EFFICIENCY OF INDIGENOUS BACTERIA FOR THE REDUCTION OF LEAD AND CADMIUM IN THE POLLUTED WATER

MD. JASIM UDDIN

DEPARTMENT OF HORTICULTURE

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

DHAKA-1207

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BY

MD. JASIM UDDIN

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Approved by:

Prof. Dr. Abul Hasnat M Solaiman Department of Horticulture Sher-e-Bangla Agricultural University **Supervisor**	**Prof. Dr. Md. Nazrul Islam** Department of Horticulture Sher-e-Bangla Agricultural University **Co-Supervisor**

Prof. Dr. Mohammad Humayun Kabir
Chairman
Examination Committee

DEPARTMENT OF HORTICULTURE
Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207
PABX: 9110351 & 9144270-79

CERTIFICATE

*This is to certify that the thesis entitled, "**EFFICIENCY OF INDIGENOUS BACTERIA FOR THE REDUCTION OF LEAD AND CADMIUM IN THE POLLUTED WATER**" submitted to the Department of Horticulture, Sher-e-Bangla Agricultural University, Dhaka, in the partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS) in HORTICULTURE,** embodies the result of a piece of bona fide research work carried out by **MD. JASIM UDDIN,** Registration No. **11-04646** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course ofthisinvestigationhasbeendulyacknowledgedandstyleofthisthesishavebeen approved and recommended forsubmission.

Dated: **June, 2018**
Dhaka, Bangladesh

Prof. Dr. Abul Hasnat M Solaiman
Department of Horticulture
Sher-e-Bangla AgriculturalUniversity
Dhaka-1207
Supervisor

DEDICATED TO

MY BELOVEDPARENTS

&

RESPECTEDRESEARCH SUPERVISOR

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ABSTRACT

The present experiment was conducted at the Laboratory of Microbiology, Department of Botany, University of Dhaka from October 2017 to February 2019 to evaluate the efficiency of indigenous bacteria for the reduction of lead and cadmium in the polluted water. The experiment consisted of two factors: Factor A: Bacterial strains (5 levels) as-DRW-20, TRS-21, TRS-24, TRS-31, TRS-32 and FactorB:Heavymetalconcentration(3levels)as-HM_0(riverwater),HM_1(500.010 ppm of Pb) and HM_2 (1000.011 ppm of Cd). The two factor experiment was laid outinRandomizedCompleteBlockDesign(RCBD)withthreereplications.Outof 5bacterialstrains,maximumreductioncapabilityfrom500.010ppmofPbwasdone by TRS-31 (238.98 ppm) while DRW-20 (221.65 ppm) showed the minimum. Whereas maximum reduction capability from 1000.011 ppm of Cd was noticed in TRS-31 (381.12 ppm) while TRS-32 (375.31 ppm) showed the minimum. In view of overall performance, the bacterial strain TRS-31 (*Pseudomonas mendocina*) showedmaximumreductioncapabilityofbothPbandCdfromthesolution.Results indicatedthattheinteractionbetweenbacterialstrainsandheavymetal(PbandCd) concentrations exerted significant influence on the bioremediation of Pb and Cd. It is expected that the results obtained from this investigation will contribute in the quality forecast of soil, water and vegetable use for everyday life and a way in remediation of Pb and Cd using indigenousbacteria.

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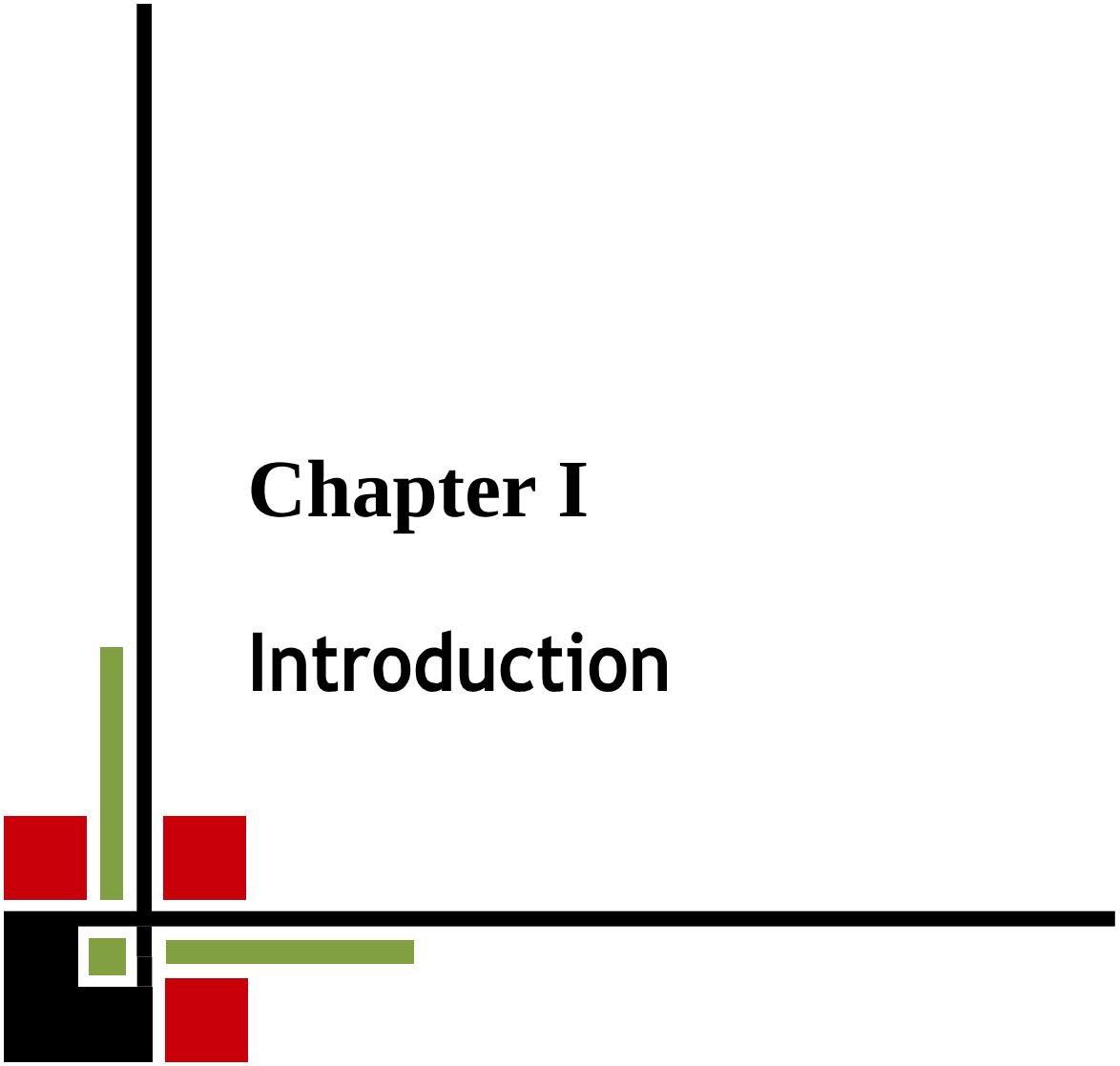
ABBREVIATIONS AND ACRONYMS

%	Percent	NB	Nutrient Broth
&	And	nm	Nanometer
^{o}C	Degree Celsius	No.	Number
Cfu	Colony forming unit	NO_2^-	Nitrite
cm	Centimeter	NO_3^-	Nitrate
Conc.	Concentration	O.D	Optical Density
e.g.	Exempli gratia	P	Phosphorus
Ed.	Editor	pH	Negative Logarithm of hydrogen ion concentration
Eds.	Editors	pp.	Pages
et al.	et alibi (with others)	Sec.	Second
etc.	Etcetera	sp.	Species (Singular)
Fig.	Figure	U.V	Ultraviolet
g.	Gram	V.P	Voges-Proskauer
hrs.	Hours	Viz.	Videli (namely)
i.e.	Id est (that is)	Vol.	Volume
Inc.	Incorporation	w/v	Weight per volume
L or l	Liter	µ	Micron/micrometer
M.R	Methyl Red	µg	Microgram
Mg	Milligram	µm	Micrometer
min.	Minute	ANOVA	Analysis of Variance
Ml	Milliliter	CV%	Percentage of Coefficient of Variation
N	Normal Concentration	Df	Degrees of freedom
NA	Nutrient agar	HSD	Highest Significant Difference
NaCI	Sodium Chloride	FAO	Food and Agricultural Organization of the United Nations



Chapter I

Introduction

CHAPTER I
INTRODUCTION

Heavy metal pollution of agricultural soil, water and vegetables is one of the most severe ecological problems in Bangladesh. Nowadays, with the growth of industrialization and extraction of natural resources, there has been a considerable increase in the discharge of industrial waste to the environment, mainly soil and water, which has led to the accumulation of heavy metals. The increasing concentration of several metals in soil and water due to industrial revolution has created an alarming situation for human life and aquatic biota. They are stable and cannot be degraded or destroyed and therefore they tend to accumulate in soils and sediments.

Dhaka city is surrounded by several rivers and canals of which Turag and Dholeshwari river receive partially treated and untreated sewage effluent, sewage polluted surface runoff and untreated industrial effluent throughdifferent government and non-government authority from nearby residence and industrial areas. Heavy metals in surface water bodies, ground water and soils can be either from natural or anthropogenic sources. Industrial wastes, atmospheric deposition from crowded cities and other domestic wastes are among the major sources of heavy metals in the urban sewage (Sorme and Lagervist, 2002). Wastewatercarries appreciable amounts of trace toxic metals which often lead to degradation of soil healthandcontaminationoffoodchainmainlythroughthevegetablegrownonsuch soils.

Irrigationofagriculturallandwithwastewaterleadstocontinuousbuildupofmetals at these sites which gets accumulated in the vegetables and crops growing on these sites. Long term use of wastewater for irrigation can cause accumulation ofthese

metals in soil which can be further translocated to food crops and thus enter food chain (Arora *et al.*, 2008; Gupta *et al.*, 2010; Singh *et al*., 2010).

Uptake of heavy metals by crops may be done through absorption from contaminated soils through roots or by deposition on foliar surfaces (Jassir *et al.*, 2005).Vegetables,especiallyleafyvegetables,accumulatehigheramountsofheavy metals (Sharma and Kansal, 1986). Roots and leaves of herbaceous plants retain higher concentration of heavy metal than stems and fruits (Yargholi and Azimi, 2008).

Heavy metals are one of a range of important types of contaminants that can be found on the surface and in the tissue of fresh vegetables (Bigdeli and Seilsepour, 2008).Severalelements,suchaslead(Pb),cadmium(Cd),nickel(Ni),cobalt(Co), chromium (Cr), Copper (Cu) and selenium (Se) (IV) can be harmful to plants and humans even at quite low concentrations (Bowen, 1979). The heavy metals are absorbed by crops along with other essential plantnutrients.

Accumulation of heavy metals beyond permissible limits affects vital organs like, kidneys, bones, liver and blood and causes serious health hazards. Health effects associated with heavy metals like, cadmium, copper, lead and chromium include gastrointestinal effects, renal impairment, neurological disorders, cardiovascular troubles, bone problems, convulsions, paralysis etc. Toxicological studies have foundheavymetalstobecarcinogenic,teratogenic,mutagenicandneurotoxic(EU, 2002).

During the recent era of environmental protection, the use of microorganisms for therecoveryofheavymetalsfromsoil,sedimentsandwateraswellasemployment of plants for landfill applications has generated growing attention. The role of microorganisms in biotransformation of heavy metals into nontoxic forms iswell-

documented and understanding the molecular mechanism of metal accumulation has numerous biotechnological implications for bioremediation of metal contaminated sites. Use of microorganisms for remediation purposes is thus a possiblesolutionforheavymetalpollutionsinceitincludessustainableremediation technologiestorectifyandre-establishthenaturalconditionofsoil.Bioremediation is a general concept that includes all those processes and actions that take place in order to improve an environment, already altered by contaminants, to its original status.

Bioremediation has been proposed as a cost effective, environmentally friendly alternative modern emerging technology which can be applied to several contaminants and site conditions.

Objectives

Considering the above findings, the present study was undertaken to achieve the following objectives:

- Determination of the pollution level of Pb and Cd in Dhaleshwari andTurag river water, soil and vegetableleaves;
- Enumeration and characterization of the bacterial isolates;and
- Bioremediation of heavy metals using metabolically active indigenous bacteria having heavy metal degradationcapabilities.

Chapter II

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

The wastes and wastewaters generated by different kinds of industries and its indiscriminate discharge into natural systems is a concerning issue all over the world.Thewastesandeffluentscontainvaryingamountsofdifferentenvironmental toxins and heavy metals such as Cu, Ni, Zn, Pb, Cd, Hg etc. They are thereby creating severe impact on the quality of water and contaminating soils and cropsof that area as well. Ahmed *et al*. (2012) and Zakir *et al*. (2015) reported that the surfacewaterandsoiloftheindustrialareainDhakaandGazipurDistrictarehighly contaminated with Zn, Cr, Cu, Pb, and Cd due to wastewater discharge from industries.Farmersusethecontaminatedwatertoirrigatevegetables,causingheavy metal contamination in vegetables in the district. In Bangladesh, industrial wastes and effluents are being randomly discharged without treatment into the natural systems and creating serious environmental hazards. But, the data regarding the extent of hazard is scarce. So, this study includes the sources, characteristics, evaluationandscenariosofindustrialwastesandeffluents,sourcesandtoxiceffects of heavy metals on soils and crops and their possible remediationtechniques.

2.1 Scenarios of industrial wastes and effluents in and aroundDhaka

The contamination of soil, water and vegetables by heavy metals is aglobal environmental issue. Heavy metals are hazardous contaminants in food and the environment and they are non-biodegradable having long biological half-lives. Heavy metal contamination may occur due to factors including irrigation with contaminatedwater,theadditionoffertilizersandmetalbasedpesticides,industrial emissions, transportation, harvesting process, storage and/or sale (Ali *et al*. 2013). According to Miah *et al*. (2010), industries around Dhaka city do not have proper waste management systems. The rivers around the industrial belts ofDhaka,

Narayanganj, Chittagong and Khulna are major receiver of untreated effluents coming from tanneries, textiles, chemicals, pesticides, medicines, foods, engineering etc. (Shabnam *et al*., 2008). Soils, water and crops contaminated by heavy metals and other environmental toxins from different industrial wastes and wastewaters in and around Dhaka are producing unhealthy food through entering into the food chain, which is consumed by human beings. The food chain contamination is the major pathway of heavy metal exposure for humans (Khan *et al*., 2008). Heavy metal pollution can originate from natural and anthropogenic sources. Activities such as mining, smelting operation and agriculture have contaminated extensive area of world (Herawati *et al*., 2000). Some trace elements or heavy metals are essential for plant nutrition but plants growing adjacent to the zone of industrial areas display increased concentration of heavy metals, serving in many cases as biomonitors of pollution loads (Mingorance *et al*., 2007). Sewages sludge containing large quantities of Pb and other metals is regularly dischargedon tofieldandgardensoilsduetoincreasingtrendsinurbanization(Paivoke,2002).

Vegetables cultivated in industrial polluted soils or used contaminated wastewater as irrigation water might be taken up heavy metals and accumulated them in food chain.Pbisnotreadilysolubleinwaterandisfoundinrelativelylowconcentration (Pais and Beaton, 1997). According to the DOE (1997), due to the increased use of fossilfuel,coalandanincreasedproductionofwastebytheindustries,automobiles exhaust accounts for about 50% of the total inorganic Pb absorbed by human body (Arora*etal.*,2008;Alam*etal*.,2003).KashemandSingh(1998)foundthattextile, tannery,dyeingandsulphuricacidproducingindustriesincreasedtheconcentration of Cu, Mn, Ni, Pb, Cr, and Cd in the vicinity of industry in Dhaka, Bangladesh. Ullah *et al*. (1995) reported that huge amounts big and small polluting industries in and around Dhaka city were discharging heavy metals like Fe, Mn, Zn, Cu, Cr, Ni, CdandPbalongsomesodiumphosphate,nitratesandnitritesandweredeteriorating

the natural water quality of Dhaka city day by day. Nuruzzaman (1995) observed thatthesoilsintanneryindustrialsitescontainedhigheramountsofCr,Zn,Cuand Pb, which exceeded the toxic levels in soils. At present the underground water is not safe for drinking purpose because of heavy metal contamination. Human activities can induce the reduction of concentration or its toxic effects in the environment (Beri and Setia, 1995). Nuruzzaman *et al*. (1993) found three times (February,MayandDecemberof1992)higherheavymetalsconcentrationsatthose locations and excessive concentrations of several heavy metals were also observed even up to 4 km away from the industries. The uptake of heavy metals by plants from contaminated soils is of great interest because an excess of dietary intake of some of these heavy metals might be deleterious to the health of the consumers (Page*etal*.,1981;Baath,1989;Roads*etal*.,1989;GerzabekandUllah,1990;Ullah *et al*.,1995).

In the low concentrations, many metals are essential to life but in excess, the same chemicalscanbeharmfulandpoisonous.Therefore,abetterunderstandingofheavy metal sources, their accumulation in the soil and the effect of their presence in soil andonplantsystemsseemtobeparticularlyimportantissuesofpresentdayresearch on riskassessment.

2.2 Pollution of Lead(Pb)

Lead is one of the well-known environmental toxic metals and is a major pollutant with increasing concern of man. Pb pollution can affect all environments, but its effects are most long lasting in soils. It is the least mobile of all heavy metals in soils. Lead is a prime pollutant in both terrestrial and aquatic ecosystems. Besides natural weathering processes, the main sources of Pb pollution are exhaust fumes of automobiles, chimneys of factories using Pb, effluents from the storage battery, industry, mining and smelting of Pb ores, metal plating and finishing operations, fertilizers, pesticides and additives in pigments and gasoline and textile (Eick *et*

al., 1999). The maximum permissible limit in earth crust is <10 mg/L and soil 100 mg/kg soil. Lead affected soils contain Pb in the range of 400 - 800 mg kg^{-1} soil, whereas in industrialized areas the level may reach up to 1000 mg Pb/kg soil. It accumulates primarily on the surface, where its increasing presence may begin to affect soil micro flora. Increasing acidity of soils from fertilizers and acid rain further increased the solubility of this metal. Plants growing near highways are usually exposed to more Pb than the localities. Nriagu (1988) considered that Pb poisoning must be regarded as the most prevalent public health problem in many parts of the world. Since microorganisms and plants show strong tendency to bio-accumulate Pb, there is a possibility that the bio-accumulated Pb may enter into the terrestrial food chains (Deuny, 1987).

In soils, this metal is largely immobile and a long half-life with very little leaching due to adsorption to soil clays, phosphates, sulfates, carbonates, hydroxides and organic matter. High concentration in soils may inhibit microbial processes and reducedecompositionprocesses.ThePbcontentofsoilsamplesfelloffrapidlywith the increase in distance from highways. However, pathogenic processes, climate and topographic effects and microbial activities influence the distribution of Pb in the soil profile. And, Pb generally accumulates in the soil surface, usually within thetopfewcentimetersanddiminisheswithdepth(Ardiano,1986andAllowayand Ayers, 1993). Most of the Pb once produced remains in soil, dust andother environments. The fate of anthropogenic Pb in soils has recently received much attention, because this metal is hazardous to human and animals from two sources such as the food chain and soil dustinhalation.

2.3 Pb in Soils andPlants

Some micro-organisms may affect heavy metal availability by the process of bio-sorption, bioaccumulation and solubilization. Bodek *et al*. (1988) reported that Pb uptakewaspassiveandthetranslocationfromrootstootherplantpartswaslowbut

aerial deposition and foliar uptake contribute significantly to leafy concentrations and that anaerobic conditions (e.g. flooding). Morel *et al*. (1986) reported that at the root surface Pb binds to carboxyl groups of mucilage uronic acids. Mucilage binding restricts metal uptake into the root and establishes an important barrier protecting the root system Low pH and low phosphate concentration promote Pb uptake. Lead is available to plants from soil and aerosol sources. Hughes *et at*. (1980) extensively reviewed the findings on Pb absorption by roots and concluded thatthemodeofitsuptakeispassiveandthattherateofuptakeisreducedbyliming andbylowtemperature.Pbuptakestudiesinplantshavedemonstratedthattheroots have an ability to take up significant quantities of Pb, whilst simultaneouslygreatly restricting its translocation to above ground parts (Lane and Martin, 1977). The extent to which Pb enters into plants via the leaves depends on the ability of leaves to absorb Pb from aerial sources, which in turn depends on the specific root morphology. Zimdahl and Koeppe (1977) showed that under certain conditions Pb ismobilewithintheplant.Pbalthoughnotreadilysolubleinsoil,isabsorbedmainly by root hairs and is stored to considerable degree in cell walls. Zimdahl (1975) described that when Pb is present in soluble forms in nutrient solutions, plant roots are able to take up great amounts of this metal, the rate increasing with increasing concentration in the solutions and with time. The translocation of Pb from roots to tops is greatly limited. Only 3% of the Pb in the root is translocated to the shoot however, that Pb from a soil source is not readily translocated to edible portion of plants. These authors stated that the main process responsible for Pb accumulation inroottissueisthedepositionofPb,especiallyasPbpyrophosphate,alongthecell walls. Malone *et al*. (1974) identified the deposit of Pb in cell walls outside the plasmalema as Pb crystals Similar deposition of Pb was observed in roots, stems and leaves suggest that Pb is transported and deposited in a similar manner in all tissues of the plant. Jones *et al*. (1973) confirmed that plant roots restrict Pb movement into shoots. Rolfe (1973) showed that Pb uptake by eight tree species grown on soils treated with five soil. Broyer *et al*. (1972) alsoagreed with the

statement that large portion of Pb taken up from solution culture is associated with roots.

2.4 Phytotoxicity of Pb

Liu *et al*., (2003) reported that Pb at low concentration could promote normal physiological and metabolic activities in plants such as the activities of nitrate reductase, the contents of soluble sugar and chlorophyll of stems and leaves, whereas at higher concentration severely affected normal physiological and metabolic activities in plants, resulting in the symptoms of leaf etiolating and withering of stems and leaves. All Pb compounds greatly enhanced Pb uptake and planttissueconcentrations.AccordingtoKannan(1997),extremelylowlevels(2to 6µg/kg)ofPbmaybenecessaryforplant,asthereissomeevidenceofastimulatory effect at low concentrations. According to him, Pb at 30 mg/L in nutrient solution has been found to be toxic to plants, with 10 mg/L slowing plants growth and 100mg/Lbeinglethal.Insometypesofplants,Pbcanbeashighas350mg/kginplant tissue without visible harm. Total Pb amounting to 400 to 500 ppm in the soil in a polluted area in Japan was found to be toxic to the plants. Lead can be readily absorbed by plant roots, but little (less than 3%) is translocated to the tops. Leafy vegetablessuchaslettuce,spinach,potatoesandbeansarelikelytoabsorbmorePb, whereas fruiting crops such as tomatoes, corn, beats, squash, eggplant and peppers donotpickupanyappreciableamountofPbthroughtheirrootsystems.According toAdriano(1986),Pbinterfereionuptakeandtranslocation,growthretardationdue to inhibition of mitochondrial respiration and inhibition of chloroplast activity.Lee *et al*. (1976) found increased respiration rate, increased activities of the enzymes, acid phosphates, peroxidase and alpha-amylase, and increased levels of soluble protein and ammonia with Pb treatment. Zimdahl (1975) reported that even a very lowPbconcentrationmayinhibitsomevitalplantprocesses.BaumhardtandWelch (1972)andRolfe(1973)reportedthatPbwastoxictoplantsexceptinverylow

concentration, and when Pb was absorbed by plants leading to reduction of growth and inhibition of cell division.

2.5 Pollution of Cadmium(Cd)

Cadmium is considered as one of the most toxic heavy metals in the environment and has no function in the plants and animals. Cd is one of the most important to consider in terms of food-chain contamination. Total Cd content in soils ranged from0.01-3.0mg/kgandsolublecontentinsoilsranged0.1-14.0mg/kg.Content in plant ranged from 0.1 - 1.0 mg/kg; reference plant, 0.05 mg/kg and 3 mg/kg will reduce plant growth. Increasing concentrations of Cd have been observed in agricultural soils due to long term application of phosphorus fertilizers and sewage sludge (Joarder, 2003 and Stephens and Calder, 2005). Cd contaminations impose an adverse effect on environmental quality and constitute a serious threat not only toplantsandanimalsbutalsotohumanlives(Martin-Garin*etal*.,2002).Thismetal is ranked number seven among the top toxins, mainly due to negative influence on the cell's enzymatic system (ATSDR, 1999) and it has been estimated that 70% of the Cd intake by humans comes from plant foods (Wagner, 1993). With the development of modern industry and agriculture, Cd has become one of the most harmful and widespread pollutants in agricultural soils, and soil-plant environment system mainly due to industrial emission, application of Cd containing sewage sludge and phosphate fertilizers and municipal waste disposal (Gupta and Gupta 1998;Wu*etal*.,2003,2010;Lima*etal*.,2006).Amongheavymetals,Cdisreadily taken up by plants and translocated to different plant parts (Florijn and Van Beusichem, 1993; Li *et al*., 1995 and Sarwar *et al*., 2010). According to Adriano (1986), Cd is twenty times more toxic than Pb. Cadmium is extremely toxic and accumulatesinthekidneysandliver,withprolongedintakeatlowlevelssometimes leading to disfunction of the kidneys. Cadmium is produced commercially as a byproduct of the Zn industry. The most important uses of Cd are as alloys in electroplating (auto industry), in pigments, cement, plastic, fertilizer, metalalloys,

asstabilizersforpolyvinylplastics,andinbatteries(Ni-Cdbatteries).Itisalsoused in photography, lithography, process engraving, rubber curing and as fungicides, primarily for golf course greens, pigments used in ceramics, paints in textiles and coatings, electronics and autos (Adriano, 1986 and Sanita di Toppi and Gabrielli, 1999).

2.6 Cadmium inSoils

Cd was relatively mobile in acid soil and its mobility increases with increasing the acidity of the soil. It was observed that the mobility of Cd was reduced in acid soil byinteractionwiththeoxides/hydroxidesofFeandAl(Bulbul,2003).Cadmiumis fairly immobile in the soil profile. The Cd level (≤1 ppm) is fairly uniform throughout the profile, and apparent mobilization also occurred in very poorly drained profiles (Adriano, 1986). Cd retention in soils is influenced by soil properties such as CEC of soil, pH, organic matter content (Adriano, 1986), and Fe_2O_3 content. Soils contaminated by smelting operations showed Cd concentrations close to background level at a depth of about 30 to 40 cm. Kuo and McNeal (1984) found that sorption of Cd by hydrous iron oxides conformed to the Langmuir isotherm. Anderson (1977) observed the effects of clay particles on the mobility of Cd in soils. He stated that the mobility of Cd mostly in soil is reduced bytheclayparticleofthesoil.Street*etal*.(1977)reportedthatCdsolubilityinsoils decreased as pH increased. The lowest values were obtained in the calcareous soils (clay loam at pH 8.4). Anderson and Nilsson (1972) indicated that practically allof Cd remained in the surface 20 cm of soil following application of 84 tones/ha of sewage sludge over a 12-yearperiod.

2.7 Phytotoxicity of Cd

Wahid *et al*. (2010) observed that the effects of Cd toxicity on above-ground parts include plant stunting, leaf rolling, chlorosis and necrosis, diminished stomatal conductance and gas exchange, perturbed leaf water and nutrient status, hormonal

imbalance,productionofoxidativestress,andenhancedperoxidationofmembrane lipids.Wang*etal*.(2009)carriedoutapotexperimentfortheselectionofpollution- safe cultivar (PSC) of water spinach (*Ipomoea aquatic* Forsk.) and found that the high-Cd accumulating ability of water spinach was a stable biological property at cultivar level and, thus, was genotype dependent. According to Dong *et al*. (2007), one of the main Cd tolerance mechanisms is involved in depressing Cdbio-availability in soils, thus reducing the amount of Cd uptake. Roots excrete some organic substances to rhizosphere during the growth, and rhizosphere controls the entrance of nutrients, water and other chemicals, beneficial or harmful to plants. In rhizosphere, a series of physical and chemical reactions of heavy metals take place that affect their transfer in soil- plant systems, which may be beneficial to decrease the metal availability and its absorption by plants. Hossain *et al*. (2007) studied on thetransferofCdfromsoiltothevegetablecropsandfoundthatthetransferfactor of Cd in roots of vegetables decreased in the order: Lettuce> Spinach> Data Sak> LalSak;whileinshoots,itwasDatasak>spinach>lettuce>lalsakandthetransfer factor varied from 2.030 to 6.785 in roots and 0.166 to 0.525 in shoot. Under Cd stress, tolerant species and genotypes in plant kingdom could reduce Cd activity to alleviate or eliminate its toxicity through regulating the physiological and biochemical metabolism. Gratao *et al*. (2005) observed that Cd induced the production of reactive oxygen species affecting important macromolecules and modifying the activity of enzymes related to the antioxidant defense system. Cadmium toxicity reduce photosynthetic rate, internal water deficit in the vascular system caused by reduced conductivity of the stems and poor root system development,ioninteractionsinplantsandpossibleinhibitionofnutrient(NandP) mineralization in soil (Belimov *et al*., 2003; Huang *et al*., 2004). Hernandez *et al*. (1996) reported that Cd reduced the absorption of nitrate and its transport from the roots to shoots, but inhibited the nitrate reductase activity in the shoots. Cadmium isanimportanttoxicantinaffectingplantproductivity(Prasad,1995;Thiebeauld*et al*.,2005;Wahid*etal*.,2008)andhasalongbiologicalhalf-life(Himly*etal*.,1985).

The critical Cd level in nutrient solution for conventional crop plants is reportedto be 8 mmol/L (Yang *et al*., 1995). In Cd-enriched soils, plants may accumulate 20 mg/kgCdintheshoots.TheuptakeofCdcanvarygreatlyamongplantspeciesand alsoamongcultivarswithinaspecies(Penner*etal*.,1995;Athur*etal*.,2000;Zhang *etal*.,2002).AllanandJarrel(1989)andWang*etal*.(2001)foundthattheactivities of respiratory enzymes were inhibited and respiration rate decreased withthe increasingconcentrationofCdinplants.Inordertosurvive,plantshavetodevelop efficient and specific heavy metal detoxification mechanisms in different plant species (Punz and Sieghardt, 1993). Cadmium is phyto-toxic interfering different morphologicalandphysiologicaldisturbancesuchasphotosyntheticandrespiratory activities, mineral nutrition, enzymatic activities, membrane functions, and hormonal balance (Clysters and Van Assche, 1985; Boussama *et al*., 1999; Chien andKao,2000;Benavides*etal*.,2005).Cadmiumistakenupthroughtherootsand accumulated mainly in the organ, but it can be also translocated to shoots, grainsor fruits (Page *et al*., 1981). Jamali *et al.* (2007) also reported from their findings that Pb concentrations in vegetables grown in agricultural sites dressed and irrigated withdomesticwastewaterweresignificantly(P<0.01)higherthancontrolvegetable samples.

2.8 Toxicity of HeavyMetals

All soils contain heavy metals. Heavy metal toxicity hinders the growth process of the underground and aboveground plant parts and the activity of thephotosynthetic apparatus (Shah *et al*., 2010). With the exception of iron, all heavy metals above a concentration of 0.1% in the soil become toxic to plants and therefore change the community structure of plants in a polluted habitat. And, each plant species has a specific threshold value for each heavy metal, where it exerts toxicity (Ernest, 1996). In non heavy metal soils, the concentrations of Zn, Cu, Pb, Ni, Cd and Cr rangebetween0.0001and0.065%,whereasMnandFecanreach0.002%and10%, respectively (Ernest, 1968). Safe values for copper, lead, and cadmium in fruitand

vegetablesrecommendedbytheWHO/FAOare40,0.3,and0.2 mg/kg,respectively (Husain *et al.*,1995).

Table 1. Guideline for safe limits of heavy metals

Sample	Standards	Cd	Pb
Soil ($\mu g\ g^{-1}$)	Indian Standard (Awasthi 2000)	3-6	250-500
	European Union (EU 2002)	3.0	300
Water ($\mu g\ ml^{-1}$)	Indian Standard (Awasthi 2000)	0.01	0.10
	FAO (1985)	0.01	5.0
Plant ($\mu g\ g^{-1}$)	Indian Standard (Awasthi 2000)	1.5	2.5
	WHO/FAO (2007)	0.2	5.0
	European Union (EU 2006)	0.2	0.30

Although many metal elements are essential for the growth of plants in low concentrations, their excessive amounts in soil above threshold values can result in toxicity. This detrimental effect varies with the nature of an element as well as plant species. The bioaccumulation of heavy metals in excessive concentrations may replace essential metals in pigments or enzymes disrupting their function and causing oxidative stress. And, the toxic concentration, normal concentration and permissible limits of heavy metals in soils and plants are presented in Table 2 and Table 3 .

Table 2. Normal concentrations of different heavy metals in soils and crops

Element	Soil (mg/kg DM)	Plants (mg/kg DM)
Zinc	20-100	20-100
Chromium	10-50	0.10-0.50
Nickel	20-60	0.2-2.0
Copper	10-50	3-12
Lead*	10-30	0.10-0.50
Cobalt	2-10	0.02-0.50
Cadmium*	0.05-1	0.05-0.50
Mercury	0.05-5.0	<0.01-0.05
Arsenic	1-10	0.1-0.50

(Source: Horak, 1996)

Table 3. Permissible limits of heavy metals in soils, sewage sludge and city wastes

Element	Soil (mg/kg)	Sewage sludge (mg/kg)	City wastes (mg/kg)
Zn	300/150	1600	1000
Cu	100	400	400
Ni	60	80	100
Cr	100	400	150
Pb*	100	400	500
Cd*	1	5	4
Hg	1	7	

(Source: Horak, 1996) for soil pH < 6.0

2.9 Pb and Cd accumulation invegetables

There were significant differences in the Pb, Cd, and Ni concentrations (µg g^{-1} dry weight basis) of different vegetable species (cabbage, cauliflower, bottle gourd, pumpkin, eggplant and tomato) in different locations of contaminated and uncontaminatedsoils(Nashir,2010).HestatedthatCdlevelasthehighestintomato witharangeof0.49to1.26µgg^{-1}followedbyeggplantwitharangeof0.34to1.02 µg g^{-1} and bottle gourd having 0.17 to 0.0.36 µg g^{-1} and other vegetables. Kannan (1997) and Dara (1998) suggested that leafy vegetables, potatoes and beans are likely to absorb more Pb than fruiting crops like tomatoes and beets. Tyksinski *et al*. (1993) analyzed Pb, Cd, Cu and Fe in vegetables grown in city Poznam and foundthat53.7%ofleafyvegetables,26.9%rootyvegetablesand40%ofvegetable crops and their fruit contained excessive concentration of Pb. Hibben *et al.* (1984) reported that radish and lettuce accumulate more Pb than other vegetables. Plants growninPbenrichedsoilareknowntoaccumulatehighlevelsofPb(RCEP,1983).

Elevated Pb contents of vegetables grown in urban and industrial areas provide a healthrisktohuman.StudiesshowedthatwithincreasingconcentrationofPbinthe soil, the uptake by the vegetable plants increased. The highest bioaccumulation of Pb generally is reported for leafy vegetables grown in surroundings of nonferrous metal smelters, where plants are exposed to Pb both from soil and air. Although market garden crops may be affected to some extent, the contamination of vegetables grown in urban gardens or municipal allotments is of much greater concern (Davies *et al*., 1979).

2.10 Bioremediation of heavymetals

Bioremediationisaninnovativeandpromisingtechnologyavailableforremovalof heavy metals and recovery of the heavy metals in polluted water and lands. Since microorganismshavedevelopedvariousstrategiesfortheirsurvivalinheavymetal-pollutedhabitats,theseorganismsareknowntodevelopandadoptdifferent

detoxifying mechanisms such as biosorption, bioaccumulation, biotransformation and biomineralization. Adhikari *et al*. (2004) defined as bioremediation is the process of cleaning up hazardous wastes with microorganisms or plants and is the safest method of clearing soil pollutants. Bioremediation uses primarily microorganisms or microbial processes to degrade and transform environmental contaminants into harmless or less toxic forms (Garbisu and Alkorta, 1997).

Remediation of heavy metals from contaminated environments using biological methodsisknownasbioremediationwhichoffershighspecificityintheremovalof particular heavy metals of interest. Bioremediation could be in-situ or ex-situ. Ex-situ bioremediation involves taking the contaminated media from its original siteto a different location for treatment based on the cost of treatment, deepness of contamination, pollutant type and the extent of pollution, geographical locality and geology of the contaminated site (Azubuike *et al.,* 2016). In-situ bioremediation is an onsite clean-up process of contaminated environments whichinvolves supplementing contaminated soils with nutrients to stimulate microorganisms in their ability to degrade contaminants, as well as add new microorganisms to the environment or improve the indigenous microorganisms to degrade specific contaminants using genetic engineering (Mani and Kumar, 2014 and Rayu *et al.,* 2012).

Bioremediation is the naturally occurring process in which microorganisms or plants either immobilize or transform environmental contaminants to innocuous stateendproducts.Duringbioremediation,microbesutilizechemicalcontaminants in the soil as an energy source and through redox-potential they can metabolize the target contaminant into usable energy for microbes. Although multitudes of reactions are adopted by microbes to degrade and transform pollutants but all the energy yielding reactions are oxidation-reduction reactions and the typical electron acceptors are oxygen, nitrates, sulfate and carbon dioxide. For bioremediation, itis

importantthateffectivemicroorganismsandplantsmaydegradethepollutantsinto harmless products by various enzymatic actions. Microbes can't degrade heavy metalsdirectlybuttheycanchangethevalencestatesofmetalswhichmayconvert them into immobile or less toxicforms.

2.11 The advantage ofbioremediation

• Itisanaturalprocess,ittakesalittletime,asanacceptablewastetreatmentprocess for contaminated material such as soil. Microbes able to degrade the contaminant and increase in numbers when the contaminant is present. When the contaminantis degraded, the biodegradative population become declines. The residues for the treatment are usually harmless product including water carbon dioxide and cell biomass.

• It requires a very less effort and can often be carried out on site, often without causing a major disruption of normal activities. This also eliminates the need to transport quantities of waste off site and the potential threats to human health and the environment that can arise duringtransportation.

• It is applied in a cost effective process as it lost less than the other conventional methods (technologies) that are used for clean-up of hazardouswaste.

• It also helps in complete destruction of the pollutants, many of the hazardous compounds can be transformed to harmless products, and this feature also eliminates the chance of future liability associated with treatment and disposal of contaminatedmaterial.

• It does not use any dangerous chemicals. Nutrients especially fertilizers added to make active and fast microbial growth. Commonly, used on lawns and gardens. Because of bioremediation change harmful chemicals into water and harmless gases, the harmful chemicals are completely destroyed.

• Simple,lesslaborintensiveandcheapduetotheirnaturalroleintheenvironment.

• Eco-friendly and sustainable.

• Contaminants are destroyed, not simply transferred to different environmental media.

• Nonintrusive, potentially allowing for continued siteuse.

• Relative ease of implementation.

• Effective way of remediating natural ecosystem from a number of contaminant and act as environment friendly options.

2.12 Microbial remediation of heavymetal

Bioremediationusingofmicroorganisms/plantstodetoxifyorremoveheavymetals from the soil is cost-effective, provides a permanent solution and is less expensive compared to physicochemical methods and has recently become prevalent in treating soils contaminated by heavy metals (Zhang Xi *et al.,* 2010). Different studies are carried out about the tolerance of bacteria to heavy metals through their abilities to adsorb, bioaccumulate and/or transform metals (Fomina *et al*., 2007, Singhal *et al.,* 2004). Bacterial cells developed numerous strategies to decrease metalpoisoning:(1)resistanttothemetalbyintra-andextracellularmechanisms; (2)metalsexcretionusingtransportsystems;(3)detoxificationofmetalsbycytosol sequestration compounds (4) formation of extracellular chelators for binding and fixing metals; (5) binding large quantities of metals by sorption o the cell walls (Haferburg and Kothe, 2007). Because of the adaptability of microbes and other biological systems, these can be used to degrade or remediate environmental hazards. Natural organisms, either indigenous or extraneous (introduced), are the primeagentsusedforbioremediation(Prescott*etal*.,2002).Theorganismsthatare utilized vary, depending on the chemical nature of the polluting agents, and are to be selected carefully as they only survive within a limited range ofchemical

contaminants(Prescott*etal*.,2002;Dubey,2004).Biologicalremediationagentwas registeredin1974,beingastrainofabletodegradepetroleum(Prescott*etal*.,2002; Glazer and Nikaido, 2007). Bioremediation techniques using either live or dead, microorganisms are of twocategories:

(1) bisorption (passive) using non-living cells, (2) bioaccumulation using living cells (Dönmez and Aksu, 2001 and Li *et al*., 2004). Because of the adaptability of microbes and other biological systems, these can be used to degrade or remediate environmental hazards. The main requirements are an energy source and a carbon source (Vidali, 2001). Since numerous types of pollutants are to be encountered in a contaminated site, diverse types of microorganisms are likely to be required for effective mediation (Watanabe *et al*., 2001). The bioremediation processes may be conducted by the autochthonous microorganisms, which naturally inhabit the soil/water environment undergoing purification, or by other microorganisms, that derivefromdifferentenvironments.Thereareanumberofmicroorganismsthatcan be used to remove metal from environment, such bacteria, fungi, yeast and algae (White *et al.*, 1997 and Vieira and Volesky, 2000). Microbial remediation means uptake, accumulate, sequester, translocate and detoxify metals which depends on numerous factors. Microbial remediation included several techniques and applications which differ greatly in the mechanism by which microbial cells can immobilize, remove, or degrade metals. Presence of heavy metals in the environment changed microbial communities and activities (Jansen *et al*., 1994, Matyar *et al*., 2008). Bioremediation has been developed to immobilize heavy metals by microorganisms. Microbial remediation by local microbes showed great use for heavy metal removal especially in harsh soil. Microorganisms can be isolated from almost any environmental conditions. Microbes can adapt and grow at subzero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen and in anaerobic conditions, with the presence of hazardous compounds or on any wastestream.

Pandit *et al.* (2013) reported that metal resistant bacterial isolates showed high degree of resistance to heavy metals ranging from 25-300 ppm. Singh *et al.* (2010) studied that*Pseudomonas aeruginosa* exhibited high resistance to heavy metals with MIC for heavy metals ranging from 50µg/ml to 300µg/ml. It has been found that large plasmids are responsible for encoding resistance to antibiotics and heavy metals (Jain *et al.*, 2009).

Various bacteria have been implicated in removal of heavy metals from industrial wastes and soil through functional groups on their cell envelops (Volesky, 1986; Brierly, 1990). The resistance mechanism and subsequently the location of accumulated metal vary with the strain. Similarly, *Bacillus* and *Pseudomonas sp*. haveabilitytoremovemaximumconcentrationofdifferentmetalslikeCd,Cu,and PbatpH7and6respectively(Rani*etal.*,2010).Metaluptakebybindingthemetal at the surface of bacterial cell is pH dependent (Wang and Chen, 2006). In *Pseudomonas sp.,* the metal removal greatly enhance above pH 5 (Pandit *et al.,* 2013). Maximum removal of cadmium by *P. aeruginosa* JN102340 was observed at 35ºC (Hussein *et al*., 2004). Many studies indicated that a number of bacterial species were capable of removing metals from aqueous environment (Manisha *et al*., 2011). Microorganisms that carry out biodegradation in many different environments are identified as active members of microbial consortiums. These microorganisms include: *Acinethobacter, Actinobacter, Acaligenes, Arthrobacter, Bacillins, Berijerinckia, Flavobacterium, Methylosinus, Mycrobacterium, Mycococcus, Nitrosomonas, Nocardia, Penicillium, Phanerochaete,Pseudomonas, Rhizoctomia, Serratio, Trametes* and *Xanthofacter.*

2.13 Factors affecting microbialbioremediation

Microorganisms act against the pollutants only when they have access to a variety of materials compounds to help them generate energy and nutrients to build more

cells. The efficiency of bioremediation depends on many factors; including, the chemicalnatureandconcentrationofpollutants,thephysicochemicalcharacteristics of the environment, and their availability to microorganisms (El Fantroussi S and Agathos SN, 2005). The reason for rate of degradation is affected due to bacteria andpollutantsdonotcontacteachother.Inadditiontothis,microbesandpollutants are not uniformly spread in the environment. The controlling and optimizing of bioremediation processes is a complex system due to many factors. These factors are included below.

2.13.1 Biologicalfactors

A biotic factors affect the degradation of organic compounds through competition between microorganisms for limited carbon sources, antagonistic interactions between microorganisms or the predation of microorganisms by protozoa and bacteriophages. The rate of contaminant degradation is often dependent on the concentration of the contaminant and the amount of “catalyst” present. In this context, the amount of “catalyst” represents the number of organisms able to metabolize the contaminant as well as the amount of enzymes(s) produced by each cell. The expression of specific enzymes by the cells can increase or decrease the rate of contaminant degradation. Furthermore, the extent to contaminant metabolism specific enzymes must be participated and their “affinity” for the contaminant and also the availability of the contaminant is largely needed. The major biological factors are included here: mutation, horizontal gene transfer, enzyme activity, interaction (competition, succession, and predation), its own growth until critical biomass is reached, population size and composition(Madhavi and Mohini, 2012; Boopathy,2000).

2.13.2 Environmental factors

The metabolic characteristics of the microorganisms and physicochemical properties of the targeted contaminants determine possible interaction

during the process. The actual successful interaction between the two; however, depends on the environmental conditions of the site of the interaction. Microorganism growth and activity are affected by pH, temperature, moisture, soil structure, solubility in water, nutrients, site characteristics, redox potential and oxygen content, lack of trained human resources in this field andPhysico-chemical bioavailability of pollutants (contaminant concentration, type, solubility, chemical structure and toxicity). These above listed factors are determining kineticsof degradation (Madhavi and Mohini, 2012; Adams, 2015). Biodegradation can occur under a wide-range of pH; however, a pH of 6.5 to 8.5 is generally optimal for biodegradation in most aquatic and terrestrial systems. Moisture influences therate of contaminant metabolism because it influences the kind and amount of soluble materialsthatareavailableaswellastheosmoticpressureandpHofterrestrialand aquatic systems (Cases and Lorenzo, 2005). Most environmental factors are listed below.

2.13.2.1 Availability ofnutrients

The addition of nutrients adjusts the essential nutrient balance for microbialgrowth and reproduction as well as having impact on the biodegradation rate and effectiveness.Nutrientbalancingespeciallythesupplyofessentialnutrientssuchas N and P can improve the biodegradation efficiency by optimizing the bacterial C: N: P ratio. To survive and continue their microbial activities microorganisms need a number of nutrients such as carbon, nitrogen, and phosphorous. In small concentrationstheextentofhydrocarbondegradationalsolimits.Theadditionofan appropriatequantityofnutrientsisafavourablestrategyforincreasingthemetabolic activity of microorganisms and thus the biodegradation rate in cold environments (Couto, 2014; Phulia, 2013). Biodegradation in aquatic environment is limited by the availability of nutrients (Thavasi, 2011). These nutrients are available in the natural environment but occur in low quantities (Macaulay,2015).

2.13.2.2 Temperature

Among the physical factors temperature is the most important one to determining the survival of microorganisms and composition of the hydrocarbons (Das N, Chandran P, 2011). In cold environments such as the Arctic, oil degradation via natural processes is very slow and puts the microbes under more pressure to clean up the spilled petroleum. The sub-zero temperature of water in this region causes the transport channels within the microbial cells to shut down or may even freeze the entire cytoplasm, thus, rendering most oleophilic microbes metabolically inactive (Macaulay, 2015; Yang, 2009). Biological enzymes participated in the degradation pathway have an optimum temperature and will not have the same metabolic turnover for every temperature. Moreover, the degradation process for specific compound needs specific temperature. Temperature also speed up or slow down bioremediation process because highly influence microbial physiological properties. The rate of microbial activities increases with temperature, and reaches to its maximum level at an optimum temperature. It became decline suddenly with further increase or decrease in temperature and eventually stop after reaching a specific temperature.

2.13.2.3 Concentration ofoxygen

Differentorganismsrequireoxygenothersalsodonotrequireoxygenbasedontheir requirementfacilitatethebiodegradationrateinabetterway.Biologicaldegradation is carried out in aerobic and anaerobic condition, because oxygen is a gaseous requirement for most living organisms. The presence of oxygen in most cases can enhance hydrocarbon metabolism (Macaulay,2012).

2.13.2.4 Moisturecontent

Microorganisms require adequate water to accomplish their growth. Thesoil moisture content has adverse effect in biodegradationagents.

2.13.2.5 pH

pHofcompoundwhichisacidity,basicityandalkalinitynatureofcompound,ithas its own impact on microbial metabolic activity and also increase and decrease removal process. The measurement of pH in soil could indicate the potential for microbialgrowth(Enim,2013).HigherorlowerpHvaluesshowedinferiorresults; metabolic processes are highly susceptible to even slight changes in pH (Wang Q, 2011).

2.14 Bioremediation of Lead and Cadmium bybacteria

2.14.1 Bioremediation ofLead

Soil bacteria could directly or indirectly interact with lead present in the contaminatedsoilandreduceditintonon-toxicforms.Someofbacteriaresponsible for the bioremediation of lead include *Bacillus* sp. and *P. aeruginosa*. These bacteriamayreducetheleadinthecontaminatedsourcethroughbiosorptionoflead by functional groups on the cell surface or by interaction and complex formation between lead and acidic sites in the cell wall. Several studies have reported the use of microorganisms to eliminate heavy metals from the environment as less expensive, cost effective and environmental friendly strategy (Yan and Viraraghavan, 2003; Feng and Aldrich, 2004; Vijayaraghavan and Yun, 2008; He *et al.*, 2011; Huang *et al.*, 2014). Bacteria have developed different defensive mechanismstocopewiththenegativeeffectscausedbyheavymetalions(Zahid*et al.*, 2012) including intracellular sequestration of metallothionein and other thiol containing compounds (Saluja and Sharma, 2014). Roane (1999) reported that the degreeandmechanismofPbresistancefortwobacterialisolatescorrespondedwith their environmental Pb exposure. *P. marginalis*, isolated from a soil contaminated with high total (but low soluble) Pb showed higher resistance and extracellular Pb exclusion with high amount of EPS production. On the other hand, *B. megaterium* isolatedfromsoilcontaininghighsolublePbshowedlowerresistanceand

intracellular accumulation of Pb. This strain produced no discernable EPS as reportedly observed by polarization microscopy. Tolerant bacteria isolated from secondaryeffluentscouldremove61.9%ofleadand74.61%ofcopper(Adel*etal*., 2014). Biosorption capability of *Sienotrophomonas maltophilia* from primary solution was 22% of copper and 42.75% of lead (Parungao *et al*., 2007). However, such methods more often than not, render the land barren, inhibiting any plant growth, as they tend to remove all microbial activities including the useful symbionts such as nitrogen-fixing bacteria and mycorrhizae as well as other fauna, during the process of decontamination, thereby further reducing the biodiversity of the area (Chaudhury *et al*., 1999). Li *et al*. (2013) evaulated another ureolytic bacteria *Sporosarcina koreensis* for removal of lead and showed 100% removal of exchangable lead fraction from contaminated soil.

Very recently, Kang *et al*. (2015) evaluated the lead remediation by altering the solubleleadtoacarbonatebiomineralfractionwiththehelpofPb-resistantbacteria.

2.14.2 Bioremediation ofCadmium

P.*aeruginosa*PU21biomassappearsaneffectivebioadsorbentfortheremovaland recoveryofCd,CuandPbfrompollutedwater(Chang*etal*.,1997).Likewise,dead cell biomass of *P*. *aeruginosa* has a high ability to adsorption of Cd and Pb in aqueous solutions (Karimpour *et al*., 2018). In contrast, live cells of *Pseudomonas* BC15wasalsocapableofbiosorbingCdalongwithothermetalssuchasPb,Niand Crinamedium(EdwardRaja*etal*.,2006).Zeng*etal*.(2009)alsoconcludedthat *P*.*aeruginosa*E1livingcellhasperformedbetterbiosorptionofCdthannon-living cells. The lyophilized cells of *P*. *aeruginosa* PAO1 adsorbed Cd from aqueous solution was estimated at acidic pH 5–6 (Peter *et al*., 2014). In another study, *P*. *aeruginosa* isolated from active sludge could effciently remove 94.7% Cd from solution within 60 min (Kermani *et al*., 2010). During biosorption studies,adapted

cells of *P. aeruginosa* strain JCM 5962 and genetically engineered (GE) *P. aeruginosa* also able to remove Cd (Bojorquez *et al*., 2016; Tang *et al*., 2018).

Recently published strain *Pseudomonas aeruginosa* is a promising candidate for cadmium bioremediation because of its large biosorption potential (Zivkovic *et al*., 2018). Interesting results on the mechanism of Cd resistance in bacterial isolates werere*vealed*bytheworkofRoane*etal*.(2001).Ofthefourisolates,*Pseudomonas* strain H1 and *Bacillus* strain H9, which were resistant to higher concentration (225 and 275 Ag/ml), appeared to use an intracellular mechanism of Cdsequestration.

The isolated *Bacillus spp.* possessed the ability to remove other heavy metals (Ni, Cr, Cu, Zn and Cd) from the tannery effluent. Zeng *et al.* (2009) isolated cadmium resistant *P. aeruginosa* E1 from metal contaminated site tolerate 360 ppm of cadmium. Cadmium has been shown to bind to capsular material in *Arthrobacter viscosus* and in *Klebsiella aerogenes* (Hrynkiewicz *et al.*, 2015). A *Citrobacter* mutant isolated from metal-polluted soil was found to accumulate Cd^{2+} asinsoluble cell-bound $CdHPO_4$ during growth in the presence of Cd^{2+} and glycerol (Macaskie *et al.*, 1987). Many studies have been undertaken with the aim of determining the mechanism of biotransformation of cadmium into cadmium sulfide. *Klebsiealla planticola (Cd-1)* grew anaerobically at a Cd concentration of 15mM and precipitated CdS (Sharma *et al.*, 2000). Bang *et al.* (2002) developed a genetically engineered bacterium capable of producing sulfide under aerobic, microaerobic, or anaerobic conditions for heavy metal precipitation. *P. aeruginosa* was found to detoxify Cd^{2+} through production of intracellular cadmium-binding proteins (Hassen *et al*., 1998). Zeng *et al.* (2009) isolated cadmium resistant *P. aeruginosa* E1 from metal contaminated site tolerate 360 ppm of cadmium. *Pseudomonas* sp. have ability to resist the metals due to the presence of intracellular metal binding proteins and multiple efflux pumps (Chovanova *et al.*,2004).

Roane and Pepper (2000) observed that different strains of Cd-resistant bacterial isolates varied in their resistance level due to potentially varied mechanism of resistance. Significant reduction of soluble Cd was observed during growth of plasmid-bearing *Bacillus* strain H9 and *Pseudomonas* strain H1. Similarly, three strains of bacteria isolated from industrial effluents (*Enterobacter cloacae* and *Klebsiellaspp*.)wereresistanttohighconcentrationsofCd,PbandCrinthegrowth media and could remove approximately 85% Cd during growth (Haq *et al.*, 1999). Baillet *et al.* (1997) adapted *Thiobacillus ferrooxidans* strain via successive exposure to higher concentrations of Cd. Kang *et al*. (2014) isolated *Lysinibacillus sphaericus* and investigated its urease activity and feasibility of Cd bioremediation viacarbonateprecipitation.Eventhoughgrowthof*L.sphaericus*hinderinpresence of Cd, urease activity did not get hampered. Study showed 99.95% removal of Cd from sand in 48 h transforming Cd into stable biomineral product. Subsequently, scanningelectronmicroscopeandX-raydiffractionanalysesconfirmedpresenceof Cd as carbonate otavite ($CdCO_3$). Similar way Kumari *et al*. (2014) conducted various experiments to investigate the effect of temperature on MICP based Cd bioremediation by an indigenous ureolytic bacterium, *Exiguobacterium undae*. Ureaseenzymeactivityandgrowthof*Exiguobacteriumundae*werenotaffectedby low temperature (10°C) compared to 25°C. Tessier sequential extraction result showed soluble Cd fraction got reduced significantly and carbonate fraction got increased by microbially induced calcite precipitation practice compared to controlled samples in both 10°C and25°C.

Some bacteria actively uptake heavy metal ions including Cd^{2+} along withessential metal ions, thus help removing heavy metal ions from aqueous environment (Rehman and Anjum,2009).

Chapter III

Materials &Methods

CHAPTER III
MATERIALS & METHODS

The present experiment was conducted at the Laboratory of Microbiology, Department of Botany, University of Dhaka from October 2017 to february 2019 to evaluate the efficiency of indigenous bacteria for the reduction of lead and cadmium in the pollutedwater.

3.1 Researchlaboratories

The collected samples, for the analysis of Pb and Cd, were done in Bangladesh Agricultural Research Institute (BARI), Gazipur. Bioremediation of Pb and Cd by indigenous bacteria were done in the Laboratory of Microbiology, Department of Botany, and University of Dhaka (Appendix II).

3.2 Samplingsite

TocompareandgetafairideaaboutthemicrobialloadandPbandCdpollution of samples, two different river sites were selected for the study situated at two different places near Dhaka city. Among them one was situated atTongi, Gazipur and the other was at Binnadangi, Manikganj (Appendix I and Plate1).

- Turag river: Tongi,Gazipur.
- Dhaleshwari river: Binnadangi,Manikganj.

3.3 Sampletypes

Three different types of samples *viz*. soil, water and bottle gourd leaves contaminated with Pb and Cd were collected from both river sites (Table 4).

3.4 Collection ofsamples

During sampling, polypropylene bottle, polythene bag, plastic jar, marker, pen, field notebook, sterilized spoon and thermometer were taken to the sampling areas. Soil was collected from the rhizosphere of bottle gourd plant grown near

Plate 1. Photographs showing sampling areas near Dhaleshwari river (A-C) and Turag river (D-F)

the riverside areas, water below the surface area of the river water and bottle gourd leaves grown near the river areas. After individual sampling, soilsamples were collected in plastic jar where water samples by polypropylene bottle and bottle gourd leaves in polythene bag. These samples were brought to the laboratoryasearlyaspossibleandimmediatelyafterthesampleswereanalyzed.

Table 4. Sampling site, date and number of samples collected from each sampling for the experiment

Sampling No.	Sampling site	Date of collection	Sample type
01	Dhaleshwari river: Binnadangi, Manikganj.	16.10.2017	Soil and Water
02	Dhaleshwari river: Binnadangi, Manikganj.	21.12.2017	Soil and Water
03	Dhaleshwari river: Binnadangi, Manikganj and Turag river: Tongi, Gazipur.	12.02.2018	Soil, Water and Bottle gourd leaves
04	Turag river: Tongi, Gazipur.	02.10.2018	Soil and Water

3.5 Heavy metal analysis (Pb and Cd) of the collectedsamples

Heavy metals like Pb and Cd content in soil, water and bottle gourd leaves collected from the sampling sites were analyzed by Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS, Thermo Scientific, USA).
The whole procedure was described below.

Soil samples collected from the rhizosphere of bottle gourd plants were put in plastic containers and transported to the laboratory for analysis. The samples

were air dried, mechanically ground using a pestle and mortar. Then 0.5 gm of soil samples were added with an aqua regia mixture of concentrated Nitric acid (HNO_3) and concentrated Perochloric acid ($HClO_4$) in a 5:1 ratio and left for overnight. After the digestion of the mixture being done for 2 hours at 180^0 C, they were left for cooling. Then 10 ml distill water was added with the mixture. After the filter being done, finally 100 ml volume was prepared. Different standards *viz*. 0.5 ppm, 1 ppm, 1.5 ppm, 2 ppm and 2.5 ppm of both Lead and CadmiumwerepreparedandthereadingwasdeterminedbyAtomicAbsorption Spectrophotometer(AAS).

Watersamplescollectedbelowthesurfaceofwaterlevelbypolypropylenebottle were transported to the laboratory for analysis. Direct sample reading was done for the analysis of water. Different standards *viz*. 0.5 ppm, 1 ppm, 1.5 ppm, 2 ppmand2.5ppmofbothLeadandCadmiumwerepreparedandthereadingwas determined by Atomic Absorption Spectrophotometer(AAS).

The leafy samples collected from the experimental sites were put in plasticbags andtransportedtothelaboratoryandthenwashedwithsteriledistillwater.After that the leaves were air dried for 24 hours. The dried leafy samples were then crushed using pestle and mortar. Then 0.5 gm of leafy samples were digested with Tri-Acid Mixture (HNO_3: H_2SO_4: $HClO_4$ = 5:1:1) until getting transparent fumes. Then the mixture was kept in normal room temperature and filteredwith Whatman (1 µm) filter paper. The final volume of 50 ml was prepared adding double distill water. Different standards *viz*. 0.5 ppm, 1 ppm, 1.5 ppm, 2 ppm and 2.5 ppm of both Lead and Cadmium were prepared and the reading was determined by Thermo Atomic Absorption Spectrophotometer (S-series, Australia).

3.6 Physical variables of the collectedsamples

3.6.1 Temperature of the sampling sites

Air, soil and water temperature was measured at the time of sampling by an alcohol thermometer. These results were immediately noted.

3.7 Chemical variables of thesamples

3.7.1 Hydrogen ion concentration (pH)

ThepHofthecollectedsampleswasmeasuredwiththehelpofdigitalpHmeter (Jenway 3510 pH meter, U.K) at the laboratory. The values were noted in pH unit.

3.8 Media and techniques for the enumeration and isolation ofbacteria

3.8.1 Culture mediaused

Peptone Yeast Extract Glucose (PYG) agar medium was used for the enumeration and isolation of aerobic heterotrophic bacteria present in samples. The pH of the medium was adjusted to 7.00 since most of the samples were within the range of 6.28-7.76. The pH was adjusted before the addition of agar and sterilization.

3.8.2 Techniquesemployed

Dilution plate technique was used for the enumeration and isolation of bacteria.

3.8.2.1 Dilution platetechnique

In case of hydrocarbon oil mixed soil sample, the serial dilution plate technique (Claus 1995) was used for the isolation of microorganisms. One ml of soil sample was transferred to 9 ml of sterile water for ten-fold (1:10) dilution and further diluted up to 10^7 dilutions. Plating in duplicate plates was made each diluted sample. One ml of each of the diluted sample was taken in a sterilized Petriplatebysterilizedpipette.Thenmoltenagarmediumwaspouredandmixed thoroughly by rotating the Petri plate, first in one direction and then in the opposite direction. After solidifying the medium, the plates were inverted and incubated at 37°C for 24 h in an incubator (Memmert GmbH + Co kg 8540 Sehwabach). Again, for the liquid oil sample one ml of liquid oil was directly takeninasterilizedPetriplate.Thenmoltenagarmediumwaspouredandmixed thoroughly by rotating the Petri plate, first in one direction and then in the oppositedirection.Aftersolidifyingthemedium,theplateswereinvertedand

incubated at 37°C for 24 h in an incubator (Memmert GmbH + Co kg 8540 Sehwabach).

3.9 Enumeration of bacteria

After 24 h of incubation the plates having well discrete colonies were selected for counting. The selected plates were placed on colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan) and the colonies were counted.

3.10 Isolation ofbacteria

Welldiscreteaerobicheterotrophicbacterialcolonieswereisolatedimmediately after counting. Based on their colonial morphology, different discrete colonies were selected for isolation. The selected colonies were marked and studied for various characters *viz.* color, form, elevation, margin surface, optical characters etc. (Eklund and Lankford 1967, Bryan 1950). Then the marked and observed bacterial colonies were transferred on nutrient agar slant for furtherstudies.

3.11 Purification of theisolates

Afterinitialselectionbasedongrowthpattern,theselectedisolateswerepurified throughrepeatedplating(bystreakinganddilutionplatemethods).Whenaplate yielded only one type of colonies the organisms were considered to bepure.

3.12 Maintenance and preservation of isolates

Thepurifiedisolateswerethentransferredonnutrientagarslant.Theslantswere kept in polythene bags and preserved as stock culture in a refrigerator at 4°Cfor further study. Periodical transfers of isolates on agar slants were donefor maintaining viability of theorganisms.

3.13 Morphological observation ofisolates

For the identification of selected isolated strains, following morphological characters were studied and recorded.

3.13.1 Colonialmorphology

The bacterial colonies on plating medium were morphologically studied astheir form, elevation, margin, surface, pigmentation, opacity, whether grown inside, at the bottom or on the surface of the medium and their rate ofgrowth.

3.13.2 Microscopic examination of isolatedstrains

Bacterial cells suspension was made by using fresh culture with physiological saline. The prepared suspension was used to make smear. A good quality glass slide was used for this purpose. Thin smear was prepared on the clean and oil freeslide.Thesmearwasallowedtodryinairandwasfixedbypassingtheslide over the flame of a spirit lamp. The following two different staining methods were employed to stain the fixedsmears.

- Simple stainingmethod
- Differential stainingmethod

3.13.2.1 Simple staining (Bryan,1950)

Manual of Microbiological Methods (SAB, 1957) was followed for simple staining. Basic dyes *viz.* crystal violet, basic fuchsin, cotton blue, safranine, mercurochrome and malachite green were used. The fixed smear was flooded withadyesolutionforoneminute.Thefloodedsmearwaswashedoffwithwater and dried inair.

3.13.2.2 Differentialstaining

Staining procedures that make visible differences between microbial cells or parts of cells were termed as differential staining (Pelczar *et al.* 1986). Differential staining uses a combination of dyes that take advantage ofchemical differences among cells (Claus 1995). The differential stains most frequently used are the simple stain, Gram stain, acid-fast stain, negative stain and spore stain (Tortora *et al*., 1998). For this purpose, fixed smear was exposed to more than one dye solution. In this study, two differential techniques were used *viz.* Gram staining and sporestaining.

3.13.2.2.1 Gramstaining

This is one of the most important and widely used differential staining technique is considered as one of the important steps in identifying an unknown bacterium. For Gram staining, method described by Claus (1995) was followed.

Fixed smear was treated with the following solutions and after application of each solution slide was gently washed off with water.

Crystal violet solution for 60 sec., Lugol's iodine solution for 60 sec, 95% Ethyl alcoholfor30secandSafraninesolutionfor60sec.Theslidewasdriedthroughair and observed under microscope (Nikon MICROPHOT, UFX-IIA, Japan).The results were recorded as Gram positive (blue-violet) and Gram negative (lightred).

3.13.2.2.2 Spore staining

The method described by Claus (1995) was applied in spore staining. Smear was madefrom24holdbacterialculture.Thefixedsmearwasfloodedwith5%aqueous solution of malachite green and heated over a brass plate for about 15-20 minutes taking care that the dye must not be dried off. Excess dye was then washed gently, and basic fuchsine was used as a counter stain for 1 minute. The slide was washed gently, dried and examined undermicroscope.

Spores were stained green and vegetative cells or sporangia were stained with red color of basic fuchsin. The shape and position of the spores within sporangia were observed. The swelling nature of the sporangium was also observed and recorded**.**

3.13.3 Measurement of bacterialcells

Themeasurementofvegetativecellsofselectedisolateswasdonebycomparingthe photographs of bacterial cells with that of the stage micrometer. The compared photographs were of same magnification. One division of micrometer is 18.5 mm equivalent to 10 µm.

3.14 Physiological and biochemical studies of theisolates

Following Bergey's Manual (Sneath *et al.,* 1986) the physiological tests of the isolated bacteria were carried out. Along with Bergey's Manual several other manualssuchasManualofMicrobiologicalMethods(SAB,1957),Microbiological Methods(CollinsandLyne,1984)andUnderstandingMicrobes(Claus,1995)were alsoconsulted.

3.14.1 Physiological studies of theisolates

3.14.1.1 Catalase test (Claus,1995)

The microbes produce the enzyme catalase to break the hydrogen peroxide into water and molecular oxygen.

$$2H_2O_2 \xrightarrow[\text{Catalase}]{} 2H_2O + O_2\uparrow$$

Catalase is an enzyme produced by and found in essentially all actively growing microorganisms capable of using oxygen for respiration.

The test for catalase in bacteria was performed by simply placing few drops of hydrogen peroxide directly on some cells on a glass slide. The evolution of oxygen bubbles indicated the positive result i.e. production of catalase**.**

3.14.1.2 Oxidase test (Claus,1995)

The enzyme oxidase in certain bacteria catalysed the transport of electron from donor bacteria to the redox dye tetra-methyl para-phenylene diaminedihydrochloride. The dye in the reduced state has a deep purple color. To perform this test filter papers were soaked in 1% aqueous tetra-methyl para-phenylene diaminedihydrochloride. Fresh young culture was rubbed on the filter paper with a clean glass rod. Results were recorded within 10 seconds. Blue color indicated a positive result.

3.14.1.3 Potassium hydroxide solubility test (Schaad,1988)

The test was done with a 3% potassium hydroxide (KOH) solution (Suslow *et al.*, 1982). One to two drops of 3% KOH were placed on a clean and dried glass slide. A loop full of the bacterial cells from the edge of a 24 h old culture was transferred and mixed thoroughly with the drops of KOH on the slide for 10 sec.

The organism was considered positive when KOH solution become viscous and showedaslimythreadanditwasnegativewhentherewasnoslime.Theorganisms showing positive reaction to KOH were Gram-negative bacteria andvice-versa.

3.14.1.4 Motility test using motility medium (Eklund and Landford, 1967)

Motilebacteriacanmovethroughsemisolid,softmotilityagarandtheirgrowthwill cloud the medium. Non motile organisms will remain and only grow near the site of inoculation. A chemical 2, 3, 5-triphenyltetrazolium chloride, which is reduced toaredcolorbygrowingbacteria,wasaddedtothemediumat0.001%tomakethe amount and extent of growth easier toobserve.

3.14.2 Biochemical studies of theisolates

3.14.2.1 Acid production from carbohydrate (Sneath *et al*.,1986)

The selected strains were tested for their ability to produce acid from different carbon source. For this purpose, basal medium was used. Bromocresol purple was added to this medium as an indicator and 10% sterilized aqueous solutions of carbohydratewereaddedtothismedium.Xylose,arabinose,mannitol,glucosewas used as carbon source Carbohydrate solution was sterilized by autoclave. The medium was poured into the sterilized Petri-plates and allowed to solidify. Inoculationwasdonebypointinoculationmethodandincubatedat37°Cfor5days. Acid production from carbohydrate was determined by yellow color around the colony.

3.14.2.2 Gas production from carbohydrates (SAB,1957)

Gas production from carbohydrates or fermentation test is of considerable significance in the identification and classification of bacteria. In the study of fermentation, D-glucose (monosaccharide) was used. Fermentation tubes with the abovecarbohydrateweremadeusingbromothymolblueasindicator.OneDurham's tube was introduced in each of the test tubes. The tubes were then inoculated in duplicates with 24 hours old culture suspension with the help of sterilized pipette and incubated at 37°C for 48 h. The change of color of the indicator from green to yellow indicated the production of acid. Presence of bubbles in the Durham's tube indicated the production of gas. No change in color indicated negativereaction.

3.14.2.3 Methyl red test (Bryan,1950)

Methyl red (M.R.) test is the test for mixed acid fermentation of glucose by microorganisms. Excreted acid contains large amount of formic, acetic, lactic and succinic acid and causes a major decrease in pH that can be detected by "Methyl Red" indicator. For this test V.P. broth was inoculated and incubated at 37°C for 5 days. After incubation, 5 drops of methyl red indicator were added to the culture broth.Redcolorthroughoutthebrothindicatedpositivereactionwhereasyellowor any yellowish red indicated negativereaction.

3.14.2.4 Voges Proskauer (V.P.) Test (SAB,1957)

For the Voges-Proskauer reaction according to the "Standard Methods" of the APHA (1946), to 1 ml of culture add 0.6 ml of 5% α-napthol in absolute alcohol and 0.2 ml of 40% KOH. It is important to shake for about 5 sec. after addition of each reagent. A recent modification of Coblenty (1943) is similar to the APHA methodbutusesaagarslantculturefollowedbyincubationofthebrothfor6hours. Also, the 40% KOH has 0.3% of creatine added to it to intensity the reaction.After additionofthereagentstheculture isshakenvigorouslyfor1minute.Positive

reaction is characterized by an intense rose-pink color developing in a few seconds to 10 min.

3.14.2.5 Utilization of Citrate (Atlas *et al.*,1995)

This test demonstrates the ability or inability of test organisms to use citrate assole source of carbon for metabolism and growth. Tubes containing Simmon's citrate agar were inoculated and incubated at 37°C for 4 days. Utilization of citrate was established by changing the color from green toblue.

3.14.2.6 Utilization of propionate (Sneath *et al.*, 1986)

Propionate agar slants were inoculated with 24 h old culture and incubated at 37°C for 3-5 days. Production of a pink color indicates the utilization of propionate by bacteria**.**

3.14.2.7 Deep glucose agar test (SAB,1957)

Microorganisms vary widely in their requirements for oxygen. The nature of microbial growth in agar deeps reflects the cells' relative need for oxygen or an oxygen free environment. In relation to free oxygen, organisms aregenerally classified as strict aerobes, microaerophiles, facultative anaerobes and strict anaerobes. A tube of deep glucose agar medium was inoculated in fluid condition approximatelyat45°C.Thetubewasrotatedtomixtheinoculumswiththemedium and was allowed to solidify. After incubation at 37°C for 7 days observation was made to find out whether the organisms grew on the surface and in the upper layer of the medium (strict aerobes), or the organisms grew just a few millimeters below the surface (microaerophiles), or the organisms grew throughout the medium (facultative anaerobes), or the organisms grew deeper in the medium (strict anaerobes).

3.14.2.8 Production of indole (Atlas *et al.*,1995)

The ability to hydrolyze tryptophan to indole is characteristic of certain entire bacteria that possess the enzyme tryptophanase. Tryptophanase catalyzes thehydrolysisoftryptophanwiththeproductionofindole,pyruvicacidandwater.The production of indole from tryptophan is useful in distinguishing *Escherichia coli* (indole positive) from other enteric bacteria that have many of the same physiological traits. The medium used to test for tryptophanase contains 1.0% tryptone, a peptone derived fromcasein.

Tryptonecontainsahighproportionoftryptophan,makingitasuitablesubstratefor thistest.

Procedures:

1. Inoculate tryptone broth tubes with the bacterialcultures.
2. Incubate the tubes at 37°C for 24-48h
3. After incubation add 10 drops of Kovoc's reagent directly to the culture tube.

3.14.2.9 Nitrate reduction test (Sneath *et al.*,1986)

Nitrate reduction is evident by complete or partial disappearance of nitrate accompanied by appearance of nitrate, ammonia or free nitrogen.

This test was performed to observe the organisms' capability on the reduction of nitratetonitrite.Theformationofnitriteindicatedthepresenceoftheenzymenitrate reductaseintheorganisms.Thefollowingthreereagentswererequiredforthistest:

Reagent A: Sulfanilic acid–acetic acid solution

Sulfanilicacid : 8.0g

5Naceticacid : 1000 ml

(Acetic acid: Distilled water = 1:2.5)

Sulfanilic acid was dissolved in acetic acid and stored in brown glass bottle.

Reagent B: Dimethyl-α-napthalamine solution

Dimethyl-α-napthalamine : 6.0 ml

5Naceticacid : 1000 ml

Reagent C: Zincdusts

Thetubesofnitratebrothinduplicateswereinoculatedwithtestorganismsandthen incubated at 37°C for 72 h. After incubation, 1 ml of reagent A was added to the incubatedtubeandshaken.Then1mlofreagentBwasalsoaddedtoeachtubeand shaken well. Formation of a distinct red or pink color indicated the reduction of nitrate to nitrite. Absence of nitrite may be due to complete conversion of nitrateas wellasnoreductionatall.Apinchofzincdustwasthenaddedtothetubeshowing absenceofnitriteanditwasallowedtostandforfewminutes.Anyremainingnitrate (in case) would be reduced to nitrite by zinc and the characteristic pink or redcolor would appear and no color indicated completereduction.

3.14.2.10 Degradation of tyrosine (Sneath *et al.*, 1986)

Tyrosine agar plates were inoculated with a single streak and were incubated for 7 days at 37°C.Clearing of tyrosine around the growth revealed the degradation of tyrosine.

3.14.2.11 Egg yolk lecithinase test (Sneath *et al.*,1986)

For this test, egg-yolk broth medium was inoculated by the selected isolates and incubated at 37°C. After 7 days incubation the appearance of a heavy white precipitation in or on the surface of the egg-yolk containing medium indicated the positive result i.e. the organisms produced lecithinase enzyme.

Lecithinase

A positive lecithinase test is noted by the appearance of a white, opaque, diffuse zone that extends into the medium surrounding the colonies. A negative lecithinase

test is indicated by the absence of a white, opaque zone extending from the edge of the colony.

Lipase

Apositivelipasetestisnotedbytheappearanceofaniridescentsheen(oilonwater) thatcanbeseenwhentheplateisheldatanangletoalightsource.Anegativelipase test is indicated by the absence of an iridescentsheen.

Proteolysis

A positive test is indicated by clear zones in the medium surrounding colonial growth. A negative test is indicated by the absence of a clear zone surrounding colonies within the medium.

3.14.2.12 Hydrolysis of casein (Collins and Lyne,1984)

Thistestdemonstratestheabilityofmicrobestodegradecaseinintosolublepeptides and amino acids by the enzyme casease. One ml of sterilized skim milk was taken in a sterilized Petri-plate and then melted agar medium was poured and mixed thoroughly. After solidifying, the plates were inoculated and incubated at 37°C for 48h.Formation of a clear, transparent zone around the growth indicated hydrolysis ofcasein**.**

3.14.2.13 Hydrolysis of starch (Claus,1995)

Organisms having enzyme amylase are capable of hydrolyzing starch to form monosaccharide or disaccharide. As an extra cellular enzyme, amylase diffuses outward from the bacterial cells and breakdown starch. This test revealed the presence or absence of the enzyme amylase in the organisms. For this test, starch-agar plates were inoculated with test organisms and the plates were incubated at 37°C for 48 h. After incubation, the surface of these plates was flooded withiodine

solution. Iodine reacts with starch and form starch iodide and gives the color deep blue. Development of a clear zone around the growth indicated starch hydrolysis.

3.14.2.14 Kligler's Iron Agar (KIA) test (WHO,1987)

Kligler's Iron Agar medium was used to differentiate Gram-negative enteric bacteria or their ability to ferment dextrose or lactose and their production of hydrogensulfide.Stabbingthebuttandstreakingtheslantthetubeswereinoculated with 24 h bacterial culture. The inoculated tubes were then incubated at 37 °C for 48 h. Yellow color in the butt and slant indicated acid production while, hydrogen sulfide production was indicated by blackening of slant. Break in the medium indicatedgasformation.Redcolorinthebuttandslantindicatealkalinereaction.

3.14.2.15 Urease Production Test (Rustigen and Stuart,1941)

A modified YS broth with 0.5% yeast extract and 0.0012% phenol red (w/v) was prepared in flask were sterilized. Urea was filter sterilized and then added to the above medium making 2% concentration of urea and finally the media was dispensed into sterile test tubes. The test cultures were inoculated and incubated at 37^0 C for 48 hours. Control tubes containing the basal medium (without urea) were inoculated and with urea not incubated. An increase in alkalinity indicated by magenta red color was regarded the presence of urease.

3.15 Optimization of the bacterial strains for the growth response at different pH and Temperature

3.15.1 Growth response of the strains at different pH (Sneath *et al.*,1986)

Fortestingthegrowthresponseofthestrainsatdifferent pH,bufferedpeptonewater broth (in 4:1 ratio) was adjusted to a wide range of pH i.e. 4.5, 6.5 and 8.5 (Table 5).Buffersolutionsusedwereofthefollowingcomposition(Williams*etal*.,1971).

Table 5. Properties of the chemicals required for different pH maintenance

pH of the buffer	Name ofChemicals	Quantity (gm)	Water(ml)
4.5	KH_2PO_4	5.45	200
6.5	KH_2PO_4	2.72	200
	K_2HPO_4	1.74	
	Na_2HPO_4	1.39	
8.5	K_2HPO_4	3.48	200
	Na_2HPO_4	2.78	

pHofthebuffersolutionswascheckedafterpreparation.Ateachstep,mediumwas adjusted with N/10 HCl or N/10 NaOH solution as required. Growth was recorded after 72 hincubation.

3.15.2 Growth response of the strains at different temperatures (Sneath *et al.*,1986)

In order to find out the optimum growth of strains in temperature, PYG broth medium was prepared. Then the broth tubes were inoculated with the isolates and wereallowedtogrowatdifferenttemperaturesuchas5,10,30,40,and65°C.The growth was observed and recorded carefully after 48h.

3.16 Growth response and bioremediation of heavy metals (Pb and Cd) bythe selected bacterialstrains

500 ppm of Pb solution and 1000 ppm of Cd solution was used with river water at the control bottles without addition of bacterial isolates, whereas, in the experimental bottles five different bacterial strains were used with said concentration of Pb and Cd, respectively. Subsamples were collected from both

control and experimental bottles after 5 days of intervals and were subsequently analyzed by monitoring the growth of bacterial isolates.

a) Five bacterial strains, considering their morphology and metabolicactivities were selected for bioremediation test were inoculated into the slants and incubated at 37^0C for 24 hours. Then the fresh subculture of 5 strains were inoculated into 5 different test tubes containing 10 ml nutrient broth and incubated at 37^0C for 24 hours. One ml of bacterial broth culture from each of5testtubeswastransferredintonew5testtubescontaining10mlnutrient broth, respectively and it was incubated at 37^0C for 24 hours. Finally, this procedure was followed for the next day and those test tubes wereincubated at 37^0C for 48hours.

b) Ten ml of bacterial broth culture from each of 5 test tubes were centrifuged at3000rpmfor10minutes.Thesupernatantwasdiscardedcarefullyandthe bacterial pellet was washed five time with sterile physiological saline water to remove the additional nutrients with the bacterialpellet.

c) Sixmlphysiologicalsalinewaterwasaddedineachof5testtubescontaining bacterial pellet to prepare the bacterial suspension by using vortex mixture device. The bacterial suspension was used as inoculum and inoculated into the experimental bottles with known concentration of Pb andCd.

d) Four ml of subculture were collected from each of control and experimental bottles and was analyzed byAAS.

Bacterialcelldensitywasmeasuredat5daysofintervalas0d,5d,10dand15d.The growth response of the selected strains were measured by usingspectrophotometer

HM_0	HM_1	HM_2
+	+	+
Bacteria	Bacteria	Bacteria

HM_0 = River water containing 0.01 ppm of Pb and 0.011 ppm of Cd,

HM_1 = 500.01 ppm of Pb

HM_2 = 1000.011 ppm of Cd

Plate 2. Preparation of solution for bioremediation of Pb and Cd

(Shimadzu,UV-120-02,Japan)at625nmwavelengthandheavymetal(PbandCd) reduction was analyzed byAAS.

3.17 Identification of the bacterialstrains

Following Bergey's Manual for Systematic Bacteriology Vol. 2, (Sneath *et al.* 1986), Gram-positive bacterial isolates were identified and Bergey's Manual for Systematic Bacteriology Vol. 1 (Krieg and Holt 1984), Gram-negative bacterial isolates were identified.

3.18 Experimentaldesign

ThepresentexperimentwaslaidoutinRandomizedCompleteBlockDesignwith2 factors in 3 replications. The experiment consisted of two factors: Factor A: Bacterial strains (5 levels) as-DRW-20, TRS-21, TRS-24, TRS-31, TRS-32 and FactorB:Heavymetalconcentration(3levels)as-HM_0(riverwater),HM_1(500.010 ppm of Pb) and HM_2 (1000.011 ppm of Cd) in thesolution.

3.19 Statisticalanalysis

The data obtained for different characters were statistically analyzed by using Statistix 10.0 computer package program to find out the significance of the difference of the Pb and Cd concentrations on growth response of bacterial strains and Pb and Cd reduction (ppm) by the bacterial strains. The significance of the difference among the treatment combinations of means was estimated by Tukey's Test at 0.05% level of significance.

Chapter IV

Results and Discussion

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Pb and Cd analysis of the collectedsamples

Pb and Cd analysis of the collected samples was presented in Table 6.

Table 6. Pb and Cd analysis of the collectedsamples

Sl No.	Sampling sites	Name of samples	Heavy metal analysis (ppm)	
			Pb	Cd
1	Dhaleshwari river: Binnadangi, Manikganj	Soil	10.89	0.345
		Water	0.006	0.004
		Bottle gourd leaf	1.290	0.011
2	Turag river: Tongi, Gazipur.	Soil	26.59	0.345
		Water	0.039	0.001
		Bottle gourd leaf	2.415	0.061

From Table 6, it clearly revealed that Pb and Cd concentrations found in the leaves ofbottlegourdfromboththesamplingsiteswerebeyondthesafelimitoftolerance as compared with the standard value recommended by FAO (2006) and EU(2007). Heavy metals found in vegetable leaves were extremely harmful to human health. Althoughthemetalconcentration(ppm)wassupposedtobehigherinsoilandwater samples based on their existing pollution level, surprisingly none of their concentrations could exceed the maximum permissible limit in agricultural land. The probable reason behind the increasing concentration in vegetable leaves was due to the use of chemical fertilizer, underground water and use of water hyacinths and other plants as mulching materials as well as composting elements during crop production in the field. Heavy metals in river water could be adsorbed by the roots of water hyacinths and through using chemical fertilizer and undergroundwater

ultimately increased the concentration of heavy metals in the vegetable leaves. Being hazardous components, Pb and Cd may cause more damage to human, animal,environmentandcropproductioninnearfutureifnotproperlyreducedtheir concentrations from sampling sides as well. So, careful bioremediation technology through using indigenous bacteria should be adapted to reduce the concentrationof heavy metals from the samplingareas.

4.2 Physical and chemical variables of thesamples

The results of the physicochemical, biological variables, bacterial abundance inthe sampling sites were describedbelow.

4.2.1 Temperature of the samplingsites

During present investigation fluctuation in the temperature was recorded in thetwo sampling sites. The air, soil and water temperature for Dhaleshwari river (Binnadangi,Manikganj)were20^0C,22^0Cand22^0CrespectivelywhereasforTurag river(Tongi,Gazipur)theywere35^0C,34^0Cand29^0Crespectively.Thetemperature difference found between the two sampling area occurred due to the change of the season and sample collection time. Temperature of the samples from different sampling sides were shown in Table7.

Table 7. Temperature (°C) of the sampling sites

Sampling sites	Temperature (^{0}C)		
	Air	Soil	Water
Dhaleshwari river	20	22	22
Turag river	35	34	29

4.2.2 Hydrogen ion concentration(pH)

The pH of the samples collected from both Dhaleshwari and Turag river was 7.40 and7.78insoiland8.10-7.19inwaterrespectively.Thistotallyindicatesthatthose bacterial strains cultured from the samples can easily survive in the alkaline pH conditions.pHofthesamplesofdifferentsamplingsiteswerepresentedinTable8.

Table 8.pH of the samples

Sampling sites	pH	
	Soil	**Water**
Dhaleshwari river	7.40	7.78
Turag river	8.10	7.19

4.3 Enumeration of aerobic heterotrophicbacteria

The heterotrophic bacterial count of two different types of samples was shown in Table 9. In case of contaminated soil sample the maximum number of bacterial count was found in Turag river and it was 7.5×10^7 cfu/g while minimum number was observed in Dhaleshwari river and it was 5.5×10^5 cfu/g. In case of water sample the maximum number of bacterial count was observed in Turag river and it was 1.1×10^6 cfu/ml while minimum number was found in Dhaleshwari river and it was 4.8×10^3cfu/ml.

Table 9. Bacterial count of the samples

Sampling sites	Colony count	
	Soil(cfu/g)	**Water(cfu/ml)**
Dhaleshwari river	5.5×10^5	4.8×10^3
Turag river	7.5×10^7	1.1×10^6

4.4 Isolation and selection of thestrains

During this study a total of 156 colonies were primarily selected. These colonies comprisedofallaerobicheterotrophicbacteria.Finally34strainswereselectedand purified for detail study towards identification. Bacterial colonies developed after applying dilution plate and streak plate techniques and the results were shown in plate 3 and plate4.

4.5 Colonial morphology of the selected strains

Colonies of the selected strains were found to be different in there for elevation, margin, surface, color and optical characteristics. The colonial morphology of the selected strains as observed on PYG agar was presented in Table 10 and Table 11.

4.6 Microscopic observation of the selectedstrains

Fromisolated34bacterialstrains,27weregrampositiveand7weregramnegative.

Among gram positive bacterial isolates 6 were long rod, spore former, occur in singly;2werelongrod,sporeformer,occurinchain;1wasrod,sporeformer,occur in singly; 1 was rod, spore former, occur in short chain; 1 was short rod, spore former, occur in singly; 9 were long rod, non-spore former, occur in singly; 5 were rod,non-sporeformer,occurinsingly;2wereshortrod,non-sporeformer,occurin singly which was described in Table 12 and Table 13. Among gram negative bacteria 1 was long rod, spore former, occur in singly; 3 were long rod, non-spore former, occur in singly; 2 were short rod, non-spore former, occur in singly and 1 was rod, non-spore former, occur in singly. Photomicrographs of the selected bacterial strains were shown in plate5.

4.7 Physiological and biochemical characteristics of the selectedstrains

Some physiological and biochemical tests of the selected bacterial strains were given in Table 14**,** Table 15 and shown in Plate 6.

Plate 3. Photographs showing bacterial colonies in PYGmedium

Plate4.Pureculture ofselectedstrainsbystreakplatetechnique

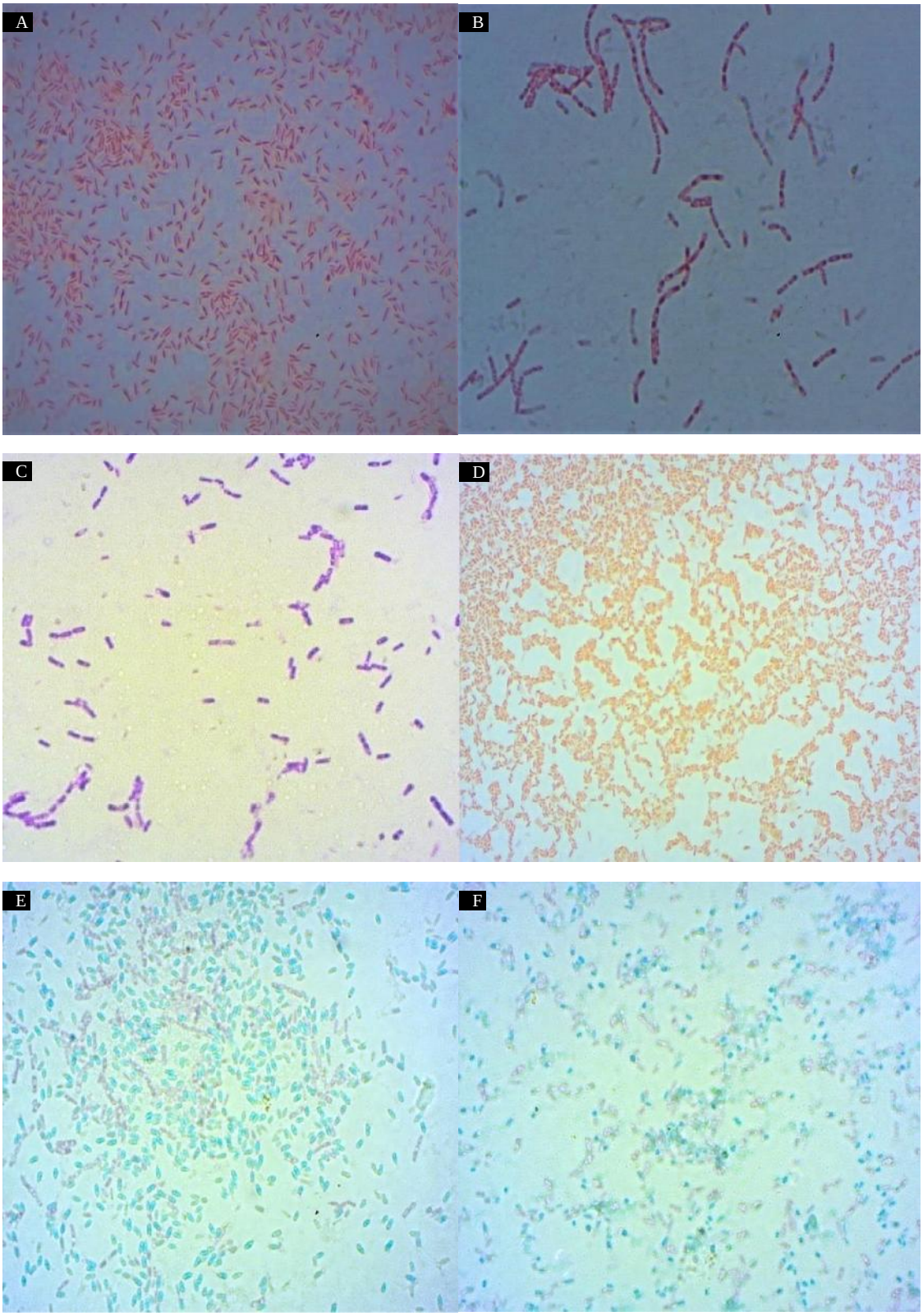


Plate 5. Photomicrograph showing (A-B) simple stain , (C) Gram positive, (D) Gram negative, (E-F) spore stain of the selected strains

V.P.test

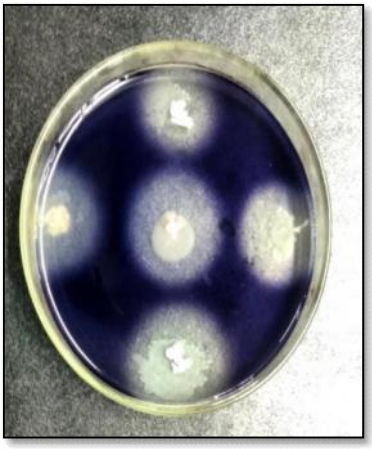
M.R. test

Utilization of citrate

Ureasetest

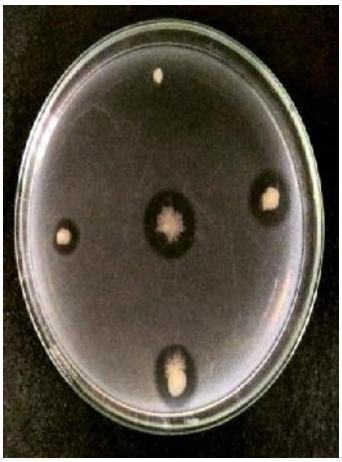
Indole test

K. I. A. test



Hydrolysisofstarch

Fermentationof Xylose



Hydrolysis ofcasein

Plate 6. Photographs showing the results of several biochemical tests

4.7.1 Physiological characteristics of the selected strains

TheresultofthephysiologicaltestswasgiveninTable14.AmongallstrainsDRS-2,DRW-7,DRW-8,TRS-24,TRW-27,TRW-28showedCatalasetestnegativeand rest of the strains were Catalase positive. 27 strains showed negative result and 7 showedpositiveresultwiththeKOHsolubilitytest.Among34testedstrains,3were Oxidase positive and 31 were negative. Out of 34 strains 11 were non-motile and rest of all weremotile.

4.7.2 Biochemical characteristics of the selectedstrains

The results of the biochemical tests were given in Table 15 and shown in Plate 5. Eighteen strains showed V.P. positive, rest 16 strains showed negative result. Twenty five strains showed Methyl red negative reaction, rest of 9 strains showed positive reaction. In the hydrolysis test of casein and starch. 22 strains showed negative effect on casein hydrolysis and rest of 12 isolates could hydrolyze casein; 11 isolates could hydrolyze starch and rest of all could not hydrolyze starch. Four strainscouldutilizecitrateand1couldutilizepropionate.No strainsshowedindole positive result and 31 strains were nitratereducer.

The result of fermentation tests with the selected carbohydrates were presented in Table15.Outof34strains,noneofthestrainscouldabletoproducegas.Fourwere able to ferment D-Glucose, 18 to L-Arabinose and D-Xylose while 20 strains were able to ferment D-Mannitol. Isolate no. DRS-1, DRS-3, DRW-6, DRW-7,DRW-8, DRW-9, DRW-17, TRW-28 were not able to ferment any tested carbohydrates. Strain no. DRS-12, TRS-21, TRS-31, TRS-33 were able to ferment all the four carbohydrates. Among 34 strains only 2 could utilize tyrosine. Only 16 strains showedlecithinasepositive.10strainsshowedthepositiveresultofeggyolklipase while 23 strains showed negative result in proteolysis test. Among gram negative bacteria, only 4 strains could produce H_2S but no strains could produce acid in butt in KIA test and none of them were gasproducer.

Table 10. Colony morphology of the selected strains on PYG agar medium from soil samples

Isolate No.	Form	Margin	Elevation	Surface	Pigmentation	Transparency	Diameter (mm)
DRS-1	Irregular	Curled	Flat	Rough	Offwhite	Opaque	4
DRS-2	Irregular	Curled	Raised	Smooth	Offwhite	Opaque	4
DRS-3	Circular	Entire	Convex	Smooth	White	Transparent	6
DRS-4	Irregular	Curled	Raised	Rough	Offwhite	Opaque	5
DRS-5	Circular	Entire	Raised	Rough	Offwhite	Opaque	6
DRS-11	Irregular	Undulate	Raised	Rough	Offwhite	Opaque	17
DRS-12	Irregular	Lobate	Raised	Rough	Offwhite	Opaque	19
DRS-13	Circular	Entire	Raised	Rough	White	Opaque	11
DRS-14	Irregular	Lobate	Raised	Rough	White	Opaque	15
DRS-15	Irregular	Curled	Raised	Rough	White	Opaque	15
TRS-21	Circular	Entire	Raised	Rough	Offwhite	Opaque	6
TRS-22	Circular	Entire	Convex	Smooth	Offwhite	Opaque	7
TRS-23	Circular	Curled	Raised	Wrinkle	White	Opaque	11
TRS-24	Irregular	Curled	Raised	Rough	White	Opaque	18

Table 10. (cont'd)

Isolate No.	Form	Margin	Elevation	Surface	Pigmentation	Transparency	Diameter (mm)
TRS-25	Circular	Entire	Flat	Smooth	White	Opaque	18
TRS-31	Circular	Entire	Flat	Smooth	Offwhite	Opaque	4
TRS-32	Circular	Entire	Raised	Smooth, Glistening	Offwhite	Opaque	5
TRS-33	Irregular	Curled	Flat	Rough	White	Opaque	6

Table 11. Colony morphology of the selected strains on PYG agar medium from water sample

Isolate No.	Form	Margin	Elevation	Surface	Pigmentation	Transparency	Diameter (mm)
DRW-6	Circular	Entire	Convex	Smooth, Glistening	Yellow	Opaque	7
DRW-7	Circular	Entire	Convex	Smooth, Glistening	Yellow	Opaque	5
DRW-8	Circular	Entire	Pulvinate	Smooth, Glistening	Yellow	Opaque	7
DRW-9	Circular	Entire	Convex	Smooth, Glistening	Yellow	Opaque	5
DRW-10	Circular	Entire	Convex	Smooth	Offwhite	Opaque	7
DRW-16	Irregular	Curled	Raised	Rough	Offwhite	Opaque	12
DRW-17	Irregular	Curled	Raised	Smooth, Glistening	White	Opaque	17
DRW-18	Irregular	Curled	Raised	Rough	White	Opaque	18
DRW-19	Irregular	Curled	Raised	Rough	Offwhite	Opaque	16
DRW-20	Irregular	Lobate	Raised	Rough	White	Opaque	17

Table 11. (cont'd)

Isolate No.	Form	Margin	Elevation	Surface	Pigmentation	Transparency	Diameter (mm)
TRW-26	Irregular	Curled	Raised	Wrinkle	Brick red	Opaque	8
TRW-27	Circular	Entire	Convex	Smooth, Glistening	White	Opaque	7
TRW-28	Circular	Entire	Convex	Smooth, Glistening	Offwhite	Opaque	6
TRW-29	Circular	Entire	Raised	Smooth	Offwhite	Opaque	6
TRW-30	Irregular	Curled	Raised	Smooth	White	Opaque	17
TRW-34	Circular	Entire	Convex	Smooth, Glistening	Yellow	Opaque	5

Table 12. Microscopic studies of the gram-positive strains

Isolates	Vegetative cell	Spore	Diameter (µm)
DRS-1	Long rod, occur in singly	Non spore former	2.12-2.31 × 1.03-1.12
DRS-2	Rod, occur in singly	Non spore former	1.81-1.54 × 0.80-0.93
DRS-4	Rod, occur in singly	Non spore former	1.34-1.53 × 0.57-0.72
DRS-5	Short rod, occur in singly	Non spore former	1.08-1.13 × 0.47-0.53
DRW-7	Long rod, occur in singly	Spore former	1.67-1.76 × 0.76-0.99
DRW-8	Long rod, occur in singly	Spore former	1.89-2.11 × 1.06-1.11
DRW-9	Long rod, occur in singly	Spore former	1.87-1.97 × 0.99-1.05
DRW-10	Long rod, occur in singly	Spore former	2.10-2.19 × 0.87-0.92
DRS-11	Short rod, occur in singly	Spore former	0.87-0.96 × 0.54-0.62
DRS-12	Rod, occur in singly	Non spore former	1.44-1.53 × 0.85-0.89
DRS-13	Rod, occur in singly	Spore former	1.43-1.54 × 0.86-0.89
DRS-14	Short rod, occur in singly	No spore former	0.88-0.94 × 0.48-0.52
DRS-15	Long rod, occur in singly	Spore former	1.87-1.98 × 0.99-1.05
DRW-16	Long rod, occur in singly	Spore former	1.77-1.89 × 0.87-0.93
DRW-17	Rod, occur in singly	Non spore former	1.67-1.78 × 0.88-0.93
DRW-18	Long rod, occur in chain	Spore former	2.03-2.14 × 1.01-1.11
DRW-19	Long rod, occur in singly	Non spore former	1.86-1.89 × 0.77-0.82
DRW-20	Long rod, occur in singly	Non spore former	1.89-1.97 × 0.67-0.75
TRS-22	Long rod, occur in singly	Spore former	1.99-2.07 × 0.79-0.83
TRS-23	Long rod, occur in singly	Non spore former	1.86-1.91 × 0.59-0.65
TRS-24	Long rod, occur in singly	Spore former	1.99-2.12 × 1.11-1.18
TRW-26	Long rod, occur in singly	Non spore former	2.11-2.17 × 1.08-1.11
TRW-27	Long rod, occur in singly	Spore former	1.88-1.96 × 0.88-0.99
TRW-29	Long rod, occur in chain	Spore former	1.77-1.86 × 0.69-0.77
TRW-30	Rod, occur in short chain	Spore former	1.70-1.85 × 1.11-1.14
TRS-32	Long rod, occur in singly	Spore former	1.64-1.78 × 0.56-0.59

Table 12. (cont'd)

Isolates	Vegetative cell	Spore	Diameter (µm)
TRW-34	Rod, occur in singly	Non spore former	1.55-1.59 × 0.49-0.56

Table 13. Microscopic studies of the gram-negative strains

Isolates	Vegetative cell	Spore	Diameter (µm)
DRS-3	Long rod, occur in singly	Non spore former	1.66-1.72 × 0.66-0.69
DRW-6	Long rod, occur in singly	Non spore former	1.78-1.89 × 0.61-0.66
TRS-21	Short rod, occur in singly	Non spore former	0.88-0.94 × 0.44-0.49
TRS-25	Long rod, occur in singly	Spore former	1.89-1.97 × 0.86-0.93
TRW-28	Long rod, occur in singly	Non spore former	1.80-1.85 × 0.45-0.49
TRS-31	Short rod, occur in singly	Non spore former	0.82-0.88 × 0.34-0.38
TRS-33	Rod, occur in singly	Non spore former	1.44-1.49 × 0.39-0.45

Table 14. Physiological characteristics of the selected strains

Isolates	Catalase Test	Oxidase Test	KOH Test	Motility Test
DRS-1	+	-	-	-
DRS-2	-	-	-	+
DRS-3	+	-	+	-
DRS-4	+	-	-	+
DRS-5	+	-	-	+
DRW-6	+	-	+	-
DRW-7	-	-	-	-
DRW-8	-	-	-	-
DRW-9	+	-	-	-
DRW-10	+	-	-	+
DRS-11	+	+	-	+
DRS-12	+	-	-	+
DRS-13	+	-	-	+
DRS-14	+	-	-	+
DRS-15	+	-	-	+
DRW-16	+	-	-	+
DRW-17	+	-	-	-
DRW-18	+	-	-	+
DRW-19	+	+	-	+
DRW-20	+	-	-	-
TRS-21	+	-	+	+
TRS-22	+	-	-	+
TRS-23	+	-	-	+
TRS-24	-	-	-	+
TRS-25	+	-	+	+
TRW-26	+	-	-	-

Table 14. (cont'd)

Isolates	Catalase Test	Oxidase Test	KOH Test	Motility Test
TRW-27	-	+	-	+
TRW-28	-	-	+	-
TRW-29	+	-	-	+
TRW-30	+	-	-	+
TRS-31	+	-	+	+
TRS-32	+	-	-	-
TRS-33	+	-	+	+
TRW-34	+	-	-	+

'+' indicate production of catalase, oxidase and gram-negative, motile bacteria

'-' indicate no production of catalase, oxidase and gram-positive, non-motile bacteria.

Table 15. Biochemical characteristics of the selectedstrains

Isolate Name	MR Test	VP Test	Deep glucose agar Test
DRS-1	-	-	A
DRS-2	+	-	FA
DRS-3	-	-	A
DRS-4	-	+	FA
DRS-5	+	-	FA
DRW-6	-	-	A
DRW-7	-	-	A
DRW-8	-	-	A
DRW-9	-	-	A
DRW-10	+	+	FA
DRS-11	+	-	A
DRS-12	-	+	FA
DRS-13	-	-	FA
DRS-14	-	+	FA
DRS-15	-	+	FA
DRW-16	-	+	FA
DRW-17	-	-	A
DRW-18	-	+	FA
DRW-19	-	+	FA
DRW-20	-	-	A
TRS-21	+	-	A
TRS-22	-	+	FA
TRS-23	-	+	FA
TRS-24	-	+	FA

Table 15. (cont'd)

Isolate Name	MR Test	VP Test	Deep glucose agar Test
TRS-25	+	+	FA
TRW-26	-	-	A
TRW-27	-	+	FA
TRW-28	-	-	A
TRW-29	+	+	FA
TRW-30	-	+	FA
TRS-31	+	+	A
TRS-32	-	-	FA
TRS-33	+	+	FA
TRW-34	-	+	FA

'+' indicate fermentation of glucose

'-' indicate glucose not fermented.

A = Aerobic, FA = Facultative anaerobic.

Table 15. (cont'd)

Isolates	Hydrolysis of	
	Starch	Casein
DRS-1	-	-
DRS-2	-	-
DRS-3	-	-
DRS-4	+	+
DRS-5	-	-
DRW-6	-	-
DRW-7	-	+
DRW-8	-	-
DRW-9	-	+
DRW-10	-	-
DRS-11	+	-
DRS-12	-	+
DRS-13	-	-
DRS-14	+	-
DRS-15	+	+
DRW-16	+	-
DRW-17	+	+
DRW-18	-	-
DRW-19	-	-
DRW-20	-	-
TRS-21	-	+
TRS-22	-	-
TRS-23	+	-
TRS-24	-	+
TRS-25	-	-

Table 15. (cont'd)

Isolates	Hydrolysis of	
	Starch	**Casein**
TRW-26	-	-
TRW-27	+	+
TRW-28	-	-
TRW-29	+	+
TRW-30	+	-
TRS-31	-	+
TRS-32	-	-
TRS-33	-	-
TRW-34	+	+

'+' Indicate hydrolysis

'-' Indicate no hydrolysis

Table 15.(cont'd)

Isolate No.	Utilization of		Formation of	
	Citrate	Propionate	Indole	Urease
DRS-1	-	-	-	-
DRS-2	-	+	-	-
DRS-3	-	-	-	-
DRS-4	-	-	-	-
DRS-5	+	-	-	+
DRW-6	-	-	-	-
DRW-7	-	-	-	-
DRW-8	-	-	-	-
DRW-9	-	-	-	-
DRW-10	-	-	-	-
DRS-11	-	-	-	-
DRS-12	-	-	-	+
DRS-13	-	-	-	-
DRS-14	-	-	-	-
DRS-15	-	-	-	-
DRW-16	-	-	-	-
DRW-17	-	-	-	-
DRW-18	-	-	-	-
DRW-19	-	-	-	-
DRW-20	-	-	-	-
TRS-21	+	-	-	+
TRS-22	-	-	-	-

Table 15.(cont'd)

Isolate No.	Utilization of		Formationof	
	Propionate	**Propionate**	**Indole**	**Urease**
TRS-23	-	-	-	+
TRS-24	-	-	-	-
TRS-25	-	-	-	-
TRW-26	-	-	-	-
TRW-27	-	-	-	-
TRW-28	-	-	-	-
TRW-29	-	-	-	-
TRW-30	-	-	-	-
TRS-31	+	-	-	+
TRS-32	-	-	-	-
TRS-33	+	-	-	+
TRW-34	-	-	-	-

''+'' indicate utilization of citrate, propionate and production of indole, urease.

''-'' indicate no utilization of citrate, propionate and non-production of indole, urease.

Table 15. (cont'd)

Isolate No.	Fermentation of Glucose	
	Acid	Gas
DRS-1	-	-
DRS-2	-	-
DRS-3	-	-
DRS-4	-	-
DRS-5	-	-
DRW-6	-	-
DRW-7	-	-
DRW-8	-	-
DRW-9	-	-
DRW-10	-	-
DRS-11	-	-
DRS-12	+	-
DRS-13	-	-
DRS-14	-	-
DRS-15	-	-
DRW-16	-	-
DRW-17	-	-
DRW-18	-	-
DRW-19	-	-
DRW-20	-	-
TRS-21	+	-
TRS-22	-	-

Table 15. (cont'd)

Isolate No.	Fermentation of Glucose	
	Acid	Acid
TRS-23	-	-
TRS-24	-	-
TRS-25	-	-
TRW-26	-	-
TRW-27	-	-
TRW-28	-	-
TRW-29	-	-
TRW-30	-	-
TRS-31	+	-
TRS-32	-	-
TRS-33	+	-
TRW-34	-	-

'+' = Gas production

'-' = No gas production

Table 15. (cont'd)

Isolate No.	Fermentation of the carbohydrates			
	D- Glucose	D-Xylose	D-Mannitol	L-Arabinose
DRS-1	-	-	-	-
DRS-2	-	-	-	+
DRS-3	-	-	-	-
DRS-4	-	+	+	+
DRS-5	-	+	+	+
DRW-6	-	-	-	-
DRW-7	-	-	-	-
DRW-8	-	-	-	-
DRW-9	-	-	-	-
DRW-10	-	-	-	+
DRS-11	-	+	+	-
DRS-12	+	+	+	+
DRS-13	-	-	-	-
DRS-14	-	+	+	+
DRS-15	-	+	+	+
DRW-16	-	+	+	+
DRW-17	-	-	-	-
DRW-18	-	+	+	-
DRW-19	-	+	+	-
DRW-20	-	-	-	-
TRS-21	+	+	+	+
TRS-22	-	+	+	+

Table 15. (cont'd)

Isolate No.	Fermentation of the carbohydrates			
	D- Glucose	**D-Xylose**	**D-Mannitol**	**L-Arabinose**
TRS-23	-	+	+	+
TRS-24	-	-	+	+
TRS-25	-	+	+	-
TRW-26	-	-	+	-
TRW-27	-	+	+	+
TRW-28	-	-	-	-
TRW-29	-	-	-	+
TRW-30	-	+	+	+
TRS-31	+	+	+	+
TRS-32	-	-	-	-
TRS-33	+	+	+	+
TRW-34	-	+	+	+

'+' = Acid production

'-' = No acid production

Table 15. (cont'd)

Isolate No.	Tyrosine degradation	Nitrate reduction
DRS-1	-	+
DRS-2	-	+
DRS-3	-	+
DRS-4	-	+
DRS-5	+	+
DRW-6	-	+
DRW-7	-	+
DRW-8	-	+
DRW-9	-	+
DRW-10	-	+
DRS-11	-	+
DRS-12	+	+
DRS-13	-	+
DRS-14	-	+
DRS-15	-	+
DRW-16	-	+
DRW-17	-	+
DRW-18	-	+
DRW-19	-	-
DRW-20	-	+
TRS-21	-	+
TRS-22	-	+
TRS-23	-	+
TRS-24	-	+

TRS-25	-	+

Table 15. (cont'd)

Isolate No.	Tyrosine degradation	Nitrate reduction
TRW-26	-	+
TRW-27	-	+
TRW-28	-	-
TRW-29	-	+
TRW-30	-	+
TRS-31	-	+
TRS-32	-	+
TRS-33	-	+
TRW-34	-	-

'+' indicate decomposition of tyrosine and reduction of nitrate.

'-' indicate tyrosine not decomposed and nitrate not reduced.

Table 15. (cont'd)

Isolates	Production of Egg Yolk		
	Lecithinase	Lipase	Proteolysis
DRS-1	+	-	-
DRS-2	-	-	+
DRS-3	+	+	-
DRS-4	+	-	-
DRS-5	-	+	+
DRW-6	+	-	-
DRW-7	+	-	-
DRW-8	+	-	-
DRW-9	+	-	-
DRW-10	-	+	-
DRS-11	-	+	-
DRS-12	-	-	+
DRS-13	-	-	-
DRS-14	+	-	-
DRS-15	+	-	-
DRW-16	-	+	+
DRW-17	+	-	+
DRW-18	+	-	-
DRW-19	-	-	-
DRW-20	+	-	-
TRS-21	-	-	+
TRS-22	-	+	-

Table 15. (cont'd)

Isolates	Production of Egg Yolk		
	Lecithinase	Lipase	Proteolysis
TRS-23	-	-	-
TRS-24	+	+	+
TRS-25	-	-	-
TRW-26	+	-	-
TRW-27	-	-	-
TRW-28	+	-	+
TRW-29	-	+	-
TRW-30	-	-	-
TRS-31	-	+	+
TRS-32	+	+	-
TRS-33	-	+	+
TRW-34	-	-	-

'+' = positive

'-' = Negative

Table 15. (cont'd)

KIA Test				
Isolates	**Slant**	**Butt**	**H_2S**	**Gas**
DRS-3	A	A	-	-
DRW-6	A	A	-	-
TRS-21	A	A	+	-
TRS-25	A	A	+	-
TRW-28	A	A	-	-
TRS-31	A	A	+	-
TRS-33	A	A	+	-

A = Alkaline red

'+' = positive

'-' = Negative

4.8 Optimization of the bacterial strains for the growth response atdifferent pH andTemperature

4.8.1 Growth response of the bacterial isolates at differentpH

All the 34 bacterial strains were tested for their growth responses at three different pH *viz*. 4.5, 6.5 and 8.5. The results were shown in Appendix III and Figure 1. The maximum growth of the strains were observed at pH 6.5. So it clearly defined that all the bacterial strains more or less liked to grow in slightly acidic to neutral environmental conditions.

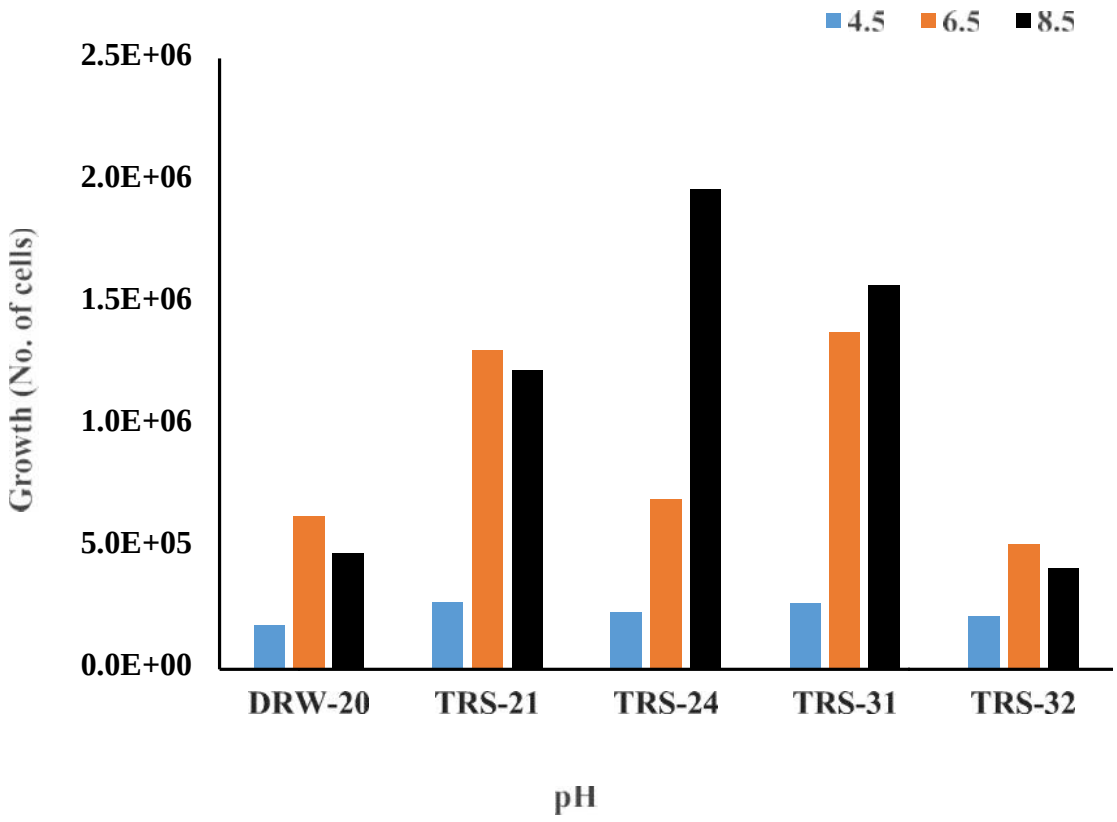


Figure 1: Growth response of the selected bacterial strains at different pH

DRW-20 = TRS-21= TRS-24= TRS-31 = TRS-32 = Baterial strain

4.8.2 Growth response of the bacterial strains at different temperature

Growth response of all the bacterial strains were tested at different temperature*viz*. 4^0C, 10^0C, 30^0C, 40^0C and 60^0C and was shown in Appendix IV and Figure 2. The maximum growth of the strains was found in between 30°C and40°C.

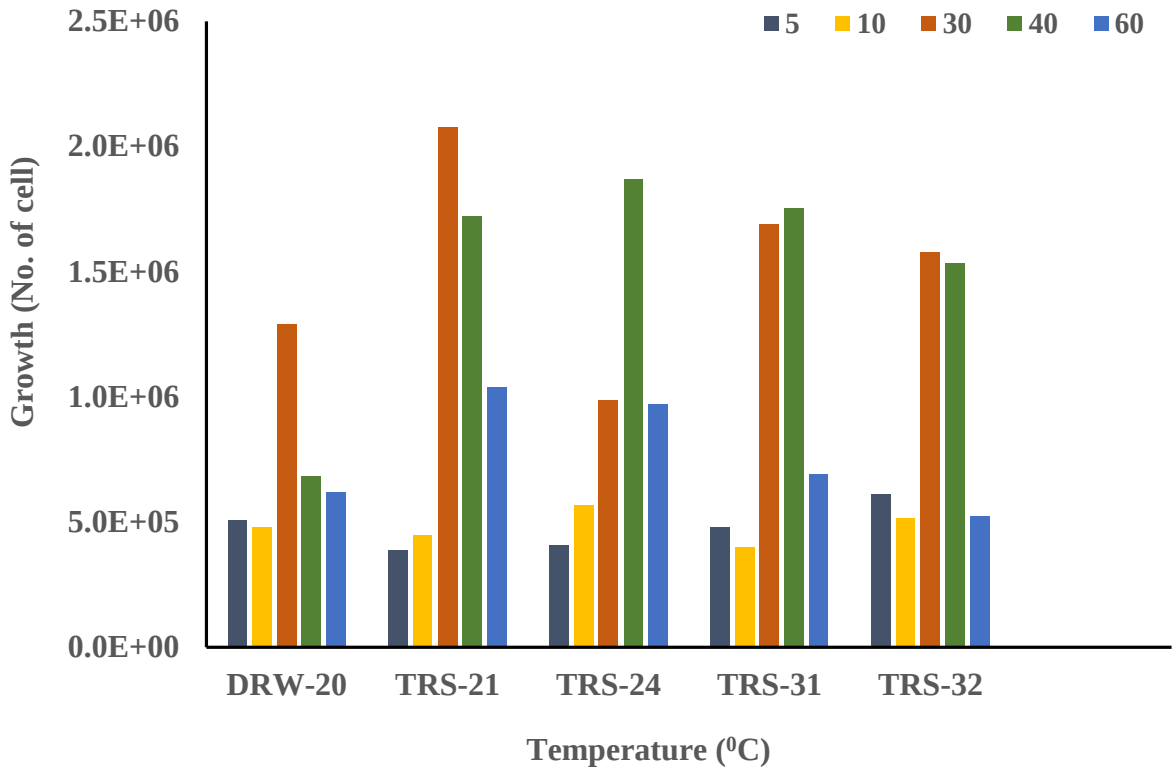


Figure 2. Growth response of the selected bacterial strains at different temperature

DRW-20 = TRS-21= TRS-24= TRS-31 = TRS-32 = Baterial strain

4.9 Growth response and bioremediation of Pb and Cd by the selected bacterialstrains

Growth response of the bacterial strains and bioremediation of Pb and Cd was presented in Appendix V, VI, VII, VIII and IX.

4.9.1 Growth response of the selected bacterial strains in Pb andCd

The data pertaining to the effect of Pb and Cd on growth response of the selected bacterial strains at day 0, 5, 10 and 15 were presented in Figure 3, Figure 4, Figure 5 & Appendix V, Appendix VI, Appendix VII and Appendix VIII.

4.9.1.1 Growth response of the selected bacterial strains inHM_0

The data pertaining to the effect of 0.01 ppm of Pb and 0.011 ppm of Cd in river water on growth response of the selected bacterial strains at day 0, 5, 10 and 15 were presented in Figure 3 & Appendix V.

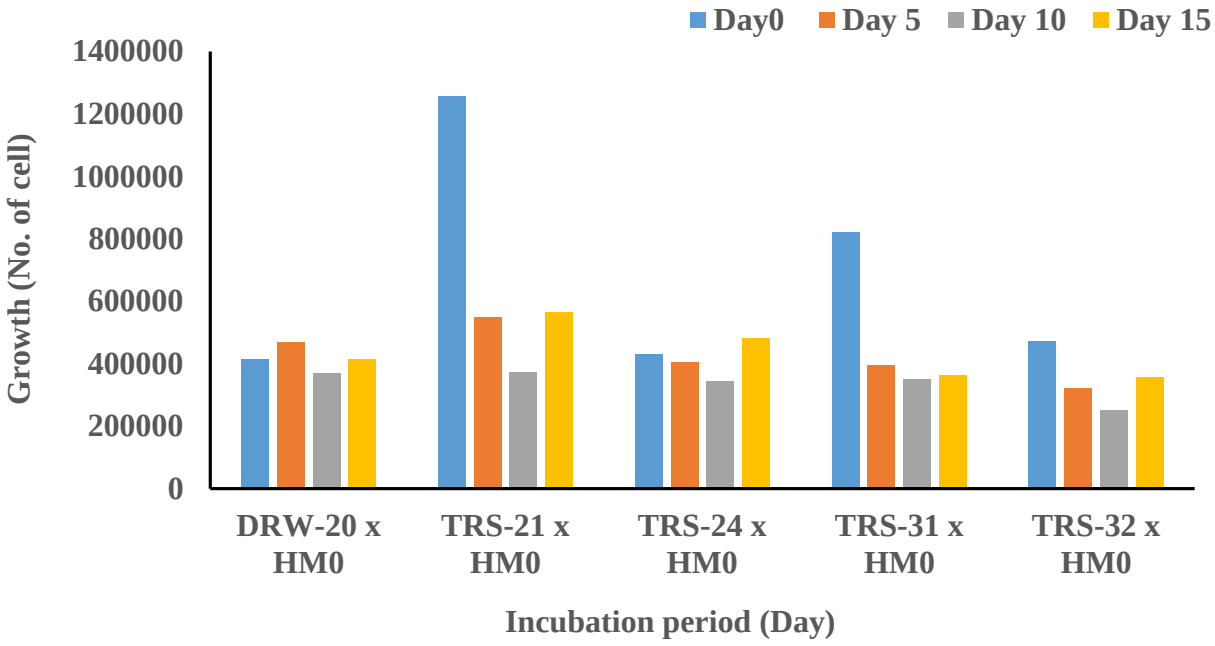


Figure 3. Growth response of the selected bacterial strains in HM_0

HM_0 = River water containing Pb and Cd
DRW-20 = TRS-21= TRS-24= TRS-31 = TRS-32 = Baterial strain

In figure 3, it was clearly visible that growth response of the bacterial strains *viz.* TRS-21, TRS-24, TRS-31 and TRS-32 in river water decreased gradually fromday 0today5andincreasedfromday10today15whileDRW-20showedtheopposite

at day 0 to day 5 by reducing the cell no. There was a significant difference in the growth response from day 0 to day 15 between TRS-21 and DRW-20.

4.9.1.2 Growth response of the selected bacterial strains inHM_1

The data pertaining to the effect of 500.01 ppm of Pb in river water on growth response of the selected bacterial strains at day 0, 5, 10 and 15 were presented in Figure 4 & Appendix VI.

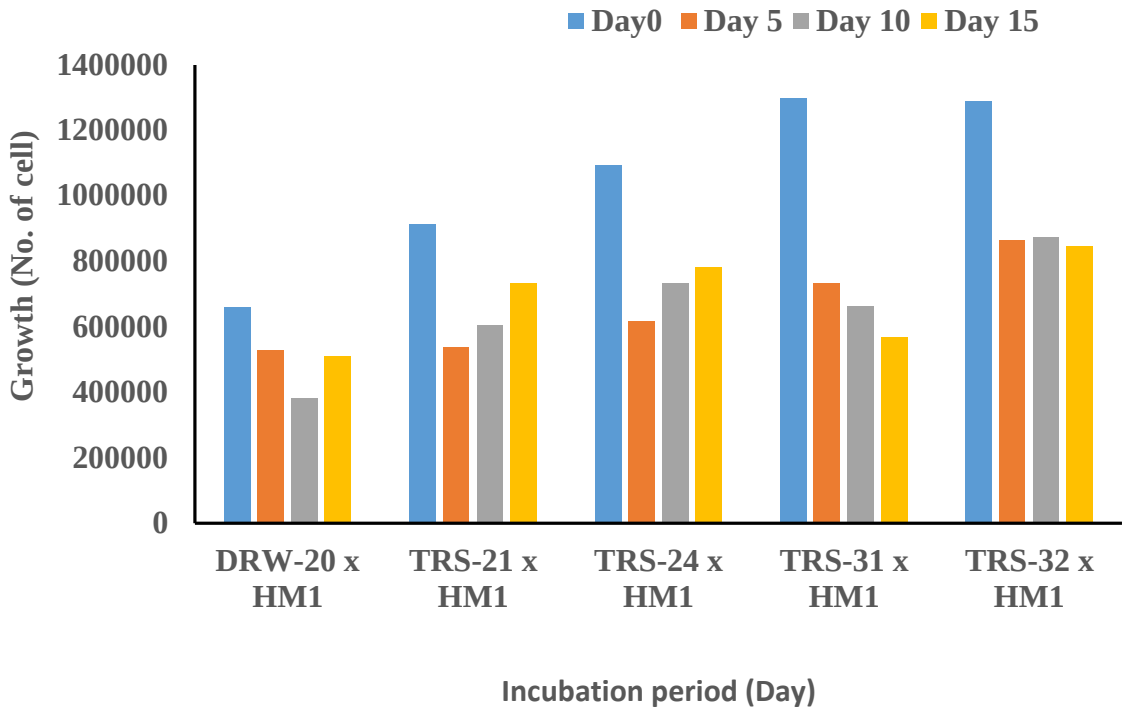


Figure 4. Growth response of the selected bacterial strains in HM_1

HM_1 = Pb concentration at 500.010 ppm,
DRW-20 = TRS-21 = TRS-24 = TRS-31 = TRS-32 = Bacterial strain

Figure 4 represented the growth response of the bacterial strains in river water treated with 500 ppm of Pb concentration. From Fig. 3, it clearly showed that the bacterial strains *viz*. DRW-20, TRS-21, TRS-24 and TRS-32 showed decreasing growth pattern from day 0 to day 5 while TRS-31 showed the opposite. From day 10 to 15, DRW-20, TRS-21 and TRS-24 showed increasing growth pattern while

TRS-31 and TRS-32 showed the opposite. There was a significant difference in the growth response at day 0 to day 15 between TRS-31 and DRW-20.

4.9.1.3 Growth response of the selected bacterial strains inHM_2

The data pertaining to the effect of 1000.011 ppm of Cd in river water on growth response of the selected bacterial strains at day 0, 5, 10 and 15 were presented in Figure 5 & Appendix VII.

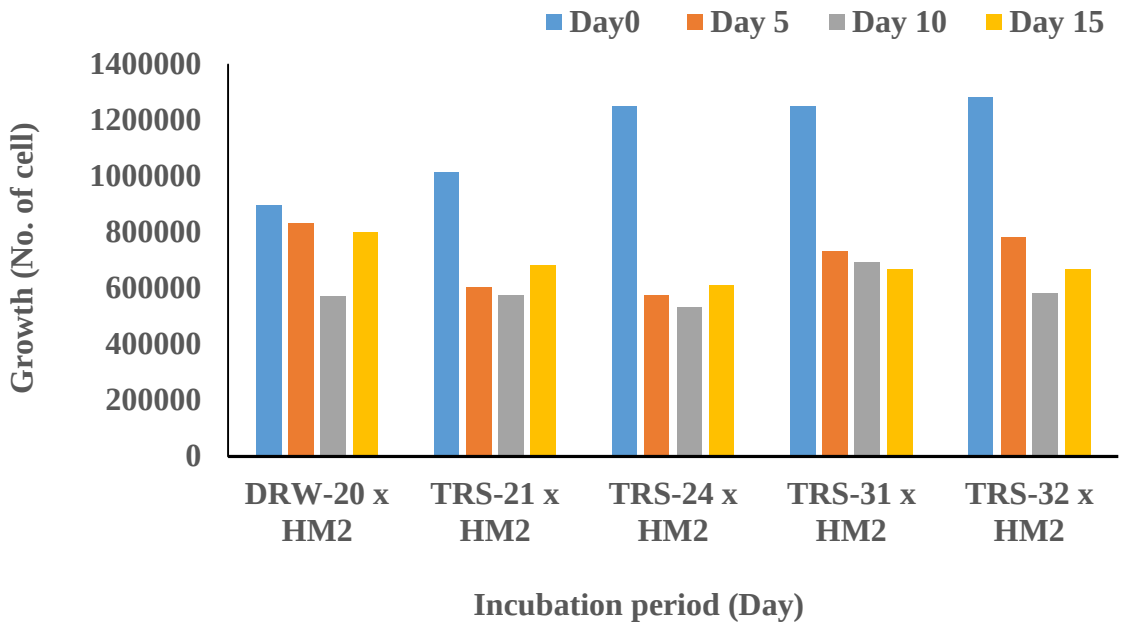


Figure 5. Growth response of the selected bacterial strains in HM_2

HM_2 = Cd concentration at 1000.011 ppm,
DRW-20 = TRS-21 = TRS-24 = TRS-31 = TRS-32 = Bacterial strain

Figure 5 represented the growth response of the bacterial strains in river water treated with 1000.011 ppm of Cadmium solution. From the above graph, it clearly showed that DRW-20, TRS-21, TRS-24, TRS-31 and TRS-32 represented decreasing growth pattern from day 0 to day 10. From day 10 to day 15 DRW-20, TRS-21, TRS-24 and TRS-32 showed increasing growth pattern whereas TRS-31

continued to decreased the growth pattern. There was a significant difference in the growth response from day 0 to day 15 between TRS-32 and DRW-20.

4.9.2 Bioremediation of Pb and Cd by the selected bacterial strains

Bioremediation of Pb and Cd in the solution by the selected bacterial strains was presented in AppendixIX.

4.9.2.1 Bioremediation of HM_0 by the selected bacterialstrains

Bioremediationof HM_0 containing0.01ppmofPband0.011ppmofCdbythe selected bacterial strains was presented in Table 16.

Table 16. Bioremediation of HM_0 by the selected bacterial strains

Treatment	Heavy metal reduction value (ppm)	
	Pb	**Cd**
DRW-20 x HM_0	-0.004 a	0.000667 a
TRS-21 x HM_0	0.00367 a	-0.005 a
TRS-24 x HM_0	0.00633 a	0.002 a
TRS-31 x HM_0	0.0167 a	0.000333 a
TRS-32 x HM_0	0.0167 a	0.00467 a
Tukey HSD $_{(0.05)}$	5.239	5.239
CV (%)	6.35	2.43

DRW-20 = TRS-21 = TRS-24 = TRS-31 = TRS-32 = Bacterial strain
HM_0 = 0.010 ppm Pb, 0.011 ppm Cd

Bioremediation of river water containing 0.010 ppm Pb and 0.011 ppm Cd was represented in Table 14. From the table, it indicated that different bacterial strains showed different pattern of heavy metal reduction. TRS-31(0.000333 ppm) and TRS-32 (0.00467 ppm) showed significant difference in Cd reduction with DRW-20 (0.000667 ppm) and TRS-24 (0.002 ppm). In contrast, TRS-21 (0.00367 ppm) and TRS-32 (0.0167 ppm) showed significant difference in Pb reduction with TRS-24 (0.00633 ppm). DRW-20 (-0.004 ppm) for Pb and TRS-21 (-0.005 ppm) for Cd showed negative reduction during the treatment respectively. There was a significant difference in the remediation of Pb and Cd between TRS-32 and TRS-24.

4.9.2.2 Bioremediation of HM_1 by the selected bacterialstrains

Bioremediation of 500.01 ppm of Pb by the selected bacterial strains was presented in Figure 6.

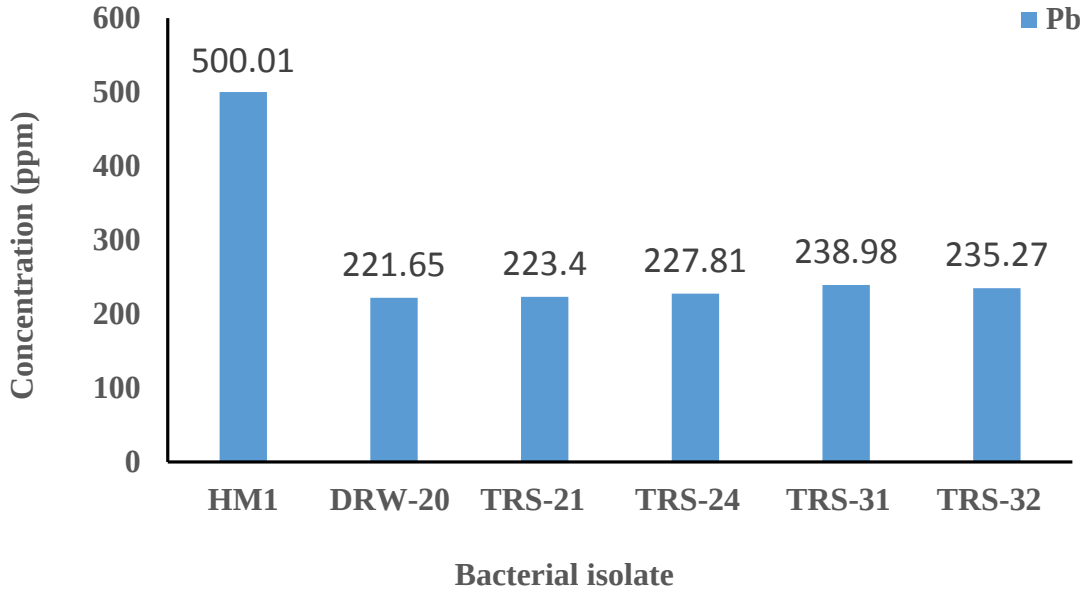


Figure 6. Bioremediation of HM_1 by the selected bacterial strains

HM_1 = 500.010 ppm Pb, DRW-20 = TRS-21 = TRS-24 = TRS-31 = TRS-32 = Bacterial strain

From Figure 6, it clearly indicated that TRS-31 (238.98 ppm) and TRS-32 (235.27 ppm) showed maximum reduction of Pb while DRW-20 (221.65 ppm) and TRS-21 (223.40 ppm) was very close to TRS-24 (227.81ppm). There was a significant difference in Pb reduction between TRS-31 (238.98 ppm) and DRW-20 (221.65ppm).

4.9.2.3 Bioremediation of HM_2 by the selected bacterial strains

Bioremediation of 1000.011 ppm of Cd by the selected bacterial strains was presented in Figure 7.

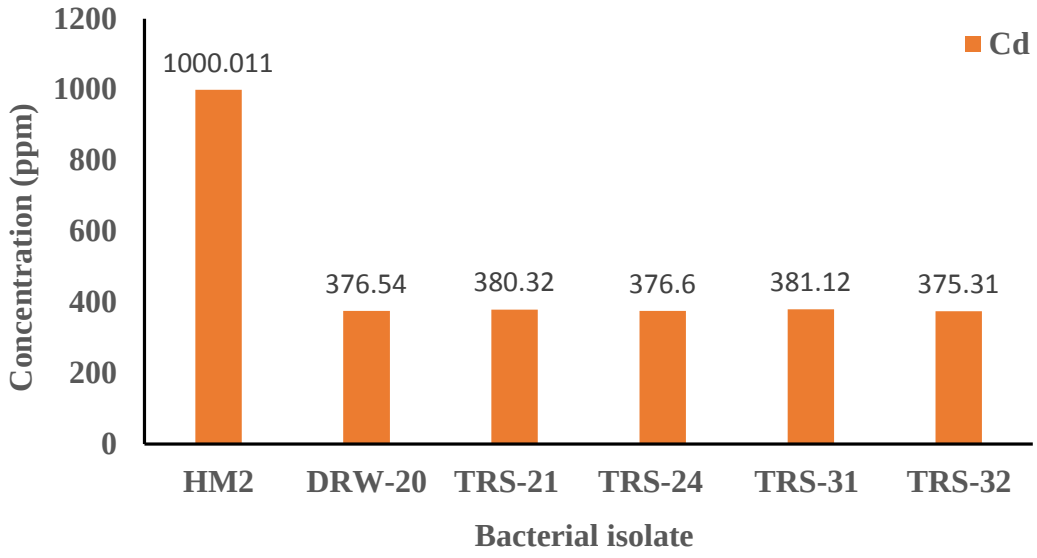


Figure 7. Bioremediation of HM_2 by the selected bacterial strains

HM_2 = 1000.011 ppm Cd, DRW-20 = TRS-21 = TRS-24 = TRS-31 = TRS-32 = Bacterial strains

FromFigure7,TRS-31(381.12ppm)andTRS-21(380.32ppm)showedsignificant difference in Cd reduction with DRW-20 (376.54 ppm), TRS-24 (376.60 ppm) and TRS-32 (375.31 ppm). There was a significant difference betweenTRS-31(381.12

ppm)andTRS-32(375.31ppm).So,there was a significant difference in the remediation of Cd between TRS-31 and TRS-32.

In view of overall performance, the strain TRS-31 showed maximum reduction capability for both Pb and Cd.

4.10 Identification of the bacterialstrains

Consulting all observed and tested characters of the isolated bacterial strains, identifications were done. The two separate groups (a) gram positive, aerobic heterotrophic bacteria and (b) gram negative bacteria were presented in two different Table 17 and Table 18 respectively. For the purpose of identification, Bergey'sManualofSystematicBacteriology(Sneath*etal*.,1986)wasfollowedfor the aerobic heterotrophic bacteria, while Manuals of WHO (1987), APHA (1989), Bergey'smanualofsystematicBacteriology(KriegandHolt,1984)wereconsulted for Gram-negativebacteria.

4.10.1 Aerobic heterotrophic gram-positivebacteria

Fromthe27aerobicheterotrophicgram-positivebacterialisolates,15belongtothe genera *Bacillus*, 8 were under the genus *Listeria* and 4 were under the genus *Caryophano*n.

4.10.2 Gram-negativebacteria

Among 7 gram-negative bacteria, 3 belong to the genera *Neisseria*, 2 were under the genus *Pseudomonas* and 2 were under the *Enterobacter*.

4.10.3 Description

In this study 34 aerobic heterotrophic bacterial isolates were selected for detailed study on the basis of their morphological and physiological characteristics. The organisms were compared with the standard description in the Bergey's Manual of Systematic Bacteriology, Vol. 1 (Krieg and Holt, 1984), Bergey's Manual of SystematicBacteriology,Vol.2(Sneath*etal.*,1986),andselectedbacterialisolates were provisionally identified. Among the bacterial isolates, 34 were selected for detailstudiesconsideringtheirmorphologicalandphysiologicalcharacteristics.Out of 34 bacterial isolates, 7 were gram negative and 27 were gram positive. Among gram positive bacterial isolates 15 were long rod, spore former where 15 belong to the genera *Bacillus*; 2 were long rod, non-spore former, 4 were rod, non-spore former and 2 were short rod, non-spore former where 8 were under the genus *Listeria*;3werelongrod,non-sporeformerand1wererod,non-sporeformerwhere 4wereunderthegenus*Caryophanon*.Thedifferentgrampositivebacterialisolates were: *Bacillus azotoformans* (5), *B. subtilis* (7), *B. alcalophilus* (2), *B. pumilus* (1), *Caryophanon latum* (4), *Listeria murrary* (6) and *L. grayi*(2).

Among gram negative bacterial isolates, 3 were long rod, non-spore former where 3 belong to the genera *Neisseria*. Two were short rod, non-spore former where 3 were under the genus *Pseudomonas*. One was long rod, spore former but the other was short rod, non-spore former where 2 were under the genus *Enterobacter*. The differentgramnegativebacterialisolateswere:*Neisseria*(3),*Pseudomonas*(2)*and Enterobacter*(2).

The isolated bacterial strains had some minor differences in biochemicalcharacters from those cited in the Bergey's Manual. Among gram positive bacteria, considering maximum similar characters DRS-1, DRW-17, DRW-20 and TRW-26 were provisionally identified as *Caryophanon latum*. The isolates were approximately similar with standard organism. DRS-2, DRS-4,DRS-5, DRS-12,

DRS-14 and TRS-23 were provisionally identified as *Listeria murrayi*. The isolate DRS-2wasdifferentfromstandardorganismbyproducingacidfromarabinoseand mannitolwhiletheVPtestshowednegativeresult.TheisolateDRS-4wasdifferent from standard organism by its ability to produce acid from arabinose and xylose; starch hydrolysis and lecithinase enzyme production in egg yolk test. The isolate DRS-5differedfromstandardorganismbyitsabilitytoproduceacidfromarabinose andxylosewhiletheVPtestshowednegativeresult.DRS-12differedfromstandard organismbyitsabilitytoproduceacidfromarabinoseandxylose.DRS-14differed from standard organism by its ability to produce acid from arabinose and xylose; starch hydrolysis and lecithinase enzyme production in egg yolk test. TRS-23 differed from standard organism by its ability to produce acid from arabinose, xylose and starch hydrolysis while the standard organism could not produce acid from arabinose, xylose and hydrolizestarch.

DRW-7, DRW-8, DRW-9, DRS-13 and TRS-32 were provisionally identified as *Bacillusazotoformans*.TheisolatesDRW-7,DRW-8,DRW-9andTRS-32differed from standard organism by their ability to produce lecithinase enzyme in egg yolk test. DRS-13 was approximately similar with standard organism. DRW-10 was provisionally identified as *Bacillus pumilus*. This isolate differed from standard organism by its inability to produce acid from xylose and mannitol. DRS-11, TRS-24 were provisionally identified as *Bacillus alcalophilus*. DRS-11 differed from standard organism by its ability to produce acid from arabinose. TRS-24 differed from standard organism by its inability to produce acid from xylose, lecithinase enzyme in egg-yolk test and starch hydrolysis. DRS-15, DRW-16, DRW-18, TRS-22, TRW-27, TRW-29 and TRW-30 were provisionally identified as*Bacillus subtilis*. DRS-15 differed from standard organism by its ability to produce lecithinase enzyme in egg yolk test while the standard organism could not. The isolatesDRW-16,TRW-27andTRW-30wereapproximatelysimilarwithstandard organism.DRW-18diferredfromstandardorganismbyitsinabilitytoproduceacid

from arabinose, lecithinase enzyme in egg yolk test while it was able to hydrolize starch. TRS-22 differed from standard organism by its inability to hydrolizestarch. TRW-29 differed from standard organism by its inability to produce acid from xylose and mannitol. DRW-19 and TRW-34 were provisionally identified as *Listeria grayi*. DRW-19 differed from standard organism by its ability to produce acid from xylose while the standard organism could not. TRW-34 differed from standard organism by its ability to produce acid from arabinose, xylose and starch hydrolysis while the standard organism showed the negativeresult.

Among gram negative bacteria, DRS-3, DRW-6 and TRW-28 were provisionally identified as *Neisseria elongata.* The isolate TRW-28 was approximately similar with standard organism. But the isolate DRS-3 and DRW-6 was different from standard organism by their ability in nitrate reduction test while the standard organismcouldnotreducenitratetonitrite.TRS-21andTRS-31wereprovisionally identified as *Pseudomonas mendocina* where both the isolates were approximately similar with standard organism. TRS-25 was provisionally identified as *Enterobacteragglomerans.*Thisisolatewasdifferentfromstandardorganismbyits inability to produce acid from glucose and arabinose while the standard organism could produce acid from both glucose and arabinose. The isolate also could not utilize citrate and showed negative VP and MR test while the standard organism could utilize citrate and also showed positive VP and MR test. TRS-33 was provisionally identified as *Enterobacter gergoviae.* The isolate TRS-33 was approximately similar with standard organism.

The provisional identified gram positive bacteria and gram negative bacteria were presented in Table 17 and 18.

Table 17. Provisional identification of the selected gram-positive strains

Isolate No	Provisionally identified name
DRS-1	*Caryophanon latum*
DRS-2	*Listeria murrayi*
DRS-4	*Listeria murrayi*
DRS-5	*Listeria murrayi*
DRW-7	*Bacillus azotoformans*
DRW-8	*Bacillus azotoformans*
DRW-9	*Bacillus azotoformans*
DRW-10	*Bacillus pumilus*
DRS-11	*Bacillus alcalophilus*
DRS-12	*Listeria murrayi*
DRS-13	*Bacillus azotoformans*
DRS-14	*Listeria murrayi*
DRS-15	*Bacillus subtilis*
DRW-16	*Bacillus subtilis*
DRW-17	*Caryophanon latum*
DRW-18	*Bacillus subtilis*
DRW-19	*Listeria grayi*
DRW-20	*Caryophanon latum*
TRS-22	*Bacillus subtilis*
TRS-23	*Listeria murrayi*
TRS-24	*Bacillus alcalophilus*
TRW-26	*Caryophanon latum*
TRW-27	*Bacillus subtilis*
TRW-29	*Bacillus subtilis*
TRW-30	*Bacillus subtilis*

Table 17. Provisional identification of the selected gram-positive strains

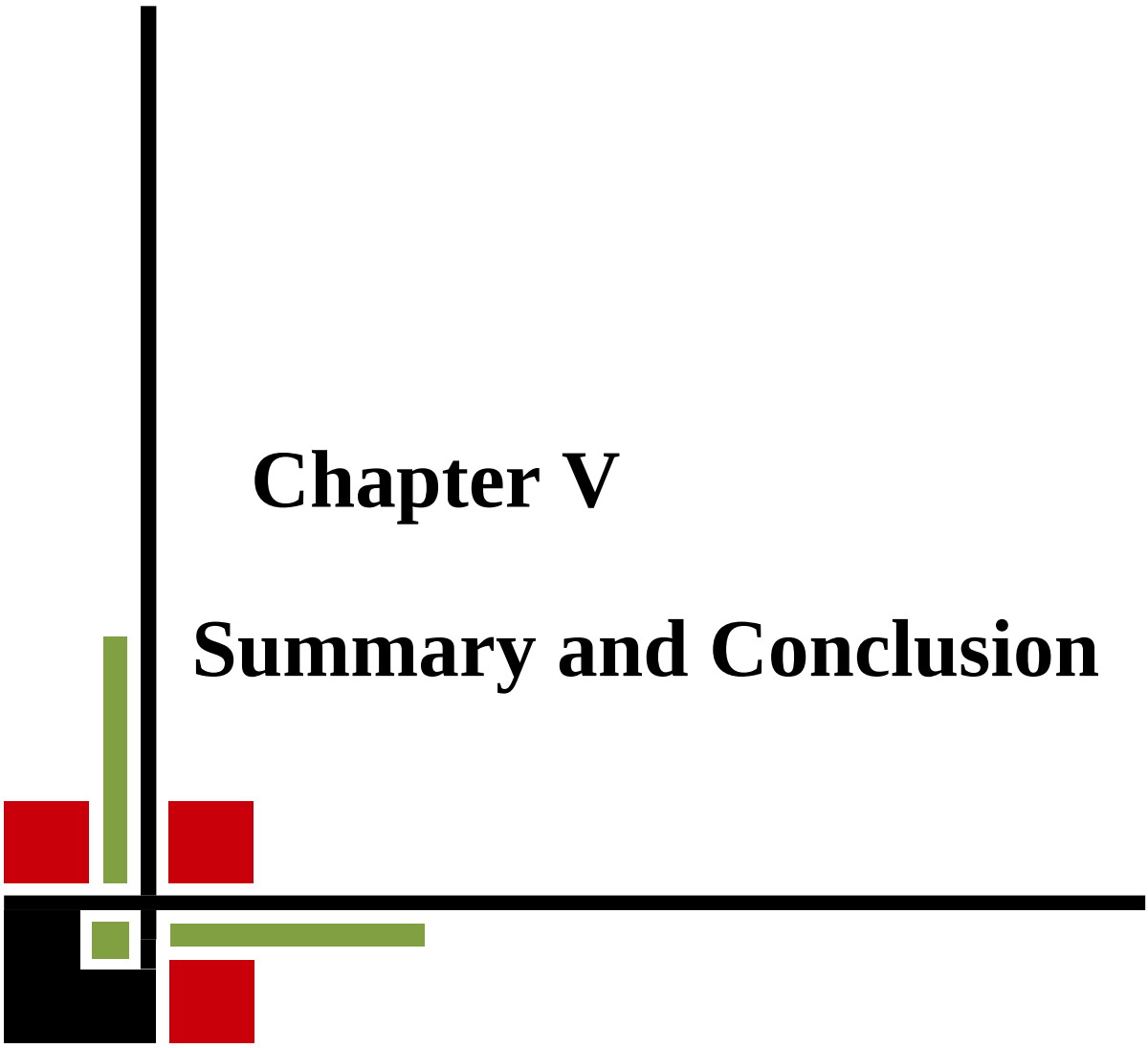
Isolate No	Provisionally identified name
TRS-32	*Bacillus azotoformans*
TRW-34	*Listeria grayi*

Table 18. Provisional identification of the selected gram-negative strains

Isolate No	Provisionally identified name
DRS-3	*Neisseria elongata*
DRW-6	*Neisseria elongata*
TRS-21	*Pseudomonasmendocina*
TRS-25	*Enterobacter agglomerans*
TRW-28	*Neisseria elongata*
TRS-31	*Pseudomonasmendocina*
TRS-33	*Enterobacter gergoviae*

Chapter V

Summary and Conclusion



CHAPTER V
SUMMARY AND CONCLUSION

The present experiment was conducted at the Laboratory of Microbiology, Department of Botany, University of Dhaka from October 2017 to February 2019 to evaluate the efficiency of indigenous bacteria for the reduction of lead and cadmium in the polluted water. The experiment consisted of two factors: Factor A: Bacterial strains (5 levels) as-DRW-20, TRS-21, TRS-24, TRS-31, TRS-32 and FactorB:Heavymetalconcentration(3levels)as-HM_0(riverwater),HM_1(500.010 ppm of Pb) and HM_2 (1000.011 ppm of Cd). The two factor experiment was laid out in Randomized Complete Block Design (RCBD) with three replications. Data were recorded for growth response of bacterial strains in Pb and Cd and bioremediationofPbandCdbythosestrainsandsignificantvariationwasrecorded for different treatment. The collected data were statistically analyzed for the evaluation of the treatment effect.

All the 34 bacterial strains were tested for their growth responses at three different pH *viz*. 4.5, 6.5 and 8.5. The maximum growth of the strains were observed at pH 6.5. So it clearly defined that all the bacterial isolates more or less liked to grow in slightly acidic to neutral environmental conditions.

Growth response of all the 34 bacterial strains were tested at different temperature *viz*.4^0C,10^0C,30^0C,40^0Cand60^0Candmaximumgrowthofthestrainswasfound in between 30°C and40°C

In case of growth response by the selected bacterial strains in river water, it was clearly visible that growth response of the bacterial strains *viz*. TRS-21, TRS-24, TRS-31 and TRS-32 in river water decreased gradually from day 0 to day 5 and increased from day 10 to day 15 while DRW-20 showed the opposite at day 0 to

day 5 by reducing the cell no. There was a significant difference in the growth response from day 0 to day 15 between TRS-21 and DRW-20.

In case of growth response of the selected bacterial strains in 500 ppm of Pb with river water, bacterial strains *viz*. DRW-20, TRS-21, TRS-24 and TRS-32 showed decreasing growth pattern from day 0 to day 5 while TRS-31 showed the opposite. From day 10 to 15, DRW-20, TRS-21 and TRS-24 showed increasing growth pattern while TRS-31 and TRS-32 showed the opposite. There was a significant difference in the growth response at day 0 to day 15 between TRS-31 and DRW-20.

In case of growth response of the selected bacterial strains in 1000 ppm of Cd with river water, DRW-20, TRS-21, TRS-24, TRS-31 and TRS-32 represented decreasing growth pattern from day 0 to day 10. From day 10 to day 15 DRW-20, TRS-21, TRS-24 and TRS-32 showed increasing growth pattern whereas TRS-31 continued to decrease the growth pattern. There was a significant difference in the growth response from day 0 to day 15 between TRS-32 and DRW-20.

DuringtheremediationofPbandCdbythestrainsinriverwater,TRS-31(0.000333 ppm) and TRS-32 (0.00467 ppm) showed significant difference in Cd reduction with DRW-20 (0.000667 ppm) and TRS-24 (0.002 ppm). In contrast, TRS-21 (0.00367 ppm) and TRS-32 (0.0167 ppm) showed significant difference in Pb reduction with TRS-24 (0.00633 ppm). DRW-20 (-0.004 ppm) for Pb and TRS-21 (-0.005 ppm) for Cd showed negative reduction during the treatment respectively. There was a significant difference in the remediation of Pb and Cd between TRS- 32 andTRS-24.

In case of remediation of Pb by the strains in Pb with river water, TRS-31 (238.98 ppm) and TRS-32 (235.27 ppm) showed maximum reduction of Pb while DRW-20(221.65ppm)andTRS-21(223.40ppm)wasveryclosetoTRS-24(227.81ppm). There was a significant difference in Pb reduction between TRS-31 (238.98 ppm) and DRW-20 (221.65ppm).

In case of remediation of Cd by the strains in Cd with river water, TRS-31(381.12 ppm)andTRS-21(380.32ppm)showedsignificantdifferenceinCdreductionwith DRW-20(376.54ppm),TRS-24(376.60ppm)andTRS-32(375.31ppm).There was a significant difference between TRS-31(381.12 ppm) and TRS-32 (375.31 ppm). There was a significant difference in the remediation of Cd between TRS-31 and TRS-32.

In view of overall performance, the strain TRS-31 showed maximum reduction capability for both Pb and Cd.

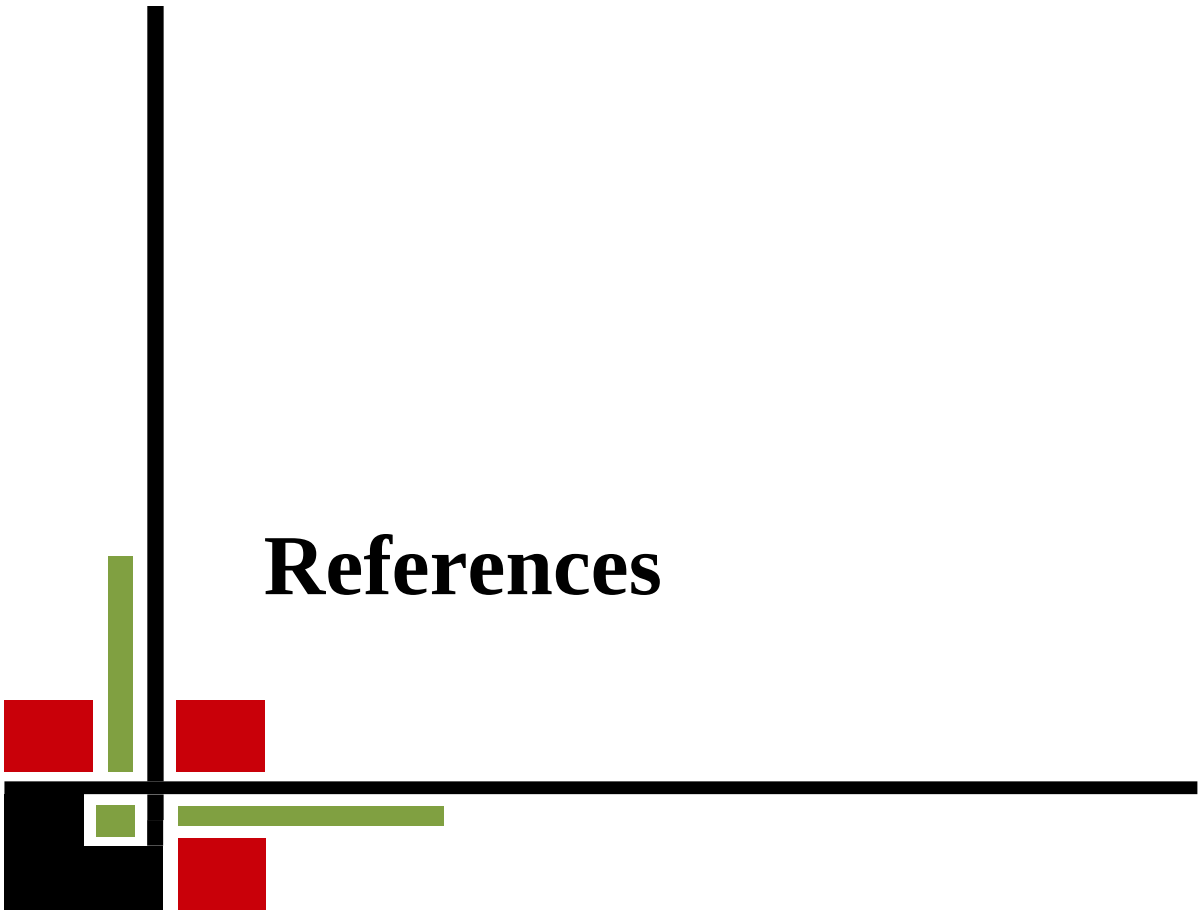
CONCLUSION

Heavymetalpoisoningoffoodisanerverackingissuefordevelopingcountrieslike Bangladesh. Unscientific waste disposal technique and injudicious application of chemical fertilizer or pesticide is one of the major cause of soil and surface water contamination with Pb andCd.

This study demonstrates that all the five bacterial strains confer a positive result in remediation of Pb and Cd concentrations in the controlled condition. This also suggest the possibility of these bacteria have the ability in the bioremediation of heavy metals in a highly polluted river water. Therefore, these bacteria can be utilized as potential bioremediation agent to eliminate or decrease the heavy metal pollutants in future.

However, more study on this matter should be executed in order to reconfirm the bioremediation activity by the bacteria, the presence of the bacteria, as well as bacteriaidentificationgenotypicallyandtheconcentrationofheavymetalspollution in the riverwater.

Thereisanurgentneedtoprotectandconserveourenvironmentbyreducingheavy metalpollutioninwaterandsoilorelseitwouldbesoonoutofourcontroltoensure safe vegetable/crop cultivation as well as to protect theenvironment.



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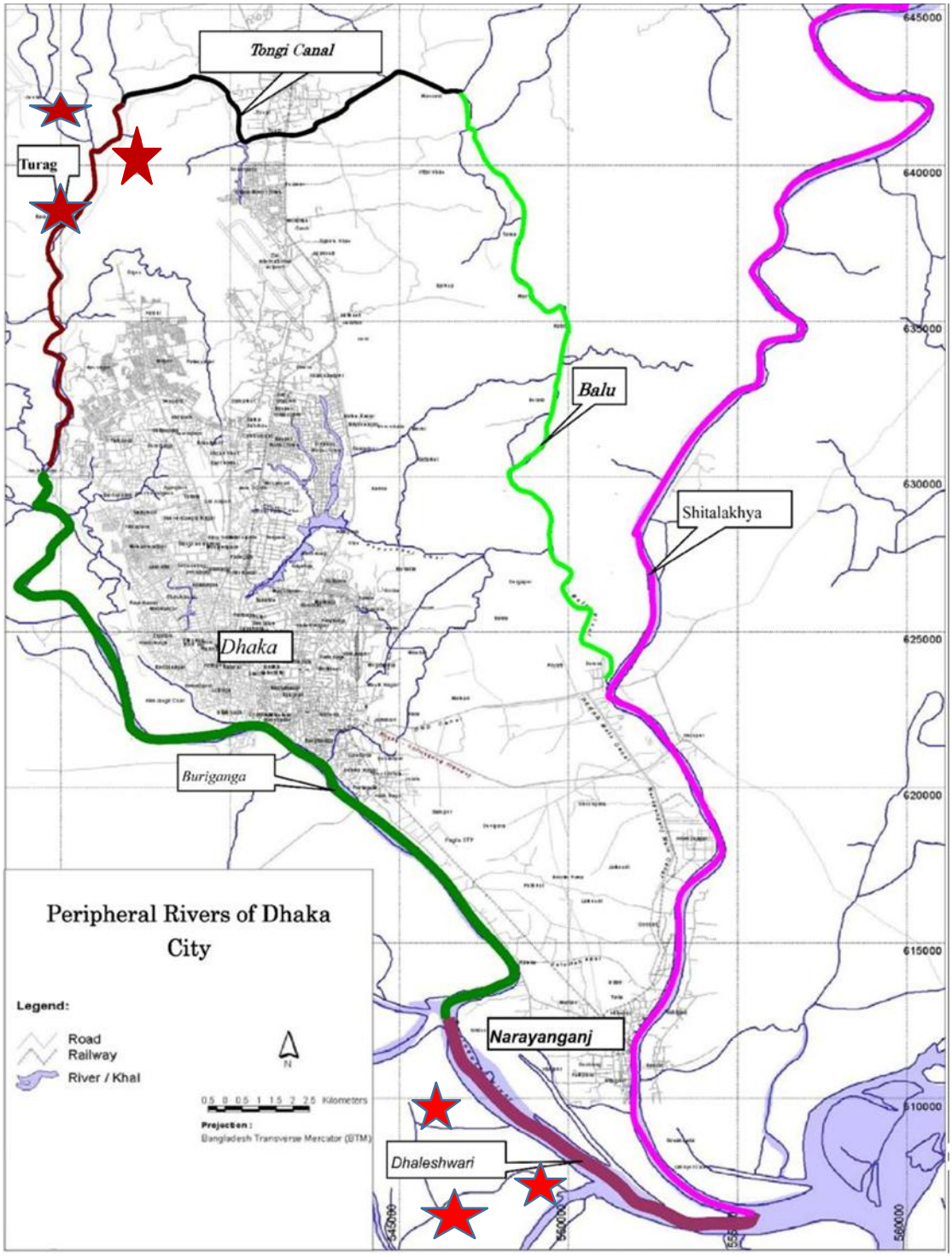
Appendices

APPENDICES

APPENDIX I. Map showing the sampling site



APPENDIX II. Map showing the experimental station

(Department of Botany, Laboratory of Microbiology,

University of Dhaka)

APPENDIX III. Growth response of the bacterial strains at different pH

Isolate No.	No. of cells in pH		
	4.5	6.5	8.5
DRS-1	281481	505556	379630
DRS-2	390741	1216667	631481
DRS-3	277778	438889	401852
DRS-4	270370	390741	1481481
DRS-5	298148	1216667	1324074
DRW-6	253704	688889	374074
DRW-7	175926	498148	1127778
DRW-8	255556	642593	446296
DRW-9	175926	757407	412963
DRW-10	250000	1437037	1429630
DRS-11	261111	1540741	2133333
DRS-12	270370	1718519	1622222
DRS-13	251852	511111	390741
DRS-14	187037	1479630	603704
DRS-15	181481	733333	409259
DRW-16	179630	914815	507407
DRW-17	238889	531481	427778
DRW-18	183333	1237037	346296
DRW-19	259259	1294444	418519
DRW-20	179630	627778	475926
TRS-21	272222	1303704	1224074
TRS-22	264815	1324074	527778
TRS-23	233333	553704	383333
TRS-24	233333	694444	1964815

APPENDIX III. (cont'd)

Isolate No.	No. of cells in pH		
	4.5	**6.5**	**8.5**
TRS-25	307407	550000	607407
TRW-26	187037	464815	320370
TRW-27	240741	644444	383333
TRW-28	179630	733333	1709259
TRW-29	181481	625926	1301852
TRW-30	237037	837037	1579630
TRS-31	270370	1377778	1568519
TRS-32	214815	509259	412963
TRS-33	259259	1596296	1507407
TRW-34	188889	746296	490741

APPENDIX IV. Growth response of the bacterial strains at different temperature

Isolate No.	No. of cells in Temperature (^{0}C)				
	5	10	30	40	60
DRS-1	472222	520370	988889	1066667	970370
DRS-2	825926	644444	1774074	1807407	687037
DRS-3	481481	490741	1572222	1448148	533333
DRS-4	550000	564815	1485185	1570370	1192593
DRS-5	440741	468519	1527778	1742593	629630
DRW-6	475926	446296	883333	1044444	548148
DRW-7	509259	403704	1790741	1253704	533333
DRW-8	475926	461111	1453704	829630	940741
DRW-9	550000	435185	1092593	1181481	522222
DRW-10	696296	466667	1620370	1268519	611111
DRS-11	729630	575926	1561111	1146296	877778
DRS-12	459259	529630	1742593	1229630	1048148
DRS-13	514815	451852	1050000	792593	1003704
DRS-14	503704	398148	1620370	1627778	1098148
DRS-15	472222	394444	1303704	1350000	1075926
DRW-16	509259	396296	903704	983333	966667
DRW-17	587037	479630	1140741	1194444	1029630
DRW-18	507407	435185	1125926	548148	629630
DRW-19	398148	474074	1279630	914815	575926
DRW-20	507407	479630	1290741	685185	620370
TRS-21	387037	446296	2075926	1718519	1038889

APPENDIX IV. (cont'd)

Isolate No.	No. of cells in Temperature (^{0}C)				
	5	**10**	**30**	**40**	**60**
TRS-22	488889	524074	1418519	1305556	666667
TRS-23	487037	538889	1072222	1307407	975926
TRS-24	405556	564815	985185	1868519	972222
TRS-25	600000	470370	898148	990741	1040741
TRW-26	590741	450000	1374074	688889	1062963
TRW-27	490741	527778	1351852	987037	948148
TRW-28	544444	572222	1461111	648148	946296
TRW-29	627778	566667	1205556	1370370	790741
TRW-30	505556	503704	1198148	1359259	566667
TRS-31	477778	398148	1687037	1750000	688889
TRS-32	611111	516667	1577778	1535185	524074
TRS-33	472222	590741	1840741	1596296	1040741
TRW-34	514815	472222	1764815	1607407	1014815

APPENDIX V: Growth response of the selected bacterial strains in HM_0

Treatment	No. of cells			
	Day 0	**Day 5**	**Day 10**	**Day 15**
DRW-20 x HM_0	414815 e	470370 d-f	370370 c-f	416667 de
TRS-21 x HM_0	1258704 ab	548704 de	372778 c-f	566667 a-e
TRS-24 x HM_0	430926 e	403704 ef	343148 ef	482778 c-e
TRS-31 x HM_0	822222 b-e	394444 ef	351852 d-f	364815 e
TRS-32 x HM_0	474074 de	324074 f	252407 f	358704 e
Tukey HSD **(0.05)**	5.239	5.239	5.239	5.239
CV (%)	15.65	10.06	15.12	16.37

DRW-20 = TRS-21 = TRS-24 = TRS-31 = TRS-32 = Bacterial strain

HM_0 = 0.01 ppm of Pb and 0.011 ppm of Cd

APPENDIX VI: Growth response of the selected bacterial strains in HM_1

Treatments	No. of cells			
	Day 0	**Day 5**	**Day 10**	**Day 15**
DRW-20 x HM_1	658704 c-e	528333 de	382037 c-f	507963 b-e
TRS-21 x HM_1	913519 a-d	538333 de	603148 bc	731481 a-c
TRS-24 x HM_1	1093148 a-c	618519 b-d	732778 ab	780185 a-c
TRS-31 x HM_1	1297593 a	731481 a-c	661667 ab	566667 a-e
TRS-32 x HM_1	1288889 a	864815 a	873519 a	843889 a
Tukey HSD **(0.05)**	5.239	5.239	5.239	5.239
CV (%)	15.65	10.06	15.12	16.37

HM_1= 500.01 ppm of Pb,

DRW-20 = TRS-21 = TRS-24 = TRS-31 = TRS-32 = Bacterial strain

APPENDIX VII: Growth response of the selected bacterial strains in HM_2

Treatments	No. of cells			
	Day 0	**Day 5**	**Day 10**	**Day 15**
DRW-20 x HM_2	899444 a-d	832778 a	574630 b-e	800556 ab
TRS-21 x HM_2	1012407 a-c	605556 b-d	576481 b-e	681481 a-d
TRS-24 x HM_2	0.6753 ab	573519 c-e	533333 b-e	611667 a-e
TRS-31 x HM_2	1250556 a	733333 a-c	693888 ab	671667 a-d
TRS-32 x HM_2	1282778 a	783333 ab	585185 b-d	667963 a-d
Tukey HSD $_{(0.05)}$	5.239	5.239	5.239	5.239
CV (%)	15.65	10.06	15.12	16.37

Cd = 1000.011 ppm of Cd,

DRW-20 = TRS-21 = TRS-24 = TRS-31 = TRS-32 = Bacterial strain

APPENDIX VIII: Analysis of variance on growth response of the selected bacterial strains in Pb and Cd

Source of variation	Degrees of freedom	Mean square			
		Day 0	**Day 5**	**day 10**	**day 15**
Replication	2	0.04710	0.00654	0.00329	0.00233
Bacterial Strain	4	0.09149	0.00634**	0.00721*	0.00631
Heavy Metal	2	0.26874	0.09578	0.12086	0.09000
Bacterial Strain x Heavy Metal	8	0.05885	0.01435	0.01335	0.01124
Error	28	0.00659	0.00105	0.00185	0.00285

APPENDIX IX: Analysis of variance on bioremediation of Pb and Cd bythe selected bacterial strains

Source of variation	Degrees of freedom	Mean square	
		Pb	Cd
Replication	2	13	17
Bacterial Strain	4	56	7
Heavy Metal	2	263160	714335
Bacterial Strain x Heavy Metal	8	56*	7
Error	28	24	9

*Significant at 5 % level of probability
** Significant at 1 % level of probability