Mass of Cr (%)	Cr Mol. (%)
S1*	6.86	56.3	0.1092	0.0762
S2*	6.85	163.1	0.2844	0.2869
B1*	6.50	256	-	-
B2*	6.77	550	1.0989	1.1726
NUST soil	7.11	412	-	-

*Pearl millet fields soil samples; S1& B1: sub surface soil, S2 & B2: upper surface soil

A standard curve for Cr (VI) was obtained using UV-Vis spectrophotometer wherein absorbance was plotted against chromium standards of concentrations 0, 0.25, 0.5, 0.75, 1 and 1.5 mg/L (Fig 4.1). Analysis and calculations for hexavalent chromium

revealed that amount of Cr (VI) in sample B2 was 177 mg/kg. This amount was higher than permissible limit for Cr (VI) in soil, i.e. 100 mg/kg, according to Dutch Standards. B2 sample, being chromium-rich, was considered appropriate for isolation of chromium tolerant bacteria.

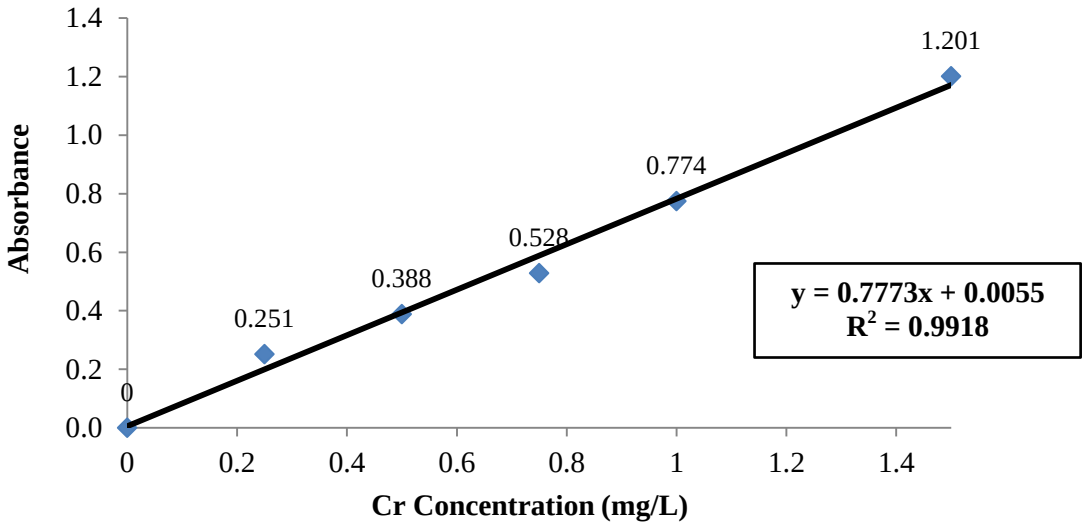

Fig 4.1 Standard curve for Cr (VI) (analysis via UV-Vis spectrophotometer)

4.2 MICROBIOLOGICAL ANALYSIS

4.2.1 Isolation and enumeration of Cr tolerant bacterial isolates

Bacterial colonies from serial dilution and spreading of soil sample B2 were analyzed and enumerated. The population density of bacterial colonies was recorded as 3.36×10^4 CFU mL^{-1} of soil, which was fairly low (Mohan *et al*., 2014). The low population density of microbes was in agreement with observations made by Revathi *et al.* (2011) who reported that heavy metals destroyed many beneficial microbes in soil. Thirteen bacterial colonies with different physical characteristics were isolated.

4.2.2 Identification of bacterial isolates

Morphological and biochemical tests on isolates revealed six strains as rod-shaped and five round-shaped. Gram staining results showed that most of isolates, nine out of eleven, were gram positive bacteria and only A5 and A13 were gram negative. These results are in accordance with earlier studies (Viti *et al*., 2002) which report high presence of tolerant gram positive bacteria in heavy metal contaminated soils. Six isolates (A2, A4, A5, A6, A10 and A13) showed were able to solubilize inorganic phosphate to varying degrees however, only one (A5) tested positive for IAA production. Table 2 shows the results of all tests conducted.

The 13 isolates were characterized at molecular level and it was found that four (A2, A4, A7, A10) belonged to *Bacillus* sp., one (A5) was attributed to the genus *Pseudomonas* and five (A3, A6, A8, A9, A11) to *Staphylococci* sp. of which three (A8, A9 and A11) were identified as *Staphylococcus aureus*. One (A13) strain was identified as *Agrobacterium tumefaciens.* Two isolates were left uncharacterized due to irregularities in their sequences. The remaining sequences obtained from 16S rRNA gene sequencing were submitted to GenBank under accession numbers listed in Table 4.2.

Table 4.2: Results of morphological and biochemical tests conducted on isolates along with their accession numbers and PGP characteristics

Strain ID	Accession Number	Catalase Test	Oxidase Test	Motility Test	Simmons' Test	MSA Test	MacConkey Test	Cell Shape	Gram Staining	IAA production	Phosphate Solubilizing
A2	KX262672	+	+	-	+	-	+	bacillus	+	-	+
A3	KX262673	+	-	-	-	-	+	coccus	+	-	-
A4	KX262674	-	-	+	-	-	+	bacillus	+	-	+
A5	KX262675	+	-	+	+	+	+	bacillus	-	+	+
A6	KX262676	+	-	-	-	+	+	coccus	+	-	+
A7	KX262677	+	-	+	+	+	+	bacillus	+	-	-
A8	KX262678	+	-	-	+	+	+	coccus	+	-	-
A9	KX262679	+	-	-	-	+	-	coccus	+	-	-
A10	KX262680	+	+	-	-	+	+	bacillus	+	-	+
A11	KX262681	+	-	-	-	-	-	coccus	+	-	-
A13	KX262682	-	-	-	-	+	-	bacillus	-	-	+

A phylogenetic tree was constructed (Fig 4.2) using the software MEGA 7. This tree shows strains isolated in this study and other related chromium-tolerant strains that have been quoted in literature (Khan *et al*., 2015; Narayani and Shetty, 2013; Suresh *et al*., 2011; Viti *et al*., 2003). This is the first time that *Pseudomonas plecoglossicida* and *Staphylococcus haemolyticus* have been reported from Pakistan and identified as chromium-tolerant bacteria.

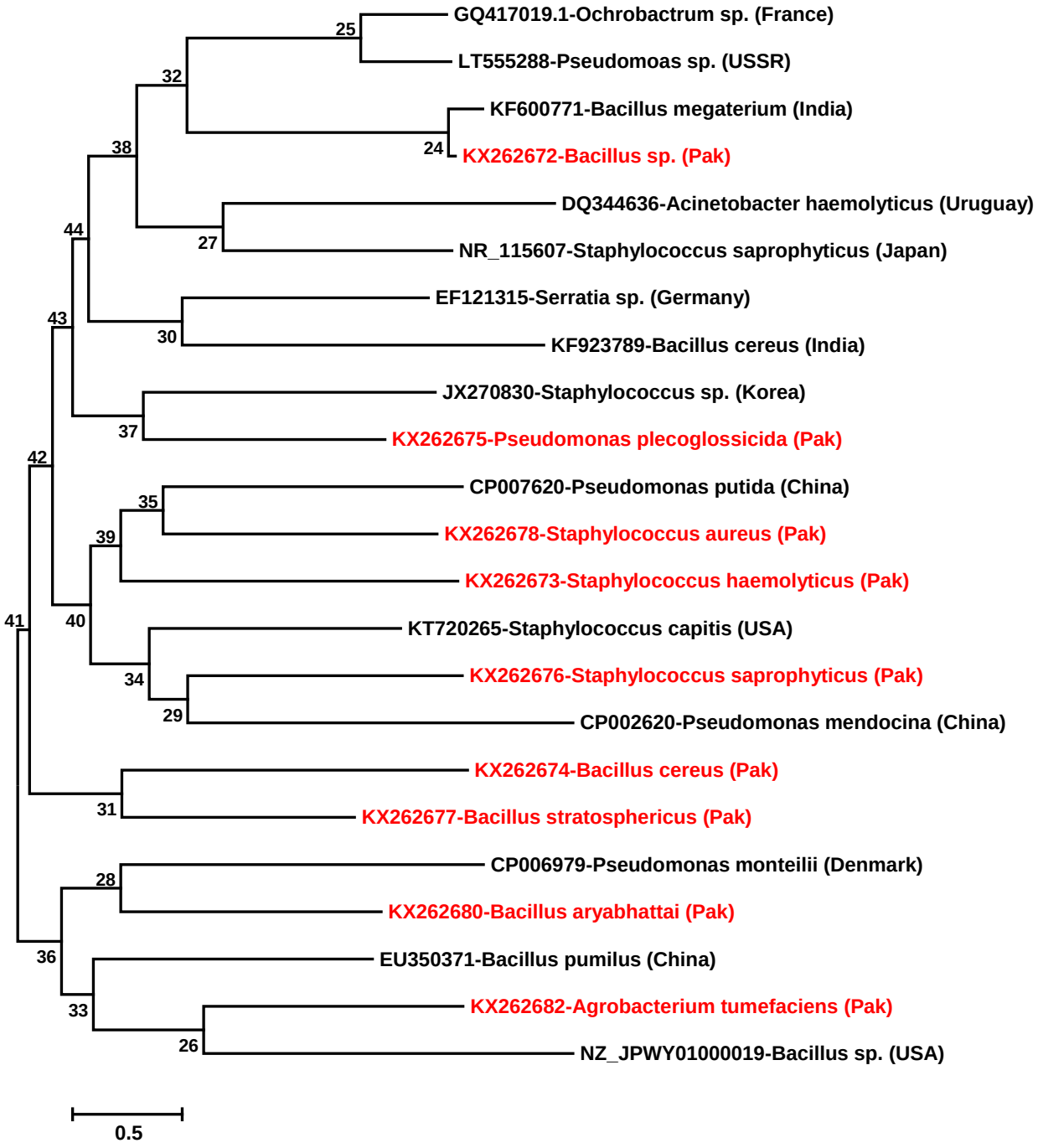

Fig 4.2 Phylogenetic neighbor-joining tree of isolates created with MEGA 7 software

4.2.3 Minimum inhibitory concentration (MIC) of Chromium (VI)

The eleven isolates were screened to determine their Cr (VI) tolerance and were subjected to MIC assays. Strains A5 and A6, from the genus *Pseudomonas* and *Staphylococcus* respectively, were found to be tolerant of Cr (VI) for concentration as high as 700 mg/L in agar plate method (Table 4.3). The next most tolerant strains, for concentration up to 350 mg/L Cr (VI), were A2 and A3 from genus *Bacillus* and *Staphylococcus* respectively.

Table 4.3: Chromium tolerance of bacterial isolates against various Cr (VI) concentrations in nutrient agar plate method

Strain ID	Chromium Concentrations (mg/L)									
	100	150	200	250	300	350	500	600	700	800
A2	+++	+++	+++	+++	+++	++	-	-	-	-
A3	+++	+++	+++	+++	+++	++	-	-	-	-
A4	+++	+++	+++	+++	++	-	-	-	-	-
A5	+++	+++	+++	+++	+++	+++	+++	++	+	-
A6	+++	+++	+++	+++	+++	+++	+++	++	+	-
A7	+++	+++	+++.	+++	++	-	-	-	-	-
A8	+++	*+++	*+++	++	-	-	-	-	-	-
A9	+++	*+++	++	-	-	-	-	-	-	-
A10	+++	+++	++	-	-	-	-	-	-	-
A11	+++	+++	+++	+++	++	-	-	-	-	-
A13	+++	+++	+++	+++	++	-	-	-	-	-

Legend: +++ maximum growth, ++ moderate growth, + less growth, - no growth
*Bacteria change color at the specific concentration

In nutrient broth method, maximum tolerance to 300 mg/L Cr (VI) was observed in A5 (0.483 OD_{600}), approximately 9% decrease in growth, and A6 (0.163 OD_{600}) with 19% decrease in growth w.r.t control after Cr (VI) addition. These were followed by A3 (0.141 OD_{600}) and A2 (0.134 OD_{600}) as illustrated in Fig 4.3. These

results conformed to those recorded on nutrient agar plate MIC assay. However, analysis in nutrient broth showed that all isolates followed a similar growth pattern i.e. growth decreased (measured as OD) as concentration of chromium increased. Past studies also confirm that *Pseudomonas* sp. is reportedly highly tolerant to Cr (VI) and effective in its reduction (Rahman *et al*., 2006) as are *Staphylococci* sp. (Kouadjo and Zeze, 2010) and *Bacillus* sp. (Camargo *et al*., 2003; Ezaka and Anyanwu, 2010).

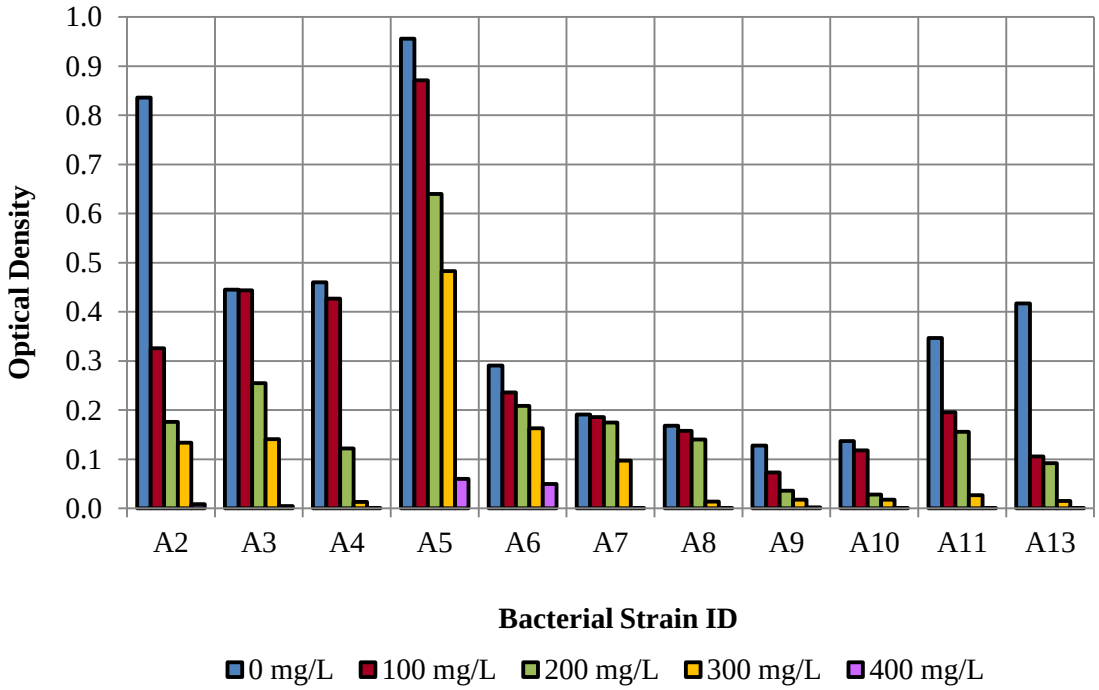

Fig 4.3 Chromium tolerance of bacterial isolates against various Cr (VI) concentrations in nutrient broth

4.3 PLANT GROWTH PROMOTING ASSAYS

4.3.1 IAA production and Phosphate solubilization efficacy

The eleven isolates were subjected to tests under *in vitro* to determine their ability to produce Indole Acetic Acid and solubilize inorganic phosphate. Six out of eleven isolates (A2, A4, A5, A6, A10, and A13) tested positive for phosphate solubilization (Fig 4.4) which included the Cr (VI) tolerant A2, A5 and A6. Maximum solubilization efficacy was found in A13 (80%) followed by A2 (77%), A5 (76%),

A10 (72%), A6 (50%) and A4 (44%). According to de Freitas et al. (1997), good phosphate solubilizers are known to produce a clearing zone of diameter more than 15 mm around their colonies. This was true for isolates A1, A2, A5, A6, A10, and A13 which may be regarded as efficient phosphate solubilizers. On the other hand, only A5 out of eleven tested positive for IAA production. Bacteria belonging to the genus *Pseudomonas* are known to be able to produce IAA and that too higher than many other species (Ahmed *et al*., 2008). The results showed that many heavy metal tolerant bacteria, like *Pseudomonas* sp. (A5), *Bacillus* sp. (A2) and *Staphylococci* sp. (A6) from this work, were also plant growth-promoting as reported earlier by Gadd (1990).

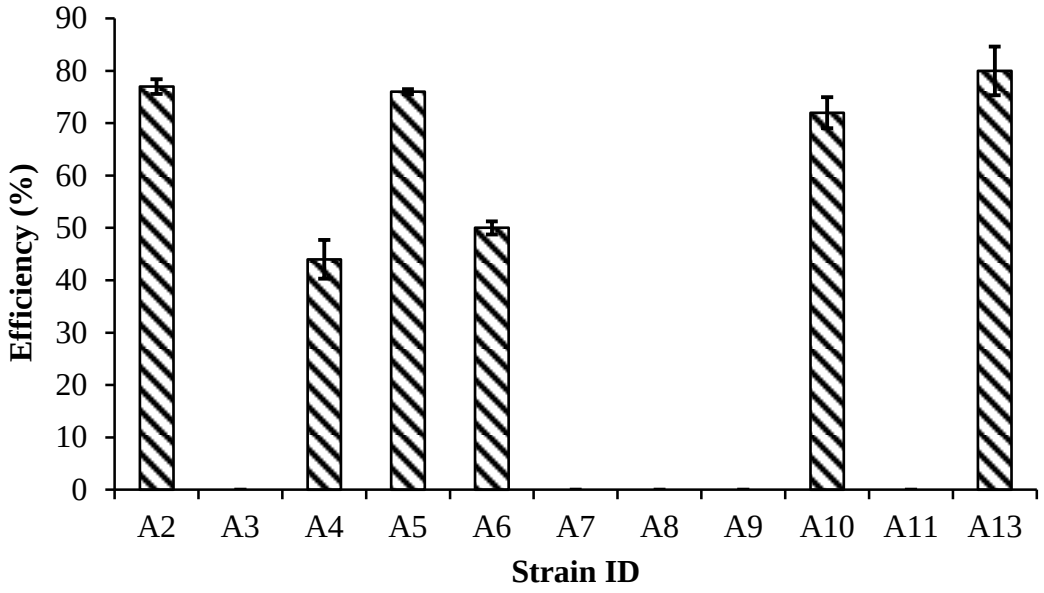

Fig 4.4 Phosphate solubilizing efficacy of Cr (VI) tolerant bacteria screened from soil

4.4 PLANT GROWTH AND YIELD

4.4.1 Germination of spinach seed

The germination percentage of spinach seeds used for experimentation was calculated as 54%. The Cr (VI) tolerant and PGP isolates A5 (*Pseudomonas* sp.) and

A6 (*Staphylococci* sp.) were selected and their effect on seed germination and plant growth was evaluated at different concentrations of Cr (VI). The results of germination tests showed a boost in germination of spinach seeds from 54 to 100% on addition of isolate A5 and from 54 to 71% on the addition of A6. The results showed percentage of germination of spinach seeds to be much higher, i.e. between 71 and 100%, for the seeds treated with the isolate A5 and various Cr (VI) treatments. Whereas, isolate A6 did not have much effect on seed germination percentage, in the presence of Cr (VI), which stayed low; ranging from 33% to 71%, as shown by Fig 4.5.

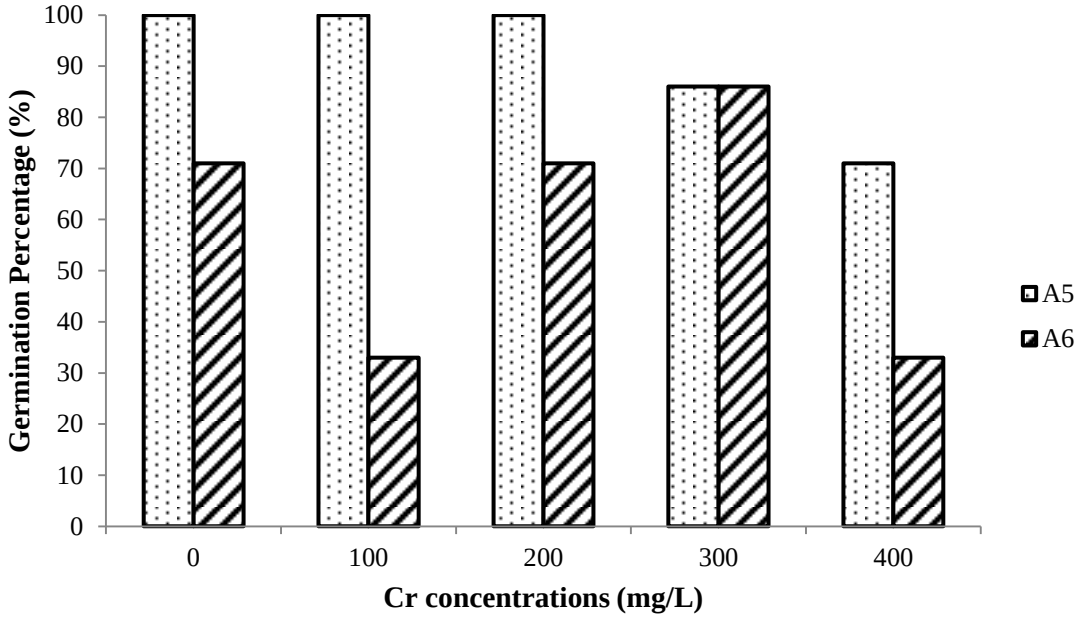

Fig 4.5 Percentage of germination of spinach seeds with PGPB and Cr (VI) treatments

Figs 4.6 and 4.7 illustrate how isolates A5 and A6 affected radicle and plumule elongation, during seed germination of spinach, treated with different concentrations of Cr (VI). Increase in concentration of chromium inhibited growth of radicle and plumule but with isolate A5, results were significant at 5% level of probability. The results are insignificant (p-value > 0.05) for isolate A6. A detailed study by Mohite

(2013) revealed that IAA producing strains, like *Pseudomonas* species, are good plant growth promoters and show significant increase in plant growth, yield and germination. This holds true as A5 (*Pseudomonas* sp.) showed similar growth enhancing traits and boosted germination.

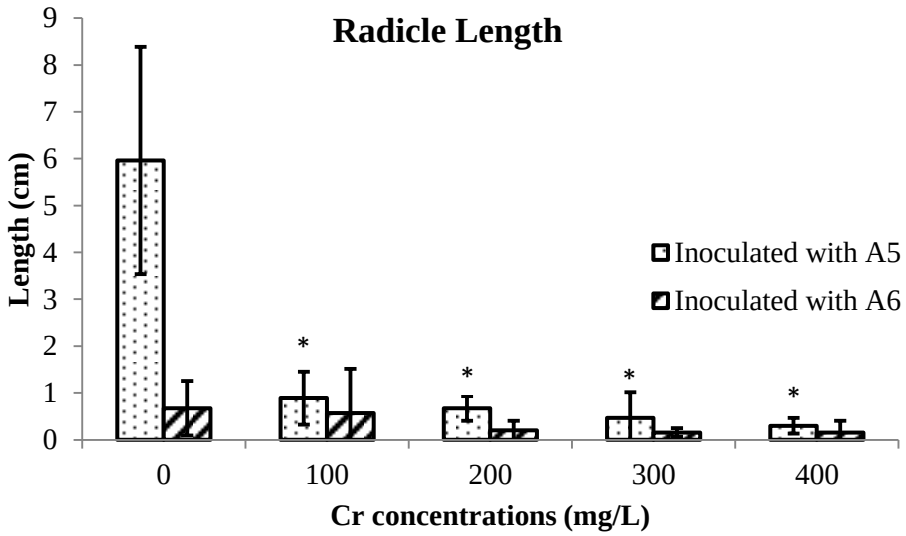

Fig 4.6 Germination of spinach seeds (radicle) with PGPB and various Cr treatments

* at 5% level of significance

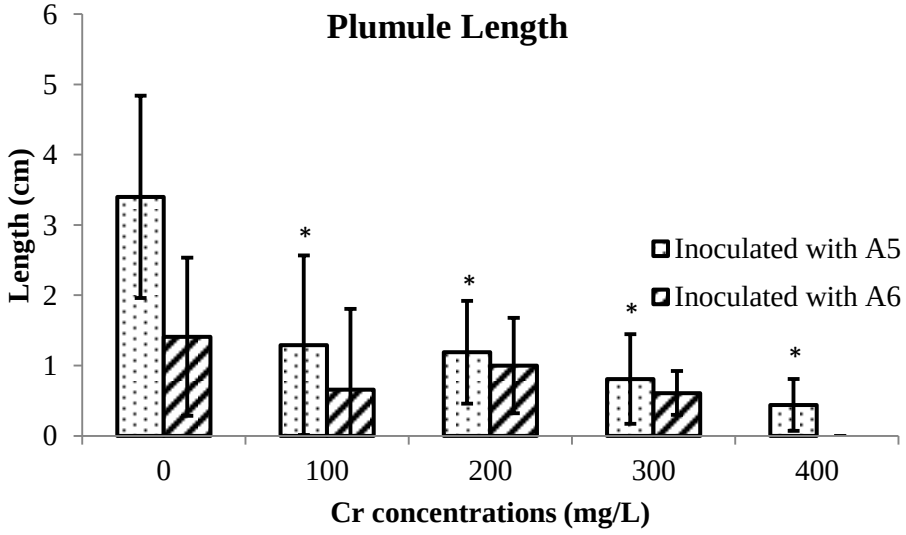

Fig 4.7 Germination of spinach seeds (plumule) with PGPB and various Cr treatments

* at 5% level of significance

4.4.2 Effect of PGPB and Cr (VI) on spinach seedlings

After 2 weeks growth in inoculated soil, the root and shoot lengths and wet and dry biomass of the spinach plants were recorded. The root and shoot length of the plants remained more or less unaffected by the presence of PGP bacteria as shown by Figs 4.8 and 4.9. Root length increased by 1% with A5 and the shoot length decreased by 13% with respect to the control. In the presence of A6, the shoot length decreased by 1% and the root length increased by 20% with respect to control but then showed inconsistent behavior with increasing Cr (VI) concentrations. Sayel *et al*. (2014) reported similar trend of decreasing root (30, 46 and 66% decrease respectively) and shoot (17, 20 and 55% decrease respectively) lengths of clover plants with the addition of 100, 200 and 300 mg/L Cr (VI). It was also reported in the same study that inoculation with *Pseudomonas* sp. enhanced the root and shoot lengths by 15 to 20% w.r.t. control which was not observed in this study.

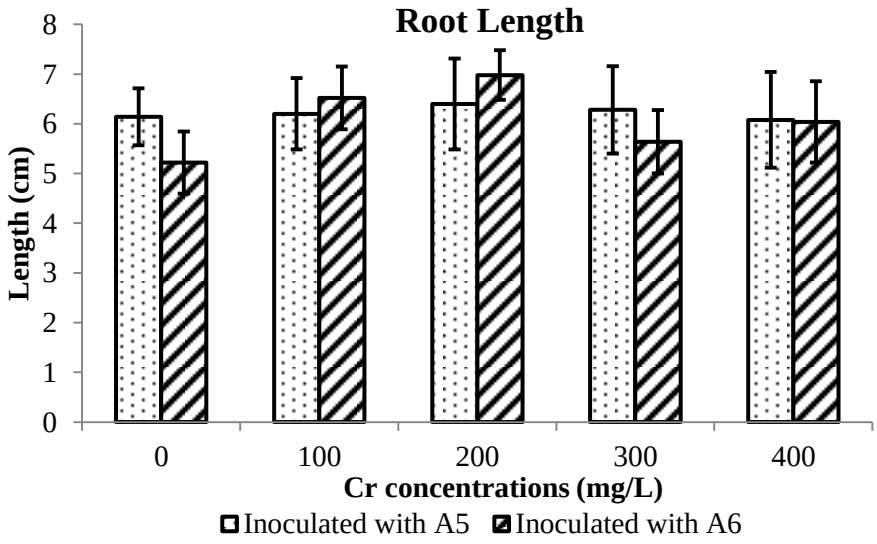

Fig 4.8 Growth of spinach seedlings (root) with various Cr treatments

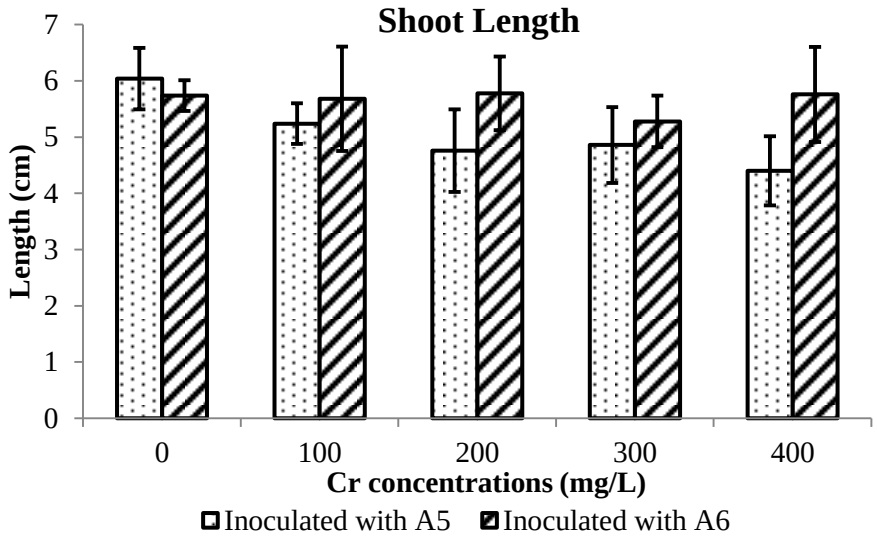

Fig 4.9 Growth of spinach seedlings (shoot) with various Cr treatments

On the other hand, the addition of isolates A5 (*Pseudomonas* sp.) and A6 (*Staphylococci* sp.) brought about changes in plant biomass. As shown in Fig 4.10, both isolates were able to enhance biomass, A5 by 44% and A6 by 7% with respect to control, in the presence of 100 mg/kg Cr (VI). As the concentrations increased the biomass decreased but trend remained at 5% level of significance with both isolates.

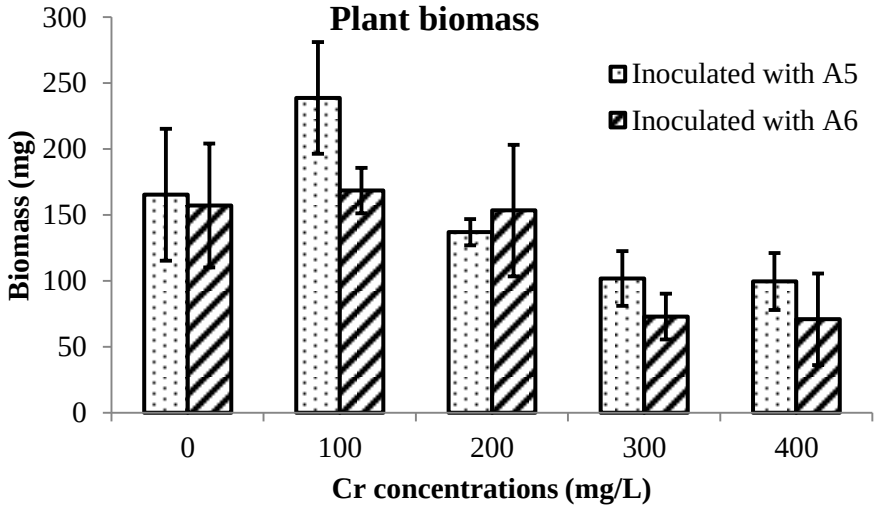

Fig 4.10 Biomass (dry) spinach seedlings with various Cr treatments

(All significant at $p<0.05$)

Application of bacterial inoculants to plants has been reported to not only improve plant growth but also enhance the yield of crops (Mirza *et al.*, 2001). Another study in which addition of PGP bacteria have been known to significantly increase fresh and dry weights of plants, maize seedlings, after 20 days of growth in chromium stressed soil (Lwin *et al*., 2012) also supported results obtained with spinach.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Based on experimental results and statistical analyses, the following conclusions were drawn from the study conducted:

i. A total of 13 bacterial strains were isolated from chromium-rich soil based on their differing morphological and biochemical characteristics. The principal species isolated included *Staphylococcus aureus, Pseudomonas plecoglossicida, Staphylococcus saprophyticus, Bacillus cereus, Bacillus stratosphericus, Staphylococcus haemolyticus, Bacillus sp., Bacillus aryabhattai* and *Agrobacterium tumefaciens*. The screening process, to ascertain chromium tolerance of these isolates, revealed that only *Pseudomonas plecoglossicida* (A5) and *Staphylococcus saprophyticus* (A6) were able to tolerate Cr (VI) concentration upto 700 mg/L followed by *Staphylococcus haemolyticus* (A3) and *Bacillus* sp. (A2) tolerating Cr (VI) upto 350 mg/L.

ii. The plant growth-promoting activity, under *in vitro*, of the isolates showed that 7 out of the 11 were able to effectively solubilize phosphate, which included both the highly tolerant species; *Pseudomonas plecoglossicida* and *Staphylococcus saprophyticus*. Only 1 isolate; *Pseudomonas plecoglossicida* was able to produce indole acetic acid.

iii. *Pseudomonas plecoglossicida* proved itself to have the potential of a prominent plant growth-promoter during the germination and pot trial experiments. It increased the percentage of seed germination by 17 to 46%

and also increased spinach plant's biomass by 44% with respect to the control.

5.2 RECOMMENDATIONS

Following recommendations are proposed for future research:

i. A study may be conducted to test the effect of a consortium of plant growth-promoting bacteria on plants grown in heavy metals-stressed soils. A consortium of bacteria, all possessing one or more PGP traits, may be more effective in enhancing growth and development of plants.

ii. Microorganisms may help reduce the uptake of heavy metals by plants. However these need to be identified in order to employ them for heavy metal reduction. Identification of genes responsible for the reduction of heavy metals in microorganisms would help distinguish them faster and also maximize their potential through modification of that particular gene expression.

iii. As of now, not much work has been done to understand the metabolism of heavy metal-tolerant bacteria. Research can be done to study the mechanism these bacteria follow while improving plant growth.

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APPENDICES

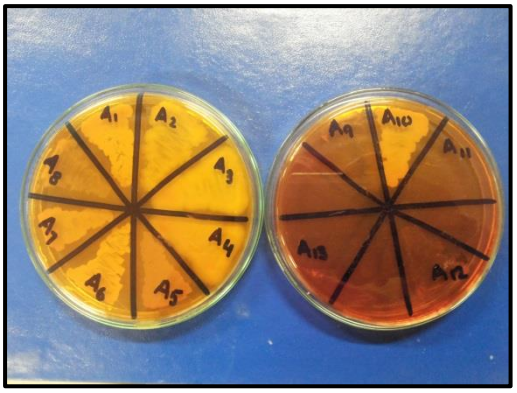
MORPHOLOGICAL ANALYSIS

Table 1 Colony morphology observed of the isolated bacterial strains

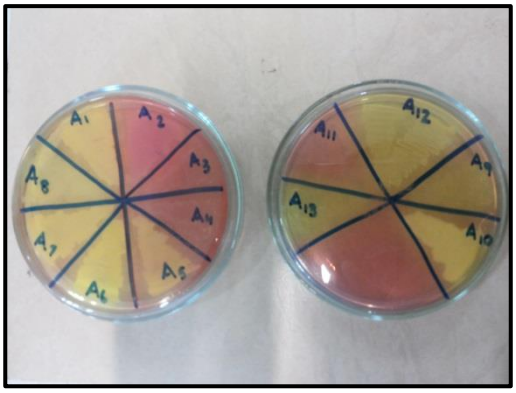
Strain ID	Colony Morphology						
	Size	**Form**	**Margin**	**Elevation**	**Surface**	**Color**	**Odor**
A1	Small	Round	Smooth	Raised	Smooth	Cream	No
A2	Pinpoint	Round	Smooth	Flat	Smooth	Cream	Yes
A3	Small	Round	Filamentous	Raised	Rough	Off-white	Yes
A4	Small	Filamentous	Filamentous	Raised	Rough	Off-white	Yes
A5	Pinpoint	Round	Smooth	Convex	Smooth	Cream-yellow	No
A6	Pinpoint	Round	Smooth	Convex	Smooth	Cream-Yellow	Yes
A7	Pinpoint	Irregular	Filamentous	Raised	Smooth	Cream-yellow	No
A8	Small	Filamentous	Filamentous	Flat	Rough	Dull pink	Yes
A9	Small	Round	Lobate	Flat	Rough	Peach-pink	No
A10	Small	Round	Smooth	Convex	Smooth	Cream	Yes
A11	Small	Round	Smooth	Raised	Smooth	Bright yellow	No
A12	Small	Irregular	Lobate	Flat	Rough	Orange-yellow	Yes
A13	Small	Irregular	Lobate	Flat	Rough	Peach	Yes

APPENDIX B

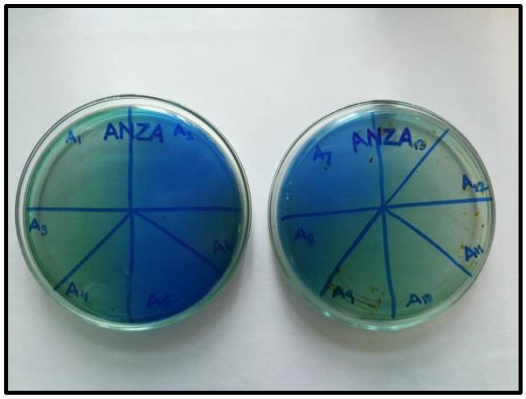
BIOCHEMICAL TESTS: a pictorial view



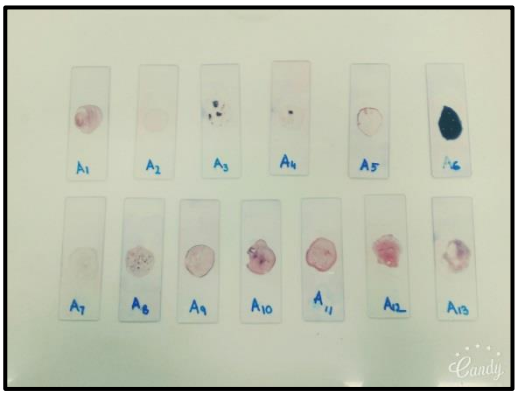
MacConkey Agar Test



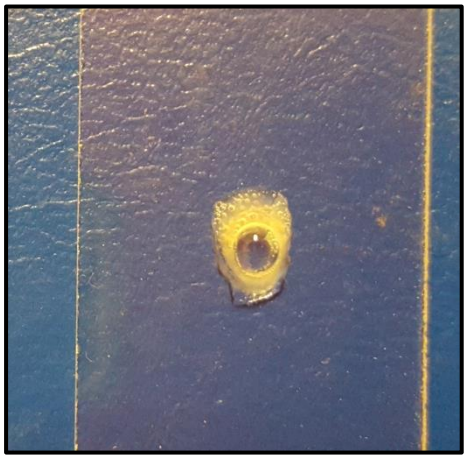
Mannitol Salt Agar Test



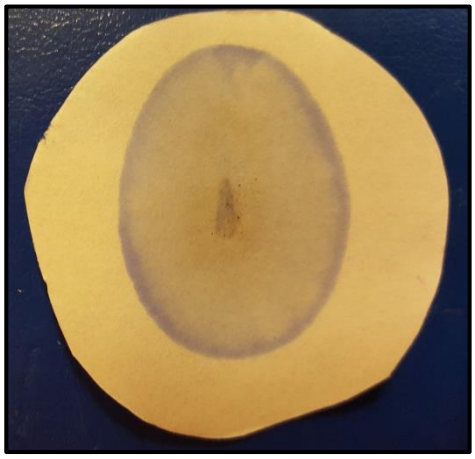
Simmons' Citrate Agar Test



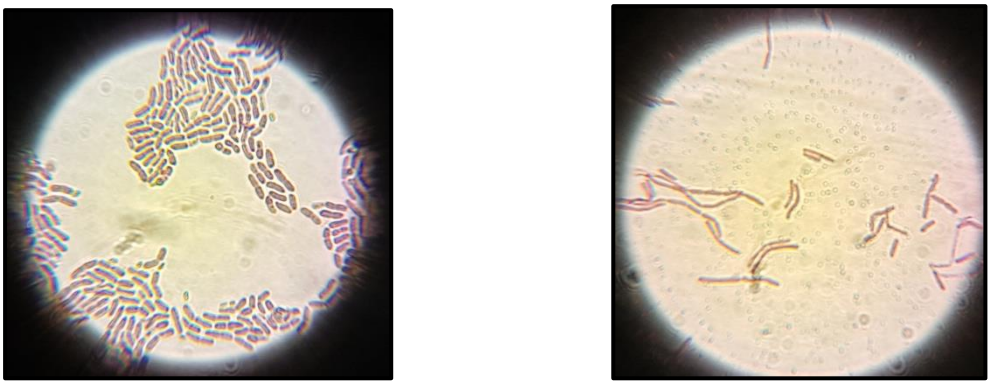
Gram Staining Slides



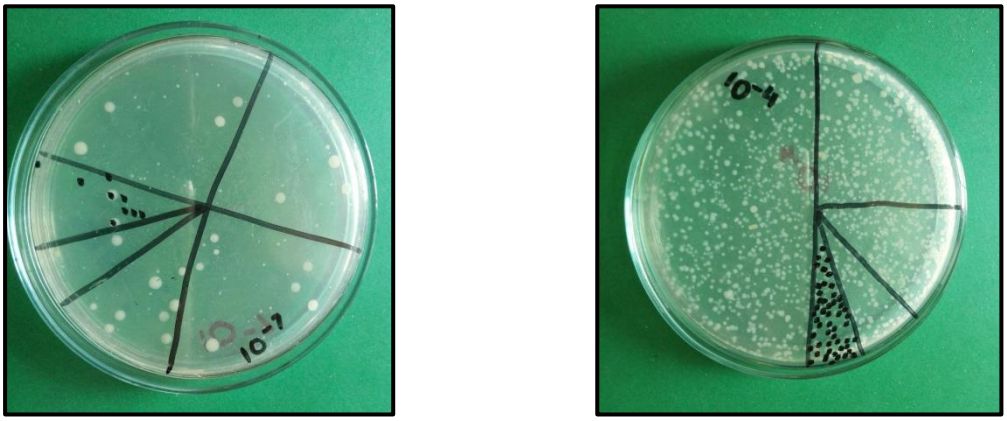
Catalase Test



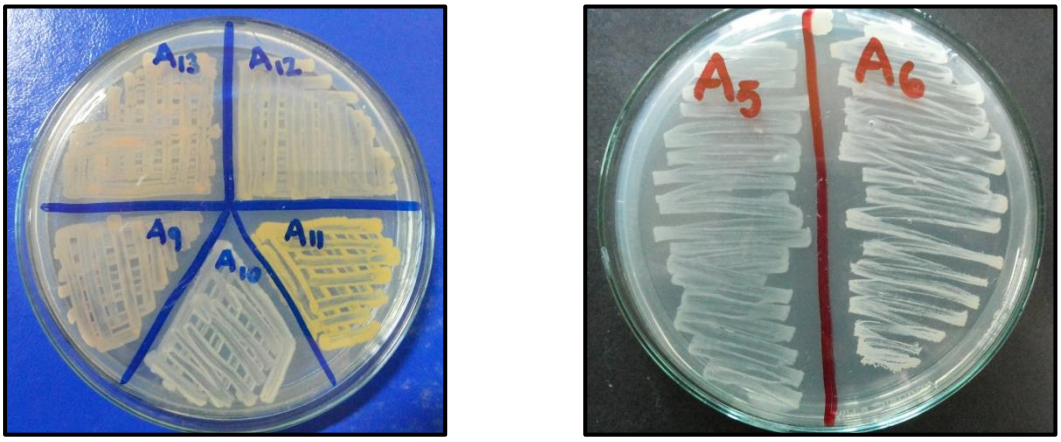
Oxidase Test



Gram Staining: rod shaped bacteria observed under the microscope



Bacterial load count after serial dilutions



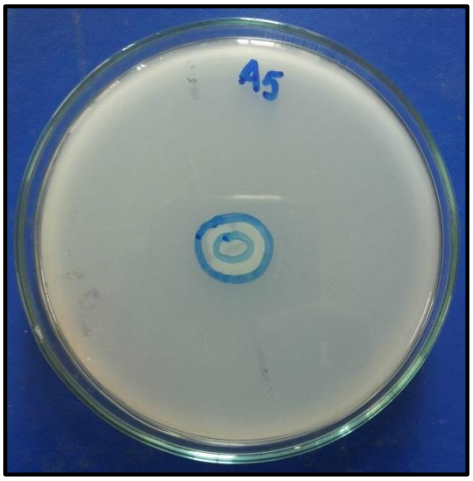
Isolated strains: streaked and preserved on nutrient agar plates

APPENDIX C

PLANT GROWTH PROMOTING ACTIVITY

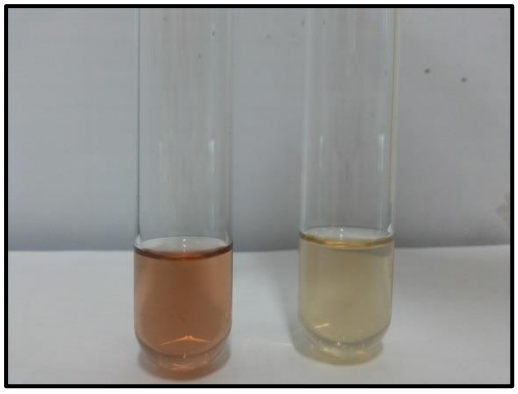
Table 2 Test for phosphate solubilizing activity

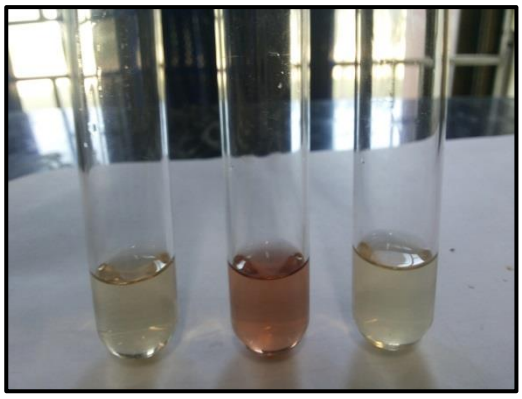
Strain ID	Solubilizing Efficiency		
	Halo zone (mm)	Colony diameter (mm)	SE (%)
A1	18	6	67
A2	26	6	77
A3	0	7	0
A4	9	5	44
A5	29	7	76
A6	18	9	50
A7	0	5	0
A8	0	4	0
A9	0	5	0
A10	18	5	72
A11	0	6	0
A12	0	5	0
A13	20	4	80



Strains placed in the center of nutrient agar plates spiked with calcium phopshate

APPENDIX C





Indole acetic acid production by bacterial isolates

APPENDIX D

PLANT GROWTH AND YIELD

Table 3 Percentage of germination of spinach seeds with PGPB and Cr (VI) treatments

Germination Percentage (%)					
Strain ID	**Chromium Concentration (mg/L)**				
	0	**100**	**200**	**300**	**400**
A5	100	100	100	86	71
A6	71	33	71	86	33

Table 4 Effect of chromium tolerant PGPB on germination at 100-400 mg/L of Cr (VI) concentrations (means of 7 replicates)

Strain ID	**Part**	**Chromium Concentration (mg/kg)**				
		0	**100**	**200**	**300**	**400**
A5	**Radicle**	5.96 ± 2.43	0.89 ± 0.56*	0.67 ± 0.26*	0.47 ± 0.54*	0.30 ± 0.17*
	Plumule	3.40 ± 1.44	1.29 ± 1.28*	1.19 ± 0.73*	0.81 ± 0.64*	0.44 ± 0.37*
A6	**Radicle**	0.67 ± 0.58	0.57 ± 0.94	0.20 ± 0.21	0.16 ± 0.09	0.16 ± 0.25
	Plumule	1.41 ± 1.13	0.66 ± 1.14	1.00 ± 0.68	0.61 ± 0.31	0.00 ± 0.00

*at 5% level of significance

Table 5 Effect of chromium tolerant PGPB on plant growth at 100-400 mg/L of Cr (VI) concentrations (means of 5 replicates)

Strain ID	**Part**	**Chromium Concentration (mg/kg)**				
		0	**100**	**200**	**300**	**400**
A5	**Root**	6.14 ± 0.57	6.20 ± 0.72	6.4 0± 0.91	6.28 ± 0.88	6.08 ± 0.96
	Shoot	6.04 ± 0.55	5.24 ± 0.36*	4.76 ± 0.74*	4.86 ± 0.67*	4.40 ± 0.62*
A6	**Root**	5.22 ± 0.62	6.52 ± 0.63*	6.98 ± 0.50*	5.64 ± 0.64*	6.04 ± 0.82*
	Shoot	5.74 ± 0.27	5.68 ± 0.93	5.78 ± 0.66	5.28 ± 0.46	5.76 ± 0.85

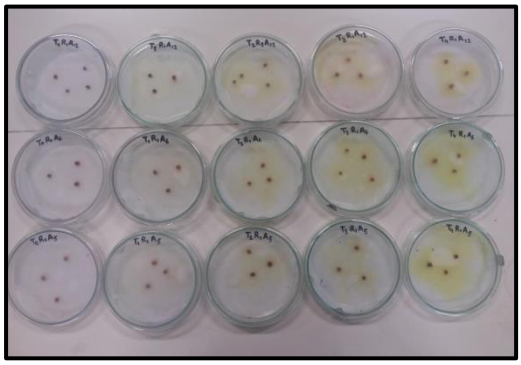
* at 5% level of significance

APPENDIX D

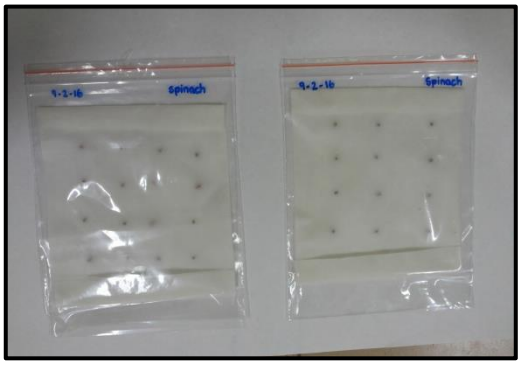
Table 6 Effect of chromium tolerant PGPB on plant biomass at 100-400 mg/L of Cr (VI) concentrations (means of 5 replicates)

Strain ID	Biomass	Chromium Concentration (mg/kg)				
		0	100	200	300	400
A5	Fresh	620 ± 79.7	317 ± 34.1*	146 ± 10.4*	110 ± 22.3*	103 ± 22.6*
	Dry	165 ± 50.0	239 ± 42.4*	137 ± 9.94*	102 ± 20.7*	100 ± 21.7*
A6	Fresh	363 ± 41.0	203 ± 39.4*	161 ± 50.8*	82 ± 18.2*	76 ± 34.7*
	Dry	157 ± 47.0	169 ± 17.3*	153 ± 49.9*	73 ± 17.3*	71 ± 34.7*

* at 5% level of significance



Germination Test (with all reagents)



Germination Test

Pots preparation

Pot experiment: spinach planted