EFFECTS OF HEAVY METAL TOXICITY ON THE BIOLOGICAL TRANSFORMATION OF PHOSPHORUS IN SOIL

NAZMUN NAHAR

Dhaka University Institutional Repository

EFFECTS OF HEAVY METAL TOXICITY ON THE BIOLOGICAL TRANSFORMATION OF PHOSPHORUS IN SOIL

A Dissertation for the Partial Fulfillment of the Requirements of the Doctor of Philosophy in Soil, Water and Environment

Submitted by

Nazmun Nahar
Regd. No. 092/2013-14

Dept. of Soil, Water and Environment
University of Dhaka

May 2016

Dhaka University Institutional Repository

Dedicated to my beloved Mother

Acknowledgement

At first I express my immeasurable praise to Allah for enabling me to prepare this thesis paper successfully.

I pleasure to express my deepest and sincerest of gratitude, heartiest appreciation, high indebtedness, and best regards to my honorable supervisor **Dr. Sirajul Hoque**, Professor and Chairman, Dept. of Soil, Water and Environment, University of Dhaka, for his excellent instruction, enthusiastic leadership, constructive criticism, and constant Suggestion, assistance encouragement, inspiration and scholastic guidance, above all, active supervision throughout the period of the research work in planning, preparation, for providing requisite laboratory facilities and completion of this thesis paper successfully.

I would like to express my sincere gratitude and gratefulness to Dr. Nurul Islam, Associate Professor, Dept. of Botany for arranging PCR amplification and DNA sequencing.

It is a great pleasure for the author to expresses his thanks to the Center for advance Researches in sciences (CARS), Dept. of Microbiology and Bangladesh Council of Scientific and Industrial Research (BCSIR) for providing laboratory facilities.

I am also grateful to my all respected teachers of the Dept. of Soil, Water and Environment, University of Dhaka, for their kind suggestions throughout the study.

I thank my senior graduate students and well wishers especially Md. Thouhidul Islam, Shumana Zaman, Rekha Rani, Md. Moniruzzaman, Sajedur Rahman, Azizul Hakim, Ferdouse Zaman, Tanjila Jesmin, Afsana Parvin, Pushpita Chowdhury, Tamjid- Us-Sakib, Maksuda hossain, Khandoker Munira Mehjabin (Dept. of Microbiology, University of Dhaka) for their constant inspiration and active cooperation, during this research work.

I would like to thank all the Laboratory assistants, Shafiqul Islam, Md. Mostafa Kamal Sarkar, Md. Allauddin, Md. Joynal Abdin, Md. Jahangir Alam Bhuiya and all office staffs of the Dept. of Soil, Water and Environment, University of Dhaka for their kind assistance in the research work.

A special thanks to my family. Words cannot express how grateful I am to my parents, parents in-laws and my dear husband for their continuous prayer for prosperity of my life in the day to come.

May, 2016 Nazmun Nahar

Certificate of Authentication

This is to certify that the thesis titled "Effects of heavy metal toxicity on the biological transformation of phosphorus in soil" submitted by Nazmun Nahar, Regd. No. 92/2013-2014, Department of Soil, Water Environment, University of Dhaka, represents an original research conducted under our supervision and has not been submitted elsewhere for any degree or diploma.

Prof. Dr. Sirajul Hoque

Thesis supervisor
Department of Soil, Water and Environment
University of Dhaka.

Expanded Contents

Chapter No	Title	Page No
1.	INTRODUCTION	1-7
2.	REVIEW OF LITERATURE	8-64
	2.1 Phosphorus Availability and Dynamics in Soil	9-11
	2.2 Factors Controlling the Transformation and Availability of Phosphorus in Soils	11
	2.2.1 Influence of pH and Different Cations	11-12
	2.2.2 Influence of Soil Texture	12
	2.2.3 Influence of Mineral Types	13
	2.2.4 Influence of Ageing	13
	2.2.5 Influence of Organic Matter	13-14
	2.2.6 Influence of Moisture	14-15
	2.2.7 Influence of Temperature	15-16
	2.2.8 Influence of Microorganisms	16-17
	2.2.9 Influence of Liming	17
	2.3 Phosphate Solubilizing Bacteria	17-18
	2.3.1 Mineral phosphate solubilization	18-21
	2.3.2 Organic phosphate solubilization	21-23
	2.4 Mechanisms of Phosphate Solubilization	24
	2.4.1 Production of organic acids	24-26
	2.4.2 Production of acid phosphatase	26-27
	2.5 Interaction of PSB with Other Microorganisms	28-29
	2.6 Phosphate Solubilizing Bacteria as Plant Growth Promoters	30-31
	2.7 Heavy Metals	31-32
	2.7.1 Sources of heavy metals	33-34
	2.8 Biological Essentiality of Heavy Metals	34-37
	2.9 The Effects of Heavy Metals on Soil Microorganisms	38-39

Expanded Contents

Chapter No	Title	Page No
	2.9.1 Cell Damage	40-41
	2.10 Heavy Metal Toxicity to Microbes	41-42
	2.11 Microbial Metal Tolerance and Resistance Mechanisms	42-47
	2.12 Cadmium in Relation to Soil Microbes	47-48
	2.12.1 Microbial Resistance to Cadmium	48-49
	2.12.2 Cadmium Toxicity to Microbes	49-50
	2.13 Lead in Relation to Soil Microbes	50-51
	2.13.1 Lead Toxicity to Microbes	52-53
	2.13.2 Microbial Resistance to Lead	53-54
	2.14 Metal Speciation in Soil	54-56
	2.14.1 Sequential extraction	56-57
	2.14.2 Sequential extraction procedures	57-58
	2.14.2.1 Tessier procedure	58-59
	2.14.2.2 Community Bureau of Reference (BCR) procedure	59-60
	2.14.2.3 Short extraction procedure by Maiz *Et Al.* (2000)	60
	2.14.2.4 Gal´An procedure	61
	2.14.2.5 Geological Society of Canada (GCS) procedure	61-62
	2.15 Future Prospectus of Researchers on Phosphate-Solubilizing Bacteria	63-64
3.	**MATERIALS AND METHODS**	65-87
	3.1 Sampling Sites	66
	3.2 Collection of Soil Samples	66
	3.3 Preservation of the Samples	66
	3.4 Preparation of Soil Sample for Analysis	66

Expanded Contents

Chapter No	Title	Page No
	3.5 Measurement of Physical and Chemical Parameters of the Samples	67
	3.5.1 Soil pH	67
	3.5.2 Measurement of Soil Moisture	67
	3.5.3 Mechanical Analysis	67
	3.5.4 Cation Exchange Capacity	67
	3.5.5 Soil Organic Carbon	67
	3.5.6 Total Nitrogen	68
	3.5.7 Nitric-Perchloric Acid Digestible Nutrients	68
	3.5.8 Determination of Lead and Cadmium of Soil	68
	3.6 Media And Techniques for Isolation and Purification of Bacterial Colony	68
	3.6.1 Culture Media Used	68-69
	3.6.2 Techniques Employed	69
	3.6.2.1 Dilution Plate Technique	69
	3.6.2.2 Pour Plate Technique	69
	3.6.2.3 Spread Plate Technique	69
	3.6.2.4 Streak Plate Technique	70
	3.7 Isolation of Different Organisms	70
	3.7.1 *Bacillus* sp., *Staphylococcus* sp. and *Pseudomonas* sp.	70
	3.7.2 *Proteus* sp. and *Serratia* sp.	70
	3.7.3 *Fungi* sp.	71
	3.8 Isolation of Colonies	71
	3.9 Isolation of Phosphate Solubilizing Microorganisms	71

Expanded Contents

Chapter No	Title	Page No
	3.10 Purification of Isolates	72
	3.11 Maintenance And Preservation of Isolates	72
	3.12 Morphological Observation of Isolated Strains	72
	3.12.1 Colonial Morphology	73
	3.12.2 Growth on Agar Slants	73
	3.12.3 Growth in Broth Media	73
	3.12.4 Motility Test	73
	3.13 Preparation of Bacterial Cells for Microscopic Examination	73
	3.13.1 Simple Staining	74
	3.13.2 Differential Staining	74
	3.13.2.1 Gram Staining	74
	3.14 Microscopic Observation of Bacteria	75
	3.15 Biochemical Test of Isolates	75
	3.15.1 Catalase Test	75
	3.15.2 Acid And Gas Production from D-Glucose	75
	3.15.3 Deep Glucose Agar Test	75
	3.15.4 Liquefaction of Gelatine	76
	3.15.5 Hydrolysis of Starch	76
	3.15.6 Production of Indole	76
	3.15.7 Methyle Red Test	76
	3.15.8 Voges Proskaur Test	76
	3.15.9 Utilization of Citrate	76
	3.15.10 Oxidase Test	77
	3.15.11 Identification of the Isolates	77
	3.16 Analytical Profile Index (API)	77
	3.16.1 API 20E	78

Expanded Contents

Chapter No	Title	Page No
	3.16.2 API 50 CHB	78
	3.16.3 API 20 NE	78
	3.16.4 API Staph	79
	3.16.5 API 20Strep	79
	3.17 Determination of Efficiency of Isolates in Dissolving Insoluble Phosphate in Liquid Culture	80
	3.18 Determination of Efficiency of Isolates in Mineralization of Organic Phosphate in Liquid Medium	80
	3.19 Screening of Lead and Cadmium Tolerant Bacteria	81
	3.19.1 Isolation of Lead Tolerant Bacteria	81
	3.19.2 Isolation of Cadmium Tolerant Bacteria	81
	3.20 Determination of Bacterial Response to Metal Tolerance	82
	3.21 Determination of Tolerance of Indigenous Soil Bacteria Amended with Heavy Metal	83
	3.22 Soil Preparation for Incubation	84
	3.23 Preparation of Stock Solution of Metals	84
	3.24 Inoculation of Phosphate-Solubilizing Microorganisms in Soil	84
	3.26 Application of Concentrated Metal Solution in Soil	85
	3.27 Determination of Bacterial Growth after Incubation	85
	3.28 Procedure of Sequential Extraction Scheme	86-87

Expanded Contents

Chapter No	Title	Page No
4.	**RESULTS AND DISCUSSION**	88-146
	4.1 Physical And Chemical Parameters Of Soils	88
	4.2 Isolation And Purification Of Bacteria	90
	4.3 Morphological Characteristics Of Isolates	92
	4.3.1 Study Of Colonial Morphology	92
	4.3.2 Study Of Cell Morphology	93
	4.4 Physiological And Biochemical Characteristics Of Isolates	93-94
	4.4.1 Motility Test	95
	4.4.2 Oxygen Requirement Test	95
	4.4.3 Catalase Test	95
	4.4.4 Oxidase Test	95
	4.4.5 Hydrolysis Of Strach	95
	4.4.6 Liquefaction Of Gelatin	95
	4.4.7 Voges Proskaur (VP) Test	95
	4.4.8 Methyl Red (MR) Test	95
	4.4.9 Indole Formation Test	99
	4.4.10 Utilization Of Citrate	99
	4.4.11 Acid And Gas Production Test	99
	4.5 Results Of APi Test	99
	4.5.1 *Bacillus* sp.	99
	4.5.2 *Pseudomonas* sp.	103
	4.5.3 *Staphylococcus* sp.	103
	4.5.4 *Proteus* sp.	104
	4.5.5 *Serratia* sp.	105
	4.6 Identification of Selected Isolates	105-106

Expanded Contents

Chapter No	Title	Page No
	4.7 Isolation of Phosphate-Dissolving Bacteria	106
	4.8 Efficiency of Isolates in Dissolving Insoluble Phosphate in Liquid Culture Medium	107
	4.8.1 Efficiency of Isolates in Dissolving Insoluble Phosphate in NBRIP and PVK Broth Medium	107-109
	4.9 Mineralizing of Organic Phosphorus in Liquid Culture Medium	110
	4.9.1 Mineralizing of Organic Phosphorus in Modified NBRIP*^{1} and NBRIP*^{2} Broth Media	110-112
	4.9.2 Mineralizing of Organic Phosphorus in Modified PVK*^{1} and PVK*^{2} Broth Media	112-114
	4.10 Primary Screening of Lead and Cadmium Tolerant Bacteria	114-115
	4.11 Bacterial Growth and Tolerance to Lead and Cadmium Ions in Liquid Media	115
	4.11.1 Bacterial Growth and Tolerance to Lead	115
	4.11.1.1 Bacterial Growth and Tolerance to Lead in Nutrient Broth (NB) Medium	115
	4.11.1.2 Bacterial Growth and Tolerance to Lead in NBRIP Broth Medium	116
	4.11.1.3 Bacterial Growth and Tolerance to Lead in PVK Broth Medium	117
	4.11.2 Bacterial Growth and Tolerance to Cadmium	117
	4.11.2.1 Bacterial Growth and Tolerance to Cadmium in Nutrient Broth (NB) Medium	117
	4.11.2.2 Bacterial Growth and Tolerance to Cadmium in liquid NBRIP broth medium	118
	4.11.2.3 Bacterial Growth and Tolerance to Cadmium in liquid PVK Broth Medium	119
	4.12 Determination of Minimum Inhibitory Concentration in Different Media	119-121
	4.13 Determination of Maximum Tolerable Concentrations in Different Growth Media	121-123
	4.14 Bacterial Growth and Tolerance to Heavy Metal Ions in Soil	123

Expanded Contents

Chapter No	Title	Page No
	4.14.1 Bacterial Growth and Tolerance to Lead	124
	4.14.1.1 Bacterial Growth and Tolerance To Pb^{2+} in Silt Loam Soil	124
	4.14.1.2 Bacterial Growth and Tolerance to Pb^{2+} in Silty Clay Loam Soil	125
	4.14.1.3 Bacterial Growth and Tolerance to Pb^{2+} in Sandy Loam Soil	127-130
	4.14.2 Bacterial Growth and Tolerance to Cadmium	130
	4.14.2.1 Bacterial Growth and Tolerance to Cd^{2+} in Silt Loam Soil	130
	4.14.2.2 Bacterial Growth and Tolerance to Cd^{2+} in Silty Clay Loam Soil	131-133
	4.14.2.3 Bacterial Growth and Tolerance to Cd^{2+} in Sandy Loam Soil	133-135
	4.15 Result of Sequential Extraction of Heavy Metal (Pb And Cd) in Soil	135-136
	4.15.1 Lead in soil fractions	136-141
	4.15.2 Cadmium in soil fractions	141-146
5.	**SUMMARY AND CONCLUSION**	147-151
6.	**REFERENCES**	152-176
	APPENDICES	A-U

List of Tables

Table No	Title	Page No
Table 2.1	List of Phosphorus solubilizing Bacterial in soil	18
Table 2.2	Total P accumulation in cultures of different bacterial species grown on insoluble mineral phosphate substrates (mg l^{-1})	21
Table 2.3	Phosphate mineralization from P-substrates by some soil bacterial species.	23
Table 2.4	Important PSM, their ecological niches and organic acids produced	25-26
Table 2.5	Effect of co-inoculation of legumes with phosphate solubilizers and N_2 Fixers	29
Table 2.6	Plant growth promotion by PSM	31
Table 2.7	Heavy metal composition of typical uncontaminated soils and agricultural crops	32
Table 2.8	Different sources of heavy metals in soils	34
Table 2.9	Microbial roles in key biogeochemical cycles of metals and other elements	36-37
Table 2.10	Mechanism of metal tolerance in microorganisms	43
Table 2.11	Lead in soils and soil solutions	51
Table 2.12	Operating conditions for sequential extraction procedures by Tessier.	59
Table 2.13	Sequential extraction procedures of BCR	60
Table 2.14	Short Sequential extraction procedures by Maiz et al., (1997)	60
Table 2.15	Sequential extraction procedures by Gal'an	61
Table 2.16	Sequential extraction procedures by Canada	62
Table 4.1	pH and metal contents of the soil samples used for isolation of bacterial strains	88
Table 4.2	Soil properties and heavy metal status of soil before metal amendments	89
Table 4.3	Phosphorus and metal contents of the phosphorus sources used in this experiment	89

List of Tables (cont.)

Table No	Title	Page No
Table 4.4	Growth of organisms on selective media for their identification	91
Table 4.5	Bacterial samples isolated from contaminated and agricultural soils	92
Table 4.6	Morphological characteristics of the bacterial colonies	93
Table 4.7	Biochemical characteristics of the selected 13 bacterial isolates	94
Table 4.8	API 50CHB result for identification of *Bacillus* sp	100
Table 4.9	API 20E result for identification of *Bacillus* sp	102
Table 4.10	Result of API 20NE for identification of *Pseudomonas* sp	103
Table 4.11	Result of API Staph for identification of *Staphylococcus* sp	104
Table 4.12	Result of API 20 E for identification of *Proteus* sp	104
Table 4.13	Result of API 20 E for identification of *Serratia* sp	105
Table 4.14	Name of the selected 13 isolates after identification	106
Table 4.15	Growth of phosphate dissolving microorganisms	106
Table 4.16	Bacterial growth in presence of lead in Nutrient Broth (NB) medium	116
Table 4.17	Bacterial growth in presence of lead in NBRIP liquid medium	116
Table 4.18	Bacterial growth in presence of lead in PVK liquid medium	117
Table 4.19	Bacterial growth in presence of cadmium in NB medium	118
Table 4.20	Bacterial growth in presence of cadmium in NBRIP medium	118
Table 4.21	Bacterial growth in presence of cadmium in PVK medium	119

List of Tables (cont.)

Table No	Title	Page No
Table 4.22	Bacterial growth in presence of Pb^{2+} in silt loam soil (after 24h of incubation)	124
Table 4.23	Bacterial growth in presence of Pb^{2+} in silt loam soil (after 15days of incubation)	125
Table 4.24	Bacterial growth in presence of Pb^{2+} in silt loam soil (after 30 days of incubation)	125
Table 4.25	Bacterial growth in presence of Pb^{2+} in silty clay loam soil (after 24h of incubation)	126
Table 4.26	Bacterial growth in presence of Pb^{2+} in silty clay loam soil (after 15 days of incubation)	126
Table 4.27	Bacterial growth in presence of Pb^{2+} in silty clay loam soil (after 30 days of incubation)	127
Table 4.28	Bacterial growth in presence of Pb^{2+} in sandy loam soil (after 24h of incubation)	128
Table 4.29	Bacterial growth in presence of Pb^{2+} in sandy loam soil (after 15 days of incubation)	128
Table 4.30	Bacterial growth in presence of Pb^{2+} in sandy loam soil (after 30 days of incubation)	128
Table 4.31	Bacterial growth in presence of Cd^{2+} in silt loam soil (after 24h of incubation)	130
Table 4.32	Bacterial growth in presence of Cd^{2+} in silt loam soil (after 15 days of incubation)	131
Table 4.33	Bacterial growth in presence of Cd^{2+} in silt loam soil (after 30 days of incubation)	131
Table 4. 34	Bacterial growth in presence of Cd^{2+} in silty clay loam soil (after 24h of incubation)	132
Table 4.35	Bacterial growth in presence of Cd^{2+} in silty clay loam soil (after 15 days of incubation)	132
Table 4.36	Bacterial growth in presence of Cd^{2+} in silty clay loam soil (after 30 days of incubation)	132
Table 4.37	Bacterial growth in presence of Cd^{2+} in sandy loam soil (after 24h of incubation)	133
Table 4.38	Bacterial growth in presence of Cd^{2+} in sandy loam soil (after 15 days of incubation)	133
Table 4.39	Bacterial growth in presence of Cd^{2+} in sandy loam soil (after 30 days of incubation)	134

List of Figures

Figure No	Title	Page No
Figure 2.1	**Schematic diagram of soil phosphorus mobilization and immobilization by bacteria**	24
Figure 2.2	**Some of the important microbial roles and activities in biogeochemical processes leading to altered metal distributions and speciation**	35
Figure 2.3	**How bacteria cope with toxic concentrations of heavy ions**	39
Figure 2.4	**Heavy metal toxicity mechanism to microbes**	42
Figure 2.5	**Mechanisms involved in the detoxification and transformation of metals**	45
Figure 2.6	**Selected mechanisms of cell protection against lead toxicity**	52
Figure 4.1	**Isolation of different bacteria by growing on different selective media.**	90
Figure 4.2	**(a) Motility test of isolates C4; (b) Catalase test of all isolates; (c) Oxidase test of isolates (A3 and A4) (-ve); (d) Oxygen requirement test are shown here, aerobic (C2, A4 and A6 isolates) right and the rest of the isolates were facultative anaerobes (left).**	96
Figure 4.3	**Hydrolysis of starch by *Bacillus* sp.**	97
Figure 4.4	**MR tests of isolates (A1, A5 and A7) were (+ve).**	97
Figure 4.5	**Utilization of citrate (+ve) by isolates (C1, C2, C4, C6, A1, A3, A4, A5, A6, and A7).**	98
Figure 4.6	**Acid production by two isolates C2 and C5 (-ve), and gas production by isolates (A4, A6 and A7) (+ve).**	98
Figure 4.7	**API 20 E (top) and API 50 CHB (bottom) test result of *Bacillus cereus* isolate.**	102
Figure 4.8	**Result of API 20 NE obtained for *Pseudomonas aeruginosa* (A6).**	103
Figure 4.9	**Result of API 20 E obtained for *Proteus mirabilis* (A7).**	104

List of Figures

Figure No	Title	Page No
Figure 4.10	**Result of API 20 E obtained for *Serratia marcescens* (A4).**	105
Figure 4.11	**Release of soluble phosphate in liquid NBRIP medium.**	108
Figure 4.12	**Release of soluble phosphate in liquid PVK medium**	109
Figure 4.13	**Release of soluble phosphate in liquid NBRIP*[1] medium.**	110
Figure 4.14	**Release of soluble phosphate in liquid NBRIP*[2] medium.**	111
Figure 4.15	**Release of soluble phosphate in liquid PVK*[1] medium.**	113
Figure 4.16	**Release of soluble phosphate in liquid PVK*[2] medium.**	113
Figure 4.17	**Comparison of MICs against Pb in three growth media (NB, NBRIP, and PVK).**	120
Figure 4.18	**Comparison of MICs against Cd in three growth media (NB, NBRIP, and PVK).**	120
Figure 4.19	**Comparison of MTCs against Pb in three growth media (NB, NBRIP, and PVK).**	122
Figure 4.20	**Comparison of MTCs against Cd in three growth media (NB, NBRIP, and PVK).**	122
Figure 4.21	**Growth of A4 isolates after 24 hours (a); 15 days (b); and 30 days (c) of incubation of Pb in sandy loam soil.**	129
Figure 4.22	**Growth of A4 isolates after 24 hours (a); 15 days (b); and 30 days (c) of incubation in sandy loam soil.**	134
Figure 4.23	**Distribution of Pb in various fractions in silt loam soil after 24 hours incubation.**	136
Figure 4.24	**Distribution of Pb in various fractions in silt loam soil after 15 days incubation.**	137
Figure 4.25	**Distribution of Pb in various fractions in silt loam soil after 30 days incubation.**	137
Figure 4.26	**Distribution of Pb in various fractions in silty clay loam soil after 24 hours incubation.**	138
Figure 4.27	**Distribution of Pb in various fractions in silty clay loam soil after 15 days incubation.**	138

List of Figures

Figure No	Title	Page No
Figure 4.28	**Distribution of Pb in various fractions in silty clay loam soil after 30 days incubation.**	138
Figure 4.29	**Distribution of Pb in various fractions in sandy loam soil after 24 hours incubation.**	140
Figure 4.30	**Distribution of Pb in various fractions in sandy loam soil after 15 days incubation.**	140
Figure 4.31	**Distribution of Pb in various fractions in sandy loam soil after 30 days incubation.**	140
Figure 4.32	**Distribution of Cd in various fractions in silt loam soil after 24 hours incubation.**	142
Figure 4.33	**Distribution of Cd in various fractions in silt loam soil after 15 days incubation.**	142
Figure 4.34	**Distribution of Cd in various fractions in silt loam soil after 30 days incubation.**	142
Figure 4.35	**Distribution of Cd in various fractions in silty clay loam soil after 24 hours incubation.**	143
Figure 4.36	**Distribution of Cd in various fractions in silty clay loam soil after 15 days incubation.**	143
Figure 4.37	**Distribution of Cd in various fractions in silty clay loam soil after 30 days incubation.**	143
Figure 4.38	**Distribution of Cd in various fractions in sandy loam soil after 24 hours incubation.**	144
Figure 4.39	**Distribution of Cd in various fractions in sandy loam soil after 15 days incubation.**	144
Figure 4.40	**Distribution of Cd in various fractions in sandy loam soil after 30 days incubation.**	144

List of Appendices

Appendix No.	Title	Page No.
Appendix A	**Composition of the Growth Media Used in this Study**	
A1	**Acid and gas production from glucose**	A
A2	**Methyl Red/Voges-proskauer broth medium**	A
A3	**Deep glucose agar medium**	B
A4	**Motility medium**	B
A5	**Nutrient agar medium**	B
A6	**Tryptic Soya Agar media**	C
A7	**Nutrient broth medium**	C
A8	**Nutrient gelatin agar medium**	C
A9	**Phenylalanine agar medium**	D
A10	**Phenol red broth**	D
A11	**Physiological saline**	D
A12	**Simmon's citrate agar**	E
A13	**Starch agar medium**	E
A14	**Tryptone broth medium**	E
A15	**NaCl Glycine Kim and Goepfert Agar (NGKG)**	F
A16	**Potato Dextrose agar(PDA)**	F
A17	**Sorbitol MacConkey's Agar (MAC)**	F
A18	**Mannitol salt agar**	G
A19	**Cetrimide agar**	G
A20	**Eosin Methylene Blue (EMB) agar**	G
A21	**Pikovskayas agar (PVK)**	H
A22	**National Botanical Research Institute's phosphate growth medium (NBRIP)**	H

List of Appendices

Appendix No.	Title	Page No.
Appendix B	Composition of the reagents used in this study	
B1	Ammonium oxalate crystal violet solution	I
B2	Alkaline Methylene blue solution	I
B3	Iodine solution	I
B4	Kovac's reagent	J
B5	Methyl red solution	J
B6	α- Napthol solution	J
B7	Oxidase test reagent	J
B8	Safranin solution	J
B9	Baritt's Reagent/ (VP1 & VP2)	K
B10	Catalase Reagent	K
B11	NIT1 and NIT2 Reagents (Reagents for Nitrate Reduction Test)	K
B12	Zinc Reagent	K
B13	Tryptophan Deaminase Reagent (TDA Reagent)	L
B14	Zym A and Zym B Reagents	L
B15	Ninhydrin Solution	L
Table No.	Appendix C	
C 1	Release of soluble phosphate and changes of pH values in NBRIP medium	M
C 2	Release of soluble phosphate and changes of pH values in PVK medium	M
C 3	Release of soluble phosphate and changes of pH values in NBRIP*1 modified medium	M
C 4	Mineralization of organic phosphate and changes of pH in NBRIP*2 modified medium	N
C 5	Release of soluble phosphate and changes of pH values in PVK*1 modified medium	N
C 6	Mineralization of organic phosphate and changes of pH values in modified PVK*2 medium	N

List of Appendices

Table No.	Title	Page No
C 7	Minimum inhibitory concentrations (MICs) against Lead and Cadmium in three broth media	O
C 8	Maximum tolerable concentrations (MTCs) concentrations against Lead and Cadmium in three media	O
C 9	Concentration of Pb^{2+} in individual fractions of silt loam soils (after 24 hours of incubation)	O
C 10	Concentration of Pb^{2+} in individual fractions of silt loam soils (after 15 days of incubation)	P
C 11	Concentration of Pb^{2+} in individual fractions of silt loam soils (after 30 days of incubation)	P
C 12	Concentration of Pb^{2+} in individual fractions of silty clay loam soils (after 24 hours of incubation)	P
C 13	Concentration of Pb^{2+} in individual fractions of silty clay loam soils (after 15 days of incubation)	Q
C 14	Concentration of Pb^{2+} in individual fractions of silty clay loam soils (after 30 days of incubation)	Q
C 15	Concentration of Pb^{2+} in individual fractions of sandy loam soils (after 24 hours of incubation)	Q
C 16	Concentration of Pb^{2+} in individual fractions of sandy loam soils (after 15 days of incubation)	R
C 17	Concentration of Pb^{2+} in individual fractions of sandy loam soils (after 30 days of incubation)	R
C 18	Concentration of Cd^{2+} in individual fractions of silt loam soils (after 24 hours of incubation)	R
C 19	Concentration of Cd^{2+} in individual fractions of silt loam soils (after 15days of incubation)	S
C 20	Concentration of Cd^{2+} in individual fractions of silt loam soils (after 30days of incubation)	S
C 21	Concentration of Cd^{2+} in individual fractions of silty clay loam soils (after 24hours of incubation)	S
C 22	Concentration of Cd^{2+} in individual fractions of silty clay loam soils (after 15days of incubation)	T

List of Appendices

Table No.	Title	Page No
C 23	Concentration of Cd^{2+} in individual fractions of silty clay loam soils (after 30days of incubation)	T
C 24	Concentration of Cd^{2+} in individual fractions of sandy loam soils (after 24hours of incubation)	T
C 25	Concentration of Cd^{2+} in individual fractions of sandy loam soils (after 15days of incubation)	U
C 26	Concentration of Cd^{2+} in individual fractions of sandy loam soils (after 30days of incubation)	U

Abstract

Experiments were carried out for the isolation, identification and characterization of heavy metal resistant phosphate dissolving bacteria, their tolerance to lead and cadmium using different liquid (NB, PVK and NBRIP) medium and three different soils (silt loam, silty clay loam and sandy loam soil). To investigate the solid-phase speciation of lead and cadmium the three soil samples were also used.

In this study, two metals, lead and cadmium were selected based on the fact that these metals are discharged from many of the industries such as tannery, textiles, dying factories and others. A total of 13 bacteria were screened from metal polluted soil from Saver EPZ zone area and uncontaminated agricultural fields from Dhamrai and Pabna. For initial isolation NA, NGKG, MAC, Mannitol salt, Cetrimied and EMB agar media were used for different group of organisms. And for final screening of the phosphate dissolving bacteria were made by using PVK and NBRIP agar medium. From primary screened 13 bacteria, 9 were found as phosphate dissolving bacteria and were used to determine their resistance to heavy metals by amended with different concentrations Pb^{2+} and Cd^{2+}.

On the basis of morphological, physiological, biochemical characteristics and API test, 13 bacteria were authentically identified as different species of *Bacillus, Micrococcus, Staphylococcus, Serratia, Pseudomonas* and *Proteus*. NB, PVK and NBRIP liquid media and three soil samples were incubated with bacterial isolates containing different metal concentrations.

Phosphate-dissolving bacterial species showed a wide range of tolerance to metals (Pb and Cd). These bacterial species showed tolerance to Pb and Cd concentrations ranging from 15 to 150 µg/ml in nutrient broth medium. In PVK broth medium, bacterial species showed tolerance to Pb and Cd concentrations ranging from 30 to 250 µg/ml and 20 to 200 µg/ml, respectively. In NBRIP broth medium, bacterial species showed tolerance to Pb and Cd concentrations ranging from 50 to 300µg/ml and 20 to 200 µg/ml, respectively.

Soils were mixed with compost and oil cake as a source of organic phosphate and rock phosphate as a source of inorganic phosphate. Bacterial tolerance to Pb and Cd at three concentrations

(15,000 µg/ml, 20,000 µg/ml, and 30,000 µg/ml) in different soils show different results after different incubation times (24hs, 15days and 30days).

Sequential extraction was used to find out the fractions of Pb and Cd from these three soils to assess metal mobility. The sequences of extractions were six operationally defined groups: water soluble (F1), exchangeable (F2), carbonate (F3), oxide (F4), organic (F5), and residual (F6). The distribution of various fractions of Cd in the three soils at different time of incubation indicated that on an average, 58% and 54% of total Cd was associated with the mobile (F1-F2) fractions in silt loam and silty clay loam soils and 23%of total Cd was associated with the mobile (F1-F3) fractions in sandy loam soil. The immobile (F4-F6) fractions were 42 %, 46 % and 77 % in silt loam soil, silty clay loam soil and sandy loam soil respectively. 26 %, 55 % and 23 % of total Pb was associated with the mobile (F1- F3) fractions in silt loam, silty clay loam and sandy loam soil. The immobile (F4-F6) fractions were 74 %, 45 % and 77 % in silt loam, silty clay loam and sandy loam soil respectively.

The behavior and bioavailability of metals in soil are affected by many chemical processes. Soil properties have profound influence of the mitigation of metal toxicity. So that organisms can survive at high concentration of Pb and Cd contaminated soil and they can participate the transformation of phosphorus in soil.

1. INTRODUCTION

Soil is one of the more complex and highly variable habitats on earth. Any organisms that make their home in soil have had to devise multiple mechanisms to cope with variability in moisture, temperature and chemical changes so as to survive, function and replicate. Within a distance of < 1 mm, conditions can vary from acid to base, from wet to dry, from aerobic to anaerobic, from reduced to oxidized, and from nutrient-rich to nutrient-poor. Along with spatial variability there is variability over time, so organisms living in soil must be able to adapt rapidly to different and changing conditions. Variations in the chemical and physical properties of the soil are thus important determinants of the presence and persistence of soil biota (Norman *et al.*, 2006).

Every small particle of soil contains numerous types of living organisms belonging both to the plant and animal kingdoms, yet so small that they cannot be recognized with the naked eye (Alexander, 1984). These organisms comprise numerous types of bacteria, fungi, algae, protozoa, nematodes and other invertebrates which vary considerably in their structure, size, mode of living and relationship to soil processes. Bacteria are very important because they are the producing, consuming and transporting members of the soil ecosystem and therefore are involved in the flow of energy and in the cycling of chemical elements. Soil would not be fertile and organic matter would accumulate within a short time without any bacteria (Bache, 1979).

Phosphorus (P) is second only to nitrogen as most essential macro-nutrient required by plants (Srinivasan *et al.*, 2012). It is a key nutrient for higher and sustained agriculture productivity (Scervino *et al.*, 2011). It plays an important role in virtually all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Khan *et al.,* 2010) and nitrogen fixation in legumes (Saber *et al.*, 2005). It is an integral part of the cellular activities of living organisms (Tandon, 1987;

Armstrong, 1988; Theodorou and Plaxton, 1993). Although P is abundant in soils in both inorganic and organic forms, it is a major limiting factor for plant growth as it is in an unavailable form for root uptake. Inorganic P occurs in soil, mostly in insoluble mineral complexes, some of them appearing after frequent application of chemical fertilizers. These insoluble precipitated forms cannot be absorbed by plants (Rengel and Marschner, 2005).

Insoluble phosphate compounds can be solubilized by organic acids and phosphatase enzymes produced by plants and microorganisms (Kucey, 1983; Duponnois *et al*., 2005). For example, phosphate solubilizing bacteria (PSB) have been shown to enhance the solubilization of insoluble P compounds through the release of low molecular weight organic acids (Sahu and Jana, 2000).

It is well known that many microorganisms isolated from the soil are able to dissolve different kinds of rock phosphates in a liquid culture (Rodriguez and Fraga, 1999). It is generally accepted that the major mechanism of the mineral phosphate solubilization is the action of organic acids synthesized by bacteria. Organic acids that solubilize phosphates are mainly: citric, lactic, gluconic, 2-ketogluconic, oxalic, tartaric, acetic, etc. (Kucey, 1983; Vazques *et al.,* 2000). These organic acids are sources of biotical generated H^+ ion able to dissolve the mineral phosphate and to make it available for the plant (Bhattacharyya and Jain, 2000). Many results indicate that the phosphate solubilization is a consequence of the decrease of pH due to the production of organic acids. However, no correlation could be established between the acidic pH and the quantity of P_2O_5 liberated (Kucey, 1983; Kim *et al.*, 2002).

Phosphorus (P) is applied to the soil in the form of phosphatic manure. However, a large portion of the applied phosphorus is rapidly immobilized, being unavailable to plants (Goldstein, 1986). On an average, the content of phosphorus of soil is about 0.05% (w/w); however, only 0.1% of them are usable for plants (Scheffer and Schachtschabel, 1992). Saline-alkali soil-based agriculture develops quickly in recent years. Similar to the fertile soil-based agriculture, the intensive

culturing of salt-tolerant and even salt-resistant plants has dramatically decreased the availability of phosphorus in saline-alkali soil. The free phosphatic ion in soil plays a crucial role; the orthophosphatic ion is the only ion which can be assimilated in an appreciable amount by plants (Beever and Burns, 1980). Soil microorganisms involve in a wide range of biological processes including the transformation of soil phosphorus. They solubilize soil phosphorus for the growth of plants (Rodriguez and Fraga, 1999). The growth of phosphate-solubilizing bacteria (PSB) often causes soil acidification, playing a key role in phosphorus solubilization (Abd-Alla, 1994).

Phosphate-solubilizing bacteria (PSB) are therefore, considered the important solubilizers of insoluble inorganic phosphate. In turn, plants reimburse PSB with carbohydrates (Goldstein, 1995). Since the beginning of last century, many phosphate-solubilizing bacteria (PSB) have been isolated including, for example, those in *Bacillus*, *Pseudomonas*, *Erwinia*, *Agrobacterium, Serratia*, *Flavobacterium*, *Enterobacter*, *Micrococcus*, *Azotobacter*, *Bradyrhizobium*, *Salmonella*, *Alcaligenes*, *Chromobacterium*, *Arthrobacter*, *Streptomyces*, *Thiobacillus,* and *Escherichia* (Zhao and Lin, 2001). The microorganisms functioning similarly also include some fungi in genus *Penicillium*, *Aspergillus*, *Rhizopus*, *Fusarium,* and *Sclerotium* (Zhao and Lin, 2001). Unfortunately, most phosphate-solubilizing bacteria (PSB) isolated previously performed relatively low salinity tolerance, being less appropriate for saline-alkali soil-based agriculture. It is urgently needed to isolate highly halophilic phosphate-solubilizing bacteria (PSB) for the development of saline-alkali soil based agriculture.

A major environmental concern due to dispersal of industrial and urban wastes generated by human activities is the contamination of soil. Metals are directly or indirectly involve in all aspects of growth, metabolism and differentiation of the biota (Beveridge and Doyle, 1989). Some of the heavy metals are essential and are required by the organisms as micro nutrients (cobalt, chromium, nickel, iron manganese and zinc etc.) and are known as ‘trace elements’ (Bruins *et al.,* 2000).

Whereas some have no biological role and are detrimental to the organisms even at very low concentration (cadmium, lead etc.). However, at high levels both of the essential and non-essential metals become toxic to the organisms and their associated activities, may directly influence the soil fertility (Kummerer, 2004).

In soil, phosphorus is sequestered by adsorption to the surface of soil particles and through precipitation reactions with soil cations, particularly iron, aluminum, and calcium (Harris *et al*., 2006). For this reason, a large amount of soluble P fertilizer is commonly applied to agricultural soils in order to increase plant growth, which is likely to adversely affect both the environment and economy. In many countries, there has been a steady increase in the use of P fertilizer (Syers *et al*., 2008), which is considered a major source of heavy metal contamination in agricultural soils (McLaughlin *et al*., 1996; Bolan *et al*., 2003).

Bacteria are among the most abundant organism that occurs everywhere on earth. Heavy metals are increasingly found in microbial habitats due to several natural and anthropogenic processes; therefore, microbes have evolved mechanisms to tolerate the presence of heavy by efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration (Gadd, 1990). The microorganisms respond to these heavy metals by several processes; including transport across the cell membrane, biosorption to the cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions (Huang *et al*., 1990; Avery and Tobin, 1993; Brady *et al.,* 1994; Veglio *et al*., 1997).

Heavy metal contamination in the environment has become a serious problem due to the increase in the addition of these metals to the environment, which cannot be degraded like organic pollutants and persist in the ecosystem having accumulated in different parts of the food chain (Igwe *et al.,* 2005). These heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity (Roane *et al.,* 2000). Toxicity of different metals may affect all forms of life including microorganisms, plants and animals, but the degree of toxicity varies

for different organisms. The immediate toxicity of metals to soil organisms is moderated by metal immobilization by soil colloidal components (Kelly *et al.*, 2003). Heavy metals may decrease metabolic activity and diversity as well as affect the qualitative and quantitative structure of microbial communities (Giller *et al.,* 1998).

The ability of microorganisms to survive toxic effects of heavy metal exposure is due to some intrinsic property and detoxification mechanisms and other resistant mechanisms (Winge *et al.*, 1989). There are, however, many reports in the literature of microbial resistance to heavy metals. Thus, metal polluted environments contain microorganisms capable of tolerating existing metals. Sampling environmental components that contains elevated concentrations of heavy metals was a potential source for toxic metal-tolerant bacteria. The adaptation to heavy metal rich environments is resulting in microorganisms which show activities for biosorption, bioprecipitation, extracellular sequestration, transport mechanisms, and/or chelation (Haferburg and Kothe, 2007).

Long-term heavy metals contamination of soils has harmful effects on soil microbial activity, especially microbial respiration (Doelman and Haanstra, 1979). Aside from long-term metal-mediated changes in soil enzyme activity activities, many reports have shown large reductions in microbial activity due to short-term exposure to toxic metals (Hemida *et al.*, 1997). Some observations revealed that heavy metal tolerance by a particular group of bacteria or an indivisual isolate in artificial media supplemented with heavy metal showed high tolerance level (Ahmad *et al.,* 2001; Hayat *et al.,* 2002) where conditions are totally different with natural condition of soil.

Microbial resistance is a fundamental importance which is particularly relevant to microbial ecology, especially in connection with the roles of microbes in polluted ecosystems and in the reclamation of metal-contaminated natural habitats. It is also important to understand the mechanisms of microbial tolerance because of

the extensive use of some metals and metal compounds as fungicides and disinfectants. The soil microbial community should be a sensitive indicator of metal contamination effects on bioavailability and biogeochemical processes. In recent years, several reports have documented the harmful effects on soil microorganisms and microbial activity of the long-term heavy metal contamination of agricultural soils (Sandaa *et al*., 2001).

Most bacterial isolates were resistant to very high concentrations of heavy metals regardless of the level of metal concentrations in their environment. It is proposed that the resistance ability of the isolates could be exploited in considering the isolates as possible candidates for the decontamination of metal-polluted sites. The most predominant isolates at high concentrations of the metal ions include *Bacillus* spp., *Pseudomonas* spp., *Corynebacterium* spp., *Micrococcus* spp., *and Flavobacterium* spp. *Proteus*, *Citrobacter*, *Alcaligenes, Enterobacter* (Anyanwu and Nwachukwu, 2011).

A study on soil bacterial tolerance to heavy metals can help to determine bacterial capacity to survive and assess the risk of deterioration of soil potentiality. It may also a purpose of the study to examine the nature of the interactions between microbes and heavy metals underlying resistance or tolerance. In this text two metals Lead and Cadmium have been chosen due to their presence in the environment from the industrial waste disposal at a large scale.

Phosphorus is almost always present in comparatively small amount in mineral soils. Moreover, a large proportion of this element at any one time is held in combinations unavailable to plants. The more simple compounds of phosphorus are relatively insoluble in most soils. As a result, this element is double critical – low total amounts and very low availability to plants. To make available of this element it is essential to bring about some microbial transformation of both organic and inorganic compounds in soil. An attempt has, therefore, been made to study the isolation and identification of phosphate dissolving micro-organisms as

well as the qualitative and quantitative distribution of different groups of these organisms in soils.

Considering all these previous details, the following objectives of the experimental studies were conducted under this present research work:

- To isolate phosphate-solubilizing organisms from metal contaminated and uncontaminated agricultural soils.
- To observe the release of available phosphate from organic and inorganic sources.
- To observe the toxic metal sensitivity of the isolated strains of microorganisms in soil.
- To determine the minimum inhibitory concentration (MIC) and maximum tolerable concentration (MTC) of identified soil bacteria.
- Soils will be treated with different concentration of Pb and Cd and transformation of these metals in the soils will be assessed through the fractionation process.

2. REVIEW OF LITERATURE

Phosphorus is one of the most limiting factors in crop production in many kinds of soils in different geographical regions as a result of high phosphorus fixation. The soil is the only source from where the plants can absorb phosphates (Islam and Ahmed, 1971). The insoluble inorganic phosphate which is not directly available to plants, many soil micro-organisms can solubilize insoluble inorganic phosphates which are largely available to plants (Hayman, 1975). Phosphate is found in plants as constituents of nucleic acids, nucleoproteins, phytin, phospholipids, phosphorylated sugars, coenzymes and related compounds (Alexander, 1961). The solubilization of inorganic P by microorganisms has been attributed to processes involving acidification, chelation and exchange reactions in the growth environment (Molla *et al.,* 1983). Several processes can work in conjunction with each other, e.g., the chelating and acidifying effects of organic acids can produce a large impact on the surrounding environment (Gaur *et al.,* 1973; Surange, 1985).

The pollution of the ecosystem by heavy metals is a real threat to the environment because metals cannot be naturally degraded like organic pollutants and persist in the ecosystem having accumulated in different parts of the food chain (Igwe *et al*., 2005). Metals are non-biodegradable, but can be transformed through sorption, methylation and complexation, and changes in valence state. These transformations affect the mobility and bioavailability of metals. Although most organisms have detoxification abilities (i.e. mineralization, transformation and/or immobilization of pollutants), particularly bacteria, play a crucial role in biogeochemical cycles and in sustainable development of the biosphere (Diaz, 2004).

The quality of life on earth is linked undeniably to the overall quality of the environment (Anonymous, 1995). The growth and activities of microorganisms in soil depends on the total content and concentrations of particular forms of heavy

metals, but it is also conditioned by several other factors, such as the granulometric composition of soil, quantity and quality of organic matter, especially carbohydrate rich organic matter, pH, total exchange capacity, nutrient availability, moisture, temperature, and oxygen availability (Das *et al*., 1997).

Metal toxicity may affect all forms of life including microorganisms, plants and animals, but the degree of toxicity varies for different organisms. The immediate toxicity of metals to soil organisms is moderated by metal immobilization by soil colloidal components however, heavy metals may be mobilized by local and global changes in soil conditions i.e., changes in physical and chemical conditions of soil environment, including decrease in pH, redox potential and enhanced decomposition of organic matter (Kelly *et al*., 2003). The impact and long-term ecological ramifications of pollution on the biosphere have resulted an increased interest to evaluate the interactions between pollutants, the environment, and the biota.

Phosphorus and heavy metals interact in soil resulting in the formation of insoluble metal phosphates which adversely affect the absorption, translocation and assimilation of phosphorus by plants. The application of phosphorus markedly counteracted the depressing effect of heavy metals on crop growth (Koshino, 1973; Gupta *et al.,* 1994).

2.1 Phosphorus Availability and Dynamics in Soil

Soil is a dynamic system and is an ecological niche of constant biological activity, influenced to a great extent by the plant and the chemical nature of its parent material and the plant growth it supports. The ability to predict long-term plant-availability of soil phosphorus provides an additional management tool for sustainable agriculture and soil management. Phosphorus availability in soils can be one of the major factors limiting growth for both natural ecosystems and agricultural systems. It is present at levels of 400–1200 mg/kg of soil. However,

95-99 % soil phosphorus is present in insoluble phosphate form and hence cannot be utilized by the plants (Sridevi *et al*., 2013). Even though some soils may have high levels of total P, they can still be P-deficient due to low levels of soluble phosphate available to plants (Gyaneshwar *et al*., 2002).

Therefore in both natural and agricultural ecosystems, the quantity of P available for plant uptake is generally low due to the low solubility of P compounds present in soils. Because much of the total soil phosphorus is unavailable for immediate plant consumption, many investigations have been conducted to quantify the relationship between unavailable and available soil phosphorus. Soil phosphorus is mainly in two forms such as inorganic and organic phosphorus (Richardson and Simpson, 2011). The proportion of different phosphorus fractions differs significantly because of soil type, soil use and management strategies (Li *et al*., 2007). These phosphorus forms differ in their behavior and fate in soils. Organic P in soil is mainly exists in stabilizing forms as inositol phosphates (soil phytate), phosphonates, and active forms as orthophosphate diesters, labile orthophosphate monoesters, and organic polyphosphates (Condron *et al*., 2005).

One unique characteristic of phosphorus is its low availability due to slow diffusion and high fixation in soils. The phenomenon of fixation and precipitation is generally highly dependent on pH and soil type (Mahatesh and Patil, 2011). Thus, in acid soils, P can be dominantly adsorbed by Fe/Al oxides by forming various complexes (Mohammadi, 2012). While in alkaline soils it is fixed by calcium (Oliveira *et al*., 2009). Therefore, efficiency of P solubilization rarely exceeding 10–20 % (Kuhad *et al*., 2011).

Hence, a holistic understanding of P dynamics from soil to plant is necessary for optimizing P management and improving P-use efficiency, aiming at reducing consumption of chemical P fertilizer, maximizing exploitation of the biological potential of root/rhizosphere processes for efficient mobilization, and acquisition of soil P by plants .Therefore, the availability of soil P is extremely complex and

needs to be systemically evaluated because it is highly associated with P dynamics and transformation among various P pools.

2.2 Factors Controlling the Transformation and Availability of Phosphorus in Soils

The availability of inorganic phosphorus is largely determined by (a) soil pH, (b) soluble iron, aluminum and manganese; (c) presence of iron, aluminum and manganese containing minerals; (d) available calcium and calcium minerals; (e) amount and decomposition of organic matter; and (f) activities of microorganisms. The first four factors are interrelated since soil pH drastically influences the reaction of phosphorus with the different ions and minerals (Brady, 1990).

2.2.1 Influence of pH and different cations

Soil pH has a profound influence on quantity of P adsorption and precipitation in soils (Tisdale *et al.,* 1985). In the absence of added organic acid, the solubility of residual orthophosphate in the soil decreased as the pH values was increased from 4 to 7 (Samuel *et al.,* 1986).

The inorganic solid phase of phosphorus may be divided into two categories: a) Sparingly soluble salts or minerals and b) Phosphates adsorbed to the surface of soil particles.

The concentration of Al^{3+} and Fe^{3+} may simply be considered as controlled by $Al(OH)_3$ and $Fe(OH)_3$. In most soils the concentration of phosphate ions in solution has a maximum in the pH range 6-7. Increasing the pH still more means precipitation of apatite, $Ca_{10}(OH)_2(PO_4)_6$ and $Ca_{10}F_2(PO_4)_6$, both having low solubilities and consequently giving low concentration of phosphate ions in the soil solution.

At intermediate pH levels, two of the phosphate ions may be present simultaneously. Thus, in solutions at pH 7.0, both $H_2PO_4^-$ and HPO_4^{2-} ions are

found. The $H_2PO_4^-$ ion is somewhat more available to plants than is the HPO_4^{2-} ion. The basic iron and aluminum phosphates have a minimum solubility around pH 3 - 4. At higher pH values some of the phosphorus is released and the fixing capacity is somewhat reduced. These facts seen to indicate that maximum phosphate availability to plants is obtained when the soil pH is maintained in the 6 - 7 ranges.

2.2.2 Influence of Soil Texture

Most of the compounds with which phosphorus reacts are in the finer soil fractions. As a consequence phosphorus fixation tends to be more pronounced in clay soils than in the coarser textured ones. Soil texture has a greater effect on P transformation (Huffman *et al.,* 1996).

Texture affects the P-supplying power in three ways:

a) The porous system diffusion coefficient of P in solution increases as the clay content increases.
b) The capacity of the solid phase P to renew the concentration in solution increases as the soil contains more clay.
c) The same rate of fertilizer P causes a smaller increment in P concentration as the clay content increases. That means more phosphorus is needed on soils with high clay content than of soils with low in order to get the same response when all other factors equal.

The most important factors governing P sorption are the surface area of the clay fraction and the type of constituents present. However, soils of similar clay contents often exhibit a marked difference in phosphorus sorbing ability, and using selective extractants, this can generally be traced to the amounts of different forms of Fe and Al in the clay fractions. Coefficients of multiple regression equations relating P sorption to the different forms of Fe and Al give estimates of their P sorbing ability (Wada *et al.,* 1989).

2.2.3 Influence of mineral types

Soil derived from minerals high in Al and Fe and low in alkaline metals as Ca and Mg by nature or as a consequence of weathering are normally poor in phosphates and fix phosphate strongly. The fixing power decreases as more Ca and Mg enter into the mineral structure. In soils containing considerable amounts of CaCO3 the phosphates will mostly be found as apatites of low solubility. Soil mineralogy is the key factor affecting P fixation (Juo and Fox, 1977).

2.2.4 Influence of ageing

With time, changes take place in the reaction products of soluble phosphates and soils. These changes generally result in a reduction in surface area of the phosphates and a similar reduction in their availability. Debnath and Mandal (1982) found that at the initial stage the distribution of added P in different products in acid and alkaline soils was in the order Al-P >Fe-P>Ca-P>RS-P and Ca-P> Al-P> Fe-P> RS-P, respectively. On ageing Fe-P increased consistently, whereas Al-P and Ca-P increased up to 15-30 days and decreased thereafter. The lability of the formed phosphates was more than that of the respective species of native phosphates. The specific activity of Al-P increased up to 15-30 days and then declined, whereas that of Fe-P showed two peaks and that of Ca-P declined consistently on ageing.

2.2.5 Influence of organic matter

Organic matter affects the phosphorus availability either directly by displacement of phosphate from iron and/or aluminum phosphate, or by influencing (a) the decrease in redox potential and (b) pH changes. Microorganisms, which secrete organic acids such as lactic, glycolic etc, have a great role in the solubilization of inorganic phosphates (Hayman, 1975). Normally organic matter increases the solubility of phosphate in soils. Two mechanisms may be responsible for that:

- Organic anions compete with phosphate ions for the binding sites on the soil particles.
- Complex organic anions chelate Al^{3+}, Fe^{3+} and Ca^{2+} and thus decrease the phosphate precipitating power of these cations.
- The organic matter in itself contains phosphorus. Roughly 50% of the total phosphorus in a soil with variations from 20 to 80% is bound up in organic compounds.

The organic phosphorus is continuously released as orthophosphate ions when microorganisms break down the organic matter. Inorganic phosphorus ions releases from organic matter equilibrate quickly with the inorganic phosphate system and it is most difficult to recognize the special effect of released organic phosphorus on the plant growth. The soils initially rich in organic matter or organic P and inositol P contents showed high mineralization rates under submerged conditions (Sood and Minhas, 1989). Humic substances associated P and available P contents increased with the addition of organic material and of insoluble phosphate compounds (Khalil and El-Shinnawi, 1989). Natural and incorporated organic matter in the soil may solubilize phosphates through formation of organic acids which complex with the phosphorus and then through the metabolism of the organic radicals, the phosphates are liberated (Malavolta, 1967). Aplication of organic matter to upland (aerobic) soils generally decreases P sorption (Meek *et al.,* 1979; Reddy *et al.,* 1980; Kuo, 1983; Frossard *et al.,* 1986).

2.2.6 Influence of moisture

Water regimes strongly influence soil P chemistry both directly through changes in water content and indirectly through changes in soil physicochemical conditions (Kirk *et al.,* 1989). The effect of wetting and drying on soil properties in general, and plant nutrient status in particular, has been the subject of a number of studies (Haynes and Swift, 1985).

Fields may remain fallow at a time for two to three months and during this period undergo alternate wetting and drying cycles consisting of high temperature and intermittent rainfall. There are possibilities that fluctuation in soil water affect the availability of phosphorus to the crops (Chhillar *et al.,* 1991).

As only dissolved phosphate ions are immediately available, certain moisture content is always necessary for plant uptake. Moisture is also necessary for the important diffusion process. It is also shown that drying sometimes decreases the solubility of phosphates more or less irreversibly. The mechanism behind this phenomenon might be the change of the redox potential and, for example, oxidizing Fe^{2+} to Fe^{3+}, the latter ion having a stronger precipitating power on phosphate ions than the former. Adverse condition due to low moisture content may to certain degree be compensated by high input of easily soluble phosphates. The high concentration of cations that develop in flooded soil solutions favors formation of soluble metal orthophosphate complexes, especially with Fe^{3+}, Ca^{2+} and Mg^{2+}. Metal P complexes that are positively charged may be more strongly adsorbed by charged surfaces than orthophosphate, which complicates the effect of pH changes on P retention by flooded soils. Concentrations of organically bound or organically complexed P may also be high in flooded soil solutions because of the high concentration of dissolved organic-P complexes are typically less significant (Kirk *et al.,* 1989).

2.2.7 Influence of temperature

Temperature is one of the most important ecological factors and influences biological as well as chemical reactions of phosphorus. The rate of most chemical and biological reactions increases with increasing temperature. Mineralization of P from soil organic matter or crop residues is dependent on soil biological activity, and increases in temperature stimulate biological activity up to the optima for the predominant biological systems. The dissolution of fertilizer P granules and resultant reactions with soil components to produce less soluble reaction products are hastened by higher temperature. For example, the solution P

concentration decreases with increasing soil temperature following the addition of several fertilizer P sources. Results of most studies show that P adsorption generally increases with higher temperatures. Phosphorus adsorption in soils of warm regions in the world is generally greater than in soils of temperate regions. Lower P concentration in the soil solution was considered to be the reason for lower P uptake under lower temperature conditions (Sumio, 2002).

2.2.8 Influence of microorganisms

Stewart (1981) has depicted microbial activity as a "wheel" that rotates in the soil, simultaneously consuming and releasing P to the soil solution. Microorganisms can affect the P supply to higher plants in three different ways:

(1) by decomposition of organic P compounds, with release of available inorganic phosphate,

(2) by immobilizing available phosphates into cellular material, and

(3) by promoting the solubilization of fixed or insoluble mineral forms of P, such as through production of chelating agents.

Biswapati *et al.* (1992) studied the effect of growth and subsequent decomposition of cyanobacteria on changes in P fractions under submerged condition. The growth of cyanobacteria in soils caused an increase in organic P with concomitant decreases in Olsen-P, Al-P, Fe-P, and Ca-P, but little change in reductant-soluble Fe-P and occluded Al-P. The decomposition of cyanobacterial biomass in soils caused an increase in Olsen-P with a simultaneous decrease in other P fixations, except the Ca-P bound.

Past studies have demonstrated the ability of soil microorganisms to solubilize phosphate rock. The soil microbiota includes chemoautotrophs capable of oxidizing S from more reduced sources causing the acidification of soil (Wainwright *et al.,* 1986). The resultant acidification can cause solubilization of phosphate rocks.

Bacteria isolated from the rhizosphere of chickpea and alkaline soil demonstrated diverse levels of phosphate solubilization activity under in vitro condition in the presence of various carbon and nitrogen sources. Acid production may have contributed to phosphate solubilization (Nautiyal *et al.,* 2000).

2.2.9 Influence of liming

Liming is one of the processes, which govern the practical control of phosphorus availability. The simplest mechanism processes is the exchange of $H_2PO^-_4$ by hydroxyl groups. With due consideration of pH, lime obviously decrease the amount of fixed phosphorus (Amarasiri and Solsen, 1973).

2.3 Phosphate Solubilizing Bacteria

Improving phosphorus nutrition is an urgent priority to meet the increasing global demand for food. A large number of autotrophic and heterotrophic soil micro-organisms demonstrate the in-vitro ability to solubilize mineral phosphorus and play a key role in the mobilization of soil P in plant-available form. Phosphorus-solubilizing fungi (PSF) and bacteria are known as effective organisms for phosphorus solubilization. In soil, phosphorus-solubilizing bacteria constitute 1–50% and fungi 0.5–0.1% of the total respective population (Chen *et al*., 2006) (Table 2.1).

Phosphorus-solubilizing bacteria's are present in almost all the soils, although their number varies depending upon the soil and climatic conditions. A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with non-rhizosphere soil (Kundu *et al*., 2009).

Table 2.1 List of phosphorus solubilizing bacteria in soil (Rodriguez *et al*., 2006).

Genera	Species	% Contribution
Bacillus	*Bacillus amyloliquefaciens*	21.95 %
	B. licheniformis	
	B. polymyxa; B. megaterium	
	B. pulvifaciens; *B. circulans*	
	B. subtilis; B. atrophaeus	
Enterobacter	*Enterobacter intermedium*	8.6 %
	E. aerogenes	
	E. tayloraer	
	E. asburiae	
Pseudomonas	*Pseudomonas fluorescens*	24.39 %
	P. putida; P. mendocina	
	P. striata; P. rathonis	
Rhizobium	*Rhizobium meliloti*	
	R. leguminosarum	
	R. loti	

2.3.1 Mineral phosphate solubilization

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986). Among the bacterial genera with this capacity are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium* and *Erwinia.*

There are considerable populations of phosphate-solubilizing bacteria in soil and in plant rhizospheres (Sperberg, 1958; Katznelson, 1962; Raghu, 1966; Alexander, 1977**)**. These include both aerobic and anaerobic strains, with a prevalence of aerobic strains in submerged soils (Raghu, 1966). A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with non-rhizosphere soil (Katznelson, 1962; Raghu, 1966 and Molla *et al.,* 1983).

Visual detection and even semi-quantitative estimation of the phosphate solubilization ability of microorganisms have been possible using plate screening methods, which show clearing zones around the microbial colonies in media containing insoluble mineral phosphates (mostly tricalcium phosphate or hydroxyapatite) as the single P source. In some cases, there have been contradictory results between plate halo detection and P solubilization in liquid cultures (Louw, 1959; Das, 1963; Ostwal and Bhide, 1972). However, the method can be regarded as generally reliable for isolation and preliminary characterization of phosphate-solubilizing microorganisms (Katznelson, 1962; Bardiya and Gaur, 1974; Goldstein and Liu, 1987; Darmwall *et al*., 1989; Illmer and Schinner, 1992; Gupta *et al.,* 1994) developed an improved procedure using a medium containing bromophenol blue. In this medium, yellow colored halos are formed around the colonies in response to the pH drop produced by the release of organic acids, which are responsible for phosphate solubilization. With this method, the authors reported more reproducible and correlated results than with the simple halo method.

In vitro studies of the dynamics of phosphate solubilization by bacterial strains have been carried out based on the measurement of P release into culture broth, from cultures developed using an insoluble compound as the only P source. The rate of P solubilization is often estimated by subtracting the final P concentration (minus that of an inoculated control) from the initial theoretical P supplied by the P substrate. This estimation has the disadvantage of not taking into account the P utilized by the cells during growth.

Species of *Bacillus, Streptomyces* and Unidentified Gm –ve short rod were to be the most efficient organisms in dissolving insoluble phosphate in liquid culture medium. Species of *Aspergillus* and *Penicillium* rank next in dissolving insoluble phosphate. Is was also found that with the increased in soluble phosphate, the pH of the media was decreased by all the species. The lowest and highest pH being

recorded as 4.6 and 6.5 by the species of *Bacillus* and *Penicillium*, respectively after 11 days of incubation.

Babenko *et al.* (1984) have isolated and grouped phosphate-solubilizing bacteria into four different types, according to kinetics and rate of P accumulation. These groups range from a linear increase of P concentration along with the growth of the culture, to oscillating behavior with variations in the soluble P levels giving rise to several peaks and troughs of P concentration. This last type of kinetic behavior has also been observed (Illmer and Schinner, 1992; Khan and Bhatnagar, 1977; Rodríguez *et al*., 1996). These changes in P concentration could be a consequence of P precipitation of organic metabolites (Babenko *et al.,* 1984; Khan and Bhatnagar, 1977) and/or the formation of organo-P compounds with secreted organic acids, which are subsequently used as an energy or nutrient source, this event being repeated several times in the culture (Illmer and Schinner, 1992).

An alternative explanation could be the difference in the rate of P release and uptake. When the rate of uptake is higher than that of solubilization, a decrease of P concentration in the medium could be observed. When the uptake rate decreases (for instance as a consequence of decreasing growth or entry into stationary phase), the P level in the medium increases again. More probably, a combination of two or more phenomena could be involved in this behavior. Thus, the P concentration in the culture broth as an indication of phosphate solubilization capacity should be viewed with caution, and a kinetic study of this parameter would offer a more reliable picture of cellular behavior toward P.

Table 2.2 summarizes the solubilization ability of different insoluble P substrates by several bacterial species. Although no accurate quantitative comparison can be made from experiments from different sources, the data suggest that *Rhizobium, Pseudomonas* and *Bacillus* species are among the most powerful solubilizers, while tricalcium phosphate and hydroxyapatite seem to be more degradable substrates than rock phosphate.

Table 2.2 Total P accumulation in cultures of different bacterial species grown on insoluble mineral phosphate substrates (mg/L)

Bacterial strain	Substrate			Reference
	$Ca_3(PO_4)_2$	Hydroxyapatite	Rock phosphate	
Pseudomonas sp.	52	nd	nd	(Illmer and Schinner, 1992)
Pseudomonas striata	156	143	22	(Arora and Gaur, 1979)
Burkholderia cepacia	35	nd	nd	(Rodríguez *et al*., 1996)
Rhizobium sp.	nd	300	nd	(Halder and Chakrabartty, 1993)
Rhizobium meliloti	nd	165	nd	(Halder and Chakrabartty, 1993)
Rhizobium leguminosarum	nd	356	nd	(Halder and Chakrabartty, 1993)
Rhizobium loti	nd	27	nd	(Halder and Chakrabartty, 1993)
Bacillus amyloliquefaciens	395	nd	nd	(Vázquez, 1996)
Bacillus polymyxa	116	87	17	(Arora and Gaur, 1979)
Bacillus megaterium	82	31	16	(Arora and Gaur, 1979)
Bacillus pulvifaciens	54	65	13	(Arora and Gaur, 1979)
Bacillus circulans	11	17	6	(Arora and Gaur, 1979)
Citrobacter freundi	16	7	5	(Arora and Gaur, 1979)
Bacillus sp.; *Pseudomonas* sp.; *Micrococcus* sp.;*Proteus* sp.; *Serratia* sp;*Streptomyces* sp.; *Aspergillus* sp.;*Penicillum* sp.	nd	nd	nd	(Molla *et al*., 1983)

'nd indicates not determined'

2.3.2 Organic phosphate solubilization

Soil contains a wide range of organic substrates, which can be a source of P for plant growth. To make this form of P available for plant nutrition, it must be hydrolyzed to inorganic P. Mineralization of most organic phosphorous compounds is carried out by means of phosphatase enzymes. The presence of a significant amount of phosphatase activity in soil has been reported (Lynch, 1990; El-Sawah *et al.*, 1993; Bishop *et al.,* 1994; Feller *et al.,* 1994; Kremer, 1994; Sarapatka and Kraskova, 1997). Important levels of microbial phosphatase

activity have been detected in different types of soils (Kirchner et al., 1993; Kucharski *et al.,* 1996). In fact, the major source of phosphatase activity in soil is considered to be of microbial origin (Garcia, 1992; Xu and Johnson, 1995). In particular, phosphatase activity is substantially increased in the rhizosphere (Tarafdar and Junk, 1987).

The presence of organic phosphate-mineralizing bacteria in soil has been surveyed by Greaves and Webley (1965) for the rhizosphere of pasture grasses, by Raghu and MacRae (1966) for rice plants, as well as by Bishop *et al.,* (1994) and Abd-Alla (1994), and others.

According to Molla *et al.* (1983); the order of sequence on the basis of efficiency of organisms in mineralizing organic phosphate in sterile soil was as follows:
Mixed culture > *Bacillus* > Unidentified Gm –ve short rod > *Streptomyces* > *Aspergillus* > *Penicillium* > *Proteus* > *Serratia* > *Micrococcus* > *Pseudomonas*. And in non-sterile soil the order of sequence was as follows: Mixed culture > *Bacillus* > *Streptomyces* > *Penicillium* > Unidentified Gm –ve short rod > *Aspergillus* > *Proteus* > *Serratia* > *Micrococcus* > *Pseudomonas.*

The pH of most soils ranges from acidic to neutral values. Thus, acid phosphatases should play the major role in this process. Significant acid phosphatase activity was observed in the rhizosphere of slash pine in two forested Spodosols (Burns, 1983; Fox and Comerford, 1992), studied the activity of various phosphatases in the rhizosphere of maize, barley, and wheat, showing that phosphatase activity was considerable in the inner rhizosphere at acidic and neutral soil pH . Soil bacteria expressing a significant level of acid phosphatases include strains from the genus *Rhizobium* (Abd-Alla, 1994), *Enterobacter*, *Serratia, Citrobacter, Proteus* and *Klebsiella* (Thaller *et al.,* 1995), as well as *Pseudomonas* (Gügi *et al.,* 1991) and *Bacillus* (Skrary and Cameron, 1998).

According to Greaves and Webley (1965), approximately 30–48% of culturable soil and rhizosphere microorganisms utilize phytate. On the other hand,

Richardson and Hadobas (1997) reported that 63% of culturable soil bacteria were able to grow on this substrate as carbon and P source on agar medium. However, of these, only 39 – 44% could utilize phytate as a P source in liquid medium, while a very low proportion could use it as a C source in this condition.

All of these studies provide evidence that support the role of bacteria in rendering organic P available to plants (Tarafdar and Claassen, 1988). Some examples of soil bacteria capable of P release from different organic sources are shown in Table 2.3.

Table 2.3 Phosphate mineralization from P-substrates by some soil bacterial species.

Bacterial strain	Substrate	Enzyme type	Reference
Pseudomonas fluorescens	Non-specific	Acid phosphatase	(Gügi *et al.,* 1991)
Pseudomonas sp.	Non-specific	Acid phosphatase	(Gügi *et al.,* 1991)
Burkholderia cepacia	Non-specific	Acid phosphatase	(Rodríguez *et al.,* 1996)
Enterobacter aerogenes	Non-specific	Acid phosphatase	(Thaller *et al.,* 1995)
Enterobacter cloacae	Non-specific	Acid phosphatase	(Thaller *et al.,* 1995)
Citrobacter freundi	Non-specific	Acid phosphatase	(Thaller *et al.,* 1995)
Proteus mirabalis	Non-specific	Acid phosphatase	(Thaller *et al.,* 1995)
Serratia marcenscens	Non-specific	Acid phosphatase	(Thaller *et al.,* 1995)
Bacillus subtilis	Inositol phosphate	Phytase	(Richardson and Hadobas, 1997)
Pseudomonas putida	Inositol phosphate	Phytase	(Richardson and Hadobas, 1997)
Pseudomonas mendocina	Inositol phosphate	Phytase	(Richardson and Hadobas, 1997)
Pseudomonas fluorescens	Phosphonoacetate	Phosphonoacetate hydrolase	(McGrath et al., 1995)
Bacillus licheniformis	D-a-glycerophosphate	D-a-glycerophosphatase	(Skrary and Cameron, 1998)
Klebsiella aerogenes	Phosphonates	C-P Lyase	(Ohtake *et al.,* 1996)
Bacillus sp.; *Pseudomonas* sp.; *Micrococcus* sp.; *Proteus* sp.; *Serratia* sp; *Streptomyces* sp.; *Aspergillus* sp.; *Penicillum* sp.	Non-specific	Acid phosphatase	(Molla *et al.*, 1983)

2.4 Mechanisms of Phosphate Solubilization

Microorganisms and their interactions in soil play a critical role in mediating the distribution of P between the available pool in soil solution and the total soil P through solubilization, mineralization and immobilization reactions of sparingly available forms of inorganic and organic soil P. As a general sketch of P solubilization in soil is shown in (Figure 2.1). Principal mechanism for phosphate solubilizing includes following:

- Production of organic acids (Hu *et al.*, 2009)
- Production of acid phosphatase. (Turner and Haygarth, 2005)

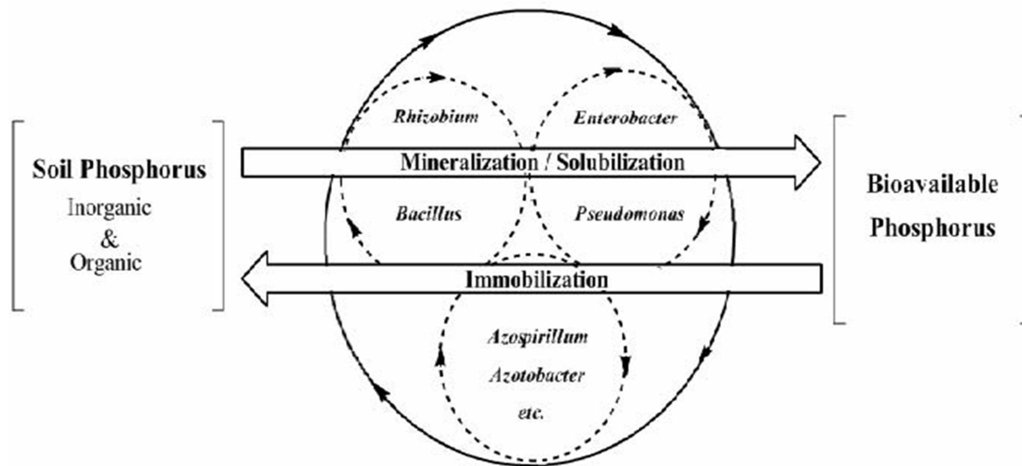


Figure 2.1 Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (Khan *et al.,* 2009).

2.4.1 Production of organic acids

A key mechanism for mineralization of Phosphates in soil is through microbial secretion of low molecular weight organic acids. The phosphate solubilizing bacteria (PSB) have ability to reduce the pH of the surroundings by the production of organic acids (Chen *et al.*, 2006) (Table 2.4). These organic acids can either dissolve phosphates as a result of anion exchange or can chelate Ca, Fe or Al ions associated with the phosphates (Gyaneshwar *et al.*, 2002). However, soil microorganisms vary considerably in their ability to secrete organic acids and,

thereby, solubilize mineral phosphates at different extent.

Table 2.4 Important phosphate solubilizing microorganism, their ecological niches and organic acids produced.

Organisms	**Ecological niche**	**Predominant acids produced**	**Reference**
Phosphate solubilizing bacteria	Soil and phosphate bearing rocks	ND (not determined)	Pikovskaya, 1948
Phosphate solubilizing bacteria	Bulk and rhizospheric soil	ND (not determined)	Gerretson 1948
Escherichia freundii	Soil	Lactic	Sperber 1958a, b
Aspergillus niger, Penicillium sp.	Soil	Citric, glycolic, succinic, gluconic, oxalic, lactic	Sperber 1958a, b
Bacillus megaterium, Pseudomonas sp., *Bacillus subtilus*	Rhizospheric soil	Lactic, malic	Taha *et al.* 1969
Arthrobacter sp., Bascillus sp., *Bacillus firmus* B-7650	Wheat and cowpea rhizosphere	Lactic, citric	Bajpai and Sundara Rao 1971
Aspergillus sp., *Penicillium* sp., *Chaetomiumnigricolor*	Lateritic soil	Oxalic, Succinic, Citric, 2-ketogluconic	Banik and Dey 1983
A. japonicus, A. foetidus	Indian Rock phosphate	Oxalic, citric, Gluconic, succinic, tartaric acid	Singal *et al.* 1994
P. radicum	Rhizosphere of wheat roots,	Gluconic	Whitelaw *et al.* 1999
Enterobacteragglomerans	Wheat rhizosphere	Oxalic, citric	Kim *et al*. 1997
Bacillus amyloliquefaciens, B. licheniformis, B. atrophaeus, Penibacillus macerans, Vibrio, proteolyticus, xanthobacter agilis, Enterobacter, aerogenes, E. taylorae, E. asburiae, Kluyvera, cryocrescens, Pseudomonas aerogenes, Chryseomonas Luteola.	Mangrove ecosystem	Lactic, itaconic, isovaleric, isobutyric, acetic	Vazquez *et al.* 2000
Penicillium rugulosum	Venezuelan phosphate rocks	Citric, gluconic acid	Reyes *et al.* 2001
Enterobacter intermedium	Grass rhizosphere	2-ketogluconic	Hwangbo *et al.* 2003
Aspergillus flavus, A . niger, Penicilliumcanescens	Stored wheat grains	Oxalic, citric, gluconic succinic	Maliha *et al.* 2004
P. fluorescens	Root fragments and rhizosphere of oil palm trees	Citric, malic, tartaric, gluconic	Fankem *et al.* 2006
Aspergillus niger	Tropical and subtropical soil	Gluconic, oxalic	Chuang *et al.* 2007

(Table 2.4 Continued)

P. trivialis	Rhizosphere of Hippophaer hamnoides growing in the cold deserts of Lahaul and Spiti in the trans-Himalayas	Lactic, formic	Vyas and Gulati 2009
*B. pumilus var.*2; *B.subtilisvar.*2; *Actinomadura oligospora; Citrobacter* sp	Giant Cardon cactus (P.pringlei) growing in ancient lava	Gluconic, Propionic, Isovaleric, Heptonic, Caproic, Isocaproic, Formic, Valeric, Succinic, Oxalic, Oxalacetic, Malonic	Puente *et al.* 2004a
B.pumilus CHOO8A; *B.fusiformis*	Cholla cactus(Opuntia Cholla)		Puente *et al.* 2004a
Bacillus sp. SENDO 6 and	Giant Cardon cactus (P.pringlei)	Gluconic, Propionic, Isovaleric, Formic, Succinic, Lactic.	Puente *et al.* 2009a, b
Pseudomonas putida M5TSA, *Enterobacter sakazakii* M2PFe, and *Bacillus megaterium* M1PCa	Wild cactus Mammillaria fraileana		Lopez *et al.* 2011

2.4.2 Production of acid phosphatase

The mineralization of phosphorus compound is carried out by the action of several phosphates (also called phosphor hydrolase), which is present in a wide variety of soil microorganism and play a significant role in assimilation of phosphate from organic compounds by plants and microorganisms (Sharma *et al.*, 2011).

These dephosphorylating reactions involve the hydrolysis of phosphoester or phosphoanhydride bonds. The phosphohydrolases are clustered in acid or alkaline. The acid phosphohydrolases, unlike alkaline phosphatases, show optimal catalytic activity at acidic to neutral pH values. Moreover, they can be further classified as specific or nonspecific acid phosphatases, in relation to their substrate specificity. Rossolini *et al.* (1998) recently published a comprehensive review of bacterial nonspecific acid phosphohydrolases. The specific phosphohydrolases with different activities include: 3'-nucleotidases and 5'-

nucleotidases; hexose phosphatases; and phytases. A specific group of P releasing enzymes are those able to cleave C-P bonds from organophosphonates (Singh *et al.,* 2011).

The decomposition of organic matter in soil is carried out by the action of numerous saprophytes, which produce the release of radical orthophosphate from the carbon structure of the molecule. The organophosphonates can equally suffer a process of mineralization when they are victims of biodegradation (Sharma *et al.,* 2010). The microbial mineralization of organic phosphorus is strongly influenced by environmental parameters; in fact, moderate alkalinity favors the mineralization of organic phosphorus (Stibal *et al.,* 2009).

The degradability of organic phosphorous compounds depends mainly on the physicochemical and biochemical properties of their molecules, e.g. nucleic acids, phospholipids, and sugar phosphates are easily broken down, but phytic acid, polyphosphates, and phosphonates are decomposed more slowly (Sharma *et al.,* 2010).

Some phosphohydrolases are secreted outside the plasma membrane, where they are either released in a soluble form or retained as membrane-bound proteins. This localization allows them to act as scavenging enzymes on organic phosphoesters that are components of high molecular weight material (i.e. RNA and DNA) and cannot cross the cytoplasmic membrane. This material can be first converted to low molecular weight components, and this process may occur sequentially i.e. the transformation of RNA and DNA to nucleoside monophosphate via RNase and DNase respectively, followed by the release of P and organic by-products via phosphohydrolases, providing the cell with essential nutrients (Singh *et al.,* 2011).

2.5 Interaction of Phosphorus Solubilizing Bacteria with Other Microorganisms

The Phosphorus Solubilizing microorganism when used with other plant growth-promoting rhizobacteria (PGPR) act synergistically to enhance crop yields (Saxena and Tilak 1994 & 1997). Coinoculation of *P. striata* and *arbuscular mycorrhizae* significantly increased the soybean yield and P uptake by plants over control. Dual inoculation of *Rhizobium* with phosphorus solubilizing microorganism (Perveen *et al.,* 2002) or *arbuscular mycorrhizae* (AM) fungi (Zaidi *et al.,* 2003) has been shown to improve plant growth more than with their sole inoculation in P-deficient soils. Synergistic interactions on plant growth have been observed by co-inoculation of phosphorus solubilizing bacteria with N_2 fixers such as *Azotobacter* (Kundu and Gaur, 1984) and *Azospirillum* (Belimov *et al.,* 1995) or with vesicular *arbuscular mycorrhizae* (Kim *et al.,* 1998). Son *et al.*, (2003 & 2006) showed that co-inoculation of *Bradyrhizobium japonicum* and *Pseudomonas* sp. enhanced the number of nodules, dry weight of nodules, yield components, soil nutrient availability and uptake in soybean crop. Research workers have reported similar results with legume crops when phosphorus solubilizing bacteria are co-inoculated with various N_2-fixing bacteria (Table 2.5).

Table 2.5 Effect of co-inoculation of legumes with phosphate solubilizers and N_2 fixers

Crop	Rhizobia	Co-inoculating PS solubilizers	Plant responses to inoculation	References
Alfalfa	*R. meliloti*	*Pseudomonas*	Plant growth, nitrogenase activity, nodule number, total nodule weight and total plant nitrogen showed significant increase	Knight and Langston-Unkefer (1988)
Chickpea	*Mesorhizobim*	*Pseudomonas*	Marked increase in nodule weight and shoot biomass when coinoculated with Mesorhizobium and Pseudomonas in sterilized chillum jar conditions. In pot experiments, co-inoculation significantly increased root and shoot biomass	Sindhu *et al.* (2002a)
	Mesorhizobium	*Bacillus*	Dual inoculation significantly increased plant dry weight, nodulation, N content, protein content and seed yield, compared to single inoculation	Wani *et al.* (2007)
	Rhizobium	*Pseudomonas, Bacillus*	Significantly increased nodule weight, root and shoot biomass and total plant nitrogen	Parmar and Dadarwal (1999)
Clover	*R. leguminosarum bv.trifolii 24*	*Pseudomonas sp*	Co-inoculation significantly increased shoot and nodule weight in comparison to plants inoculated with R. leguminosarum bv. Trifolii	Derylo and Skorupska (1993)
Common bean	*Rhizobium*	*A. brasilense*	Co-inoculation promoted root hair formation and an increase in secretion of the nod gene induced flavonoids resulting in greater number of nodules	Burdman *et al.* (1996)
Soybean	*B. japonicum*	*P. fluorescens*	Co-inoculation increased colonization of B. japonicum on soybean roots, nodule number and the acetylene reduction assay	Chebotar *et al.* (2001), Son *et al.* (2006)
Green Gram	*Bradyrhizobium sp (Vigna)*	*Bacillus*	Co-inoculation enhanced nodulation and growth of greengram	Sindhu *et al.* (2002b)
Wheat	*R. leguminismarum*	*Pseudomonas* sp.	Dual inoculation along with P fertilizer increase yield by 30–40%	Afzal and Asghari (2008)

2.6 Phosphate Solubilizing Bacteria as Plant Growth Promoters

Although several phosphate solubilizing bacteria occur in soil, usually their numbers are not high enough to compete with other bacteria commonly established in the rhizosphere. Thus, the amount of P liberated by them is generally not sufficient for a substantial increase in in-situ plant growth. Therefore, inoculation of plants by a target microorganism at a much higher concentration than that normally found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement. The microorganisms have enormous potential in providing soil P for plant growth. Use of phosphate-solubilizing bacteria microorganisms can increase crop yields up to 70 percent (Mohammadi, 2012**)**.

The phosphate-solubilizing bacteria as inoculants simultaneously increases P uptake by the plant and crop yield. The phosphate-solubilizing bacteria exhibiting multiple plants growth promoting traits on soil-plant system is needed to uncover their efficacy as effective bio-inoculants. The inoculation of phosphate-solubilizing bacteria and plant growth-promoting *rhizobacteria* (PGPR) together could reduce 50% of P fertilizer application without any significant decrease of crop yield (Sharma *et al*., 2011). Combined inoculation of *Arbuscular mycorrhiza* and phosphate-solubilizing bacteria's give better uptake of both native P from the soil and P coming from the phosphatic rock and enhance plant growth by solubilizing P from different fractions of soil (Ahmed *et al.,* 2008). The phosphate-solubilizing bacteria's are able to synthesize phyto-hormones like Indole Acetic Acid (IAA), Gibberellic Acid (GA) (Ramkumar and Kannapiran, 2011) and siderophore (Babna *et al*., 2013). Phosphate-solubilizing bacteria's are also enhances plant growth by increasing the efficiency of biological nitrogen fixation or enhancing the availability of other trace elements such as iron, zinc, etc. (Ponmurugan and Gopi, 2006).

These Bacteria enhance the growth and grain yield of different plants as reported in *Zea mays* (Yang *et al*., 2012) and wheat (Afzal *et al*., 2005*)* (Table 2.6).

Moreover, phosphate-solubilizing bacteria and NPK had a positive effect on germination and seed quality of some plants like radish that directly improved vigour index (Lamo *et al*., 2012). The effect of phosphate-solubilizing microorganisms is also known on growth of medicinal plants and their biosynthesis of specific drugs (Gupta *et al*., 2012). Field experiments revealed that P-solubilizing bacteria (PSB) not only improved the growth, yield and quality of crops but also drastically reduced (1/3-1/2) the usage of chemical or organic fertilizers (Yasmin and Bano, 2011). The application of these inoculants by different methods can be positive to enhance the efficiency of naturally and synthetically produced P resources and thus, optimize the crop production.

Table 2.6 Plant growth promotion by phosphate-solubilizing microorganism (Patil *et al.,* 2002)

PSM Bioinoculant	Crop benefited
B. firlmus NCIM 2636	Paddy in acid soils
G. faciculatum	Banana
B. megaterium+G. faciculatum	Banana
Phosphobacterium	Sword bean variety SBS 1
P. Striata	Soybean in sandy alluvial soil
P. Striata	Chick pea

2.7 Heavy Metals

Areas surrounding historic mining and smelting operations represent some of the most highly contaminated and destructed soil habitats due to the high toxicity and widespread ecological effects associated with metal contamination. The term 'heavy metal' although often not rigidly defined is commonly held for those metals, which have specific weights more than 5 g/cm^3 (Holleman and Wiberg, 1985). There are about 40 elements that fall into this category. Heavy metals are largely found in disperse form in rock formations. Industrialization and urbanization have increased the anthropogenic contribution of heavy metals in biosphere. Heavy metals have largest availability in soil and aquatic ecosystems and to a relatively smaller proportion in atmosphere as particulate or vapour.

Soils normally contain low background levels of heavy metals (Table 2.7). However, in areas where agricultural, industrial or municipal wastes are land-applied as fertilizer, concentrations may be much higher. Industrial inputs and the agronomic application of fertilizers, pesticides and metal-contaminated sewage continue to contribute the metal accumulation in the soil (Herland *et al*., 2000). Metals most commonly associated with metal pollution include arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), mercury (Hg), lead (Pb) and zinc (Zn) (Maier *et al*., 2000).

Table 2.7 Heavy metal composition of typical uncontaminated soils and agricultural crops (Modified from Allaway, 1968).

Heavy metals	Range in soil (ppm d.wt)	Range in agricultural crops (ppm d.wt)
Cd	0.01-0.7	0.2-0.8
Co	1-40	0.05-0.5
Cr	5-3000	0.2-1.0
Cu	2-100	4-15
Fe	7000-550000	-
Mn	100-4000	15-100
Mo	0.2-5	1-100
Ni	10-1000	1.0
Pb	2-200	0.1-10
Zn	10-300	15-200

The recent rapid development and growth of Asian countries is likely to result in heavy metal pollution. Soil pollution is generally site specific, and various factors contribute to soil pollution, including factors related to the pollutants (such as the source, the transport and degradation pathways, and the chemical form of heavy metals) and factors related to soils (such as adsorption capacity, buffering capacity, and clay mineral content and the chemical forms of heavy metals present in soils). Various Asian countries have established laws regulating heavy metals in soils, although regulations differ between countries. In Japan, rapid industrialization in the 1960s brought about dangerous levels of soil pollution by heavy metals such as cadmium (MoE, 1970).

2.7.1 Sources of heavy metals

Chemical compounds, entering the ecosystem as a result of different human activities, may accumulate in soil and water environments. Therefore, soil may be regarded as a long-term reservoir of pollutants, from which these compounds may be introduced to food chains or groundwater (Pečiulyté and Dirginčiuté-Volodkiené, 2009). Inappropriate and careless disposal of industrial waste often results in environmental pollution (Table 2.8). The pollution includes point sources are as follows

- Fossil fuel combustion
- Agricultural and horticultural materials
- Vehicle exhaustion and metal smelting or mining
- Industries-manufacture, use and disposal of metal commodities
- Electronics-manufacture, use and disposal of metal commodities
- Chemical and other manufacturing industries
- Waste disposal and sewage sludge
- Volcanic eruption

Although under some circumstances, there are direct atmospheric inputs to plants, and soils, subsequent transfer from soil to plant is for the most heavy metals the major route of entry into human and animals.

Table 2.8 Different sources of heavy metals in soils (Lone *et al.,* 2008).

Heavy metals	Sources
As	Semiconductors, petroleum refining, wood preservatives, animal feed additives, coal power plants, herbicides, volcanoes, mining and smelting
Cu	Electroplating industry, smelting and refining, mining, biosolids
Cd	Geogenic sources, anthropogenic activities, metal smelting and refining, fossil fuel burning, application of phosphate fertilizers, sewage sludge
Cr	Electroplating industry, sludge, solid waste, tanneries
Pb	Mining and smelting of metalliferous ores, burning of leaded gasoline, municipal sewage, industrial wastes enriched in Pb, paints
Hg	Volcano eruptions, forest fire, emissions from industries producing caustic soda, coal, peat and wood burning
Se	Coal mining, oil refining, combustion of fossil fuels, glass manufacturing industry, chemical synthesis (e.g., varnish, pigment formulation)
Ni	Volcanic eruptions, land fill, forest fire, bubble bursting and gas exchange in ocean, weathering of soils and geological materials
Zn	Electroplating industry, smelting and refining, mining, biosolids

2.8 Biological Essentiality of Heavy Metals

Metals are directly and/or indirectly involved in all aspects of microbial growth, metabolism and differentiation (Gadd, 1992a). Metals and their compounds interact with microbes in various ways depending on the metal species, organism and environment, while structural components and metabolic activity also influence metal speciation and therefore solubility, mobility, bioavailability and toxicity (Gadd & Griffiths, 1978; Gadd, 1992a, 1993a, 2004, 2005, 2007) (Table 12). Many metals are essential for life, e.g. Na, K, Cu, Zn, Co, Ca, Mg, Mn and Fe, but all can exert toxicity when present above certain threshold concentrations.

Other metals, e.g. Cs, Al, Cd, Hg and Pb, have no known essential metabolic functions but all can be accumulated. Microbes are intimately associated with the biogeochemical cycling of metals, and associated elements, where their activities can result in mobilization and immobilization depending on the mechanism involved and the microenvironment where the organism(s) are located (Gadd, 2004, 2007; Violante *et al.,* 2008; Ehrlich & Newman, 2009).

Metal– mineral–microbe interactions are of key importance within the framework of geomicrobiology and also fundamental to microbial biomineralization processes. The term biomineralization represents the collective processes by which organisms form minerals (Bazylinski, 2001; Dove *et al.,* 2003), a phenomenon widespread in biology and mediated by bacteria, protists, fungi, plants and animals. Most biominerals are calcium carbonates, silicates and iron oxides or sulfides (Baeuerlein, 2000; Bazylinski, 2001). Biomineralization is itself an important interdisciplinary research area, and one that overlaps with geomicrobiology (Banfield & Nealson, 1997; Dove *et al.,* 2003; Banfield *et al.,* 2005; Konhauser, 2007).

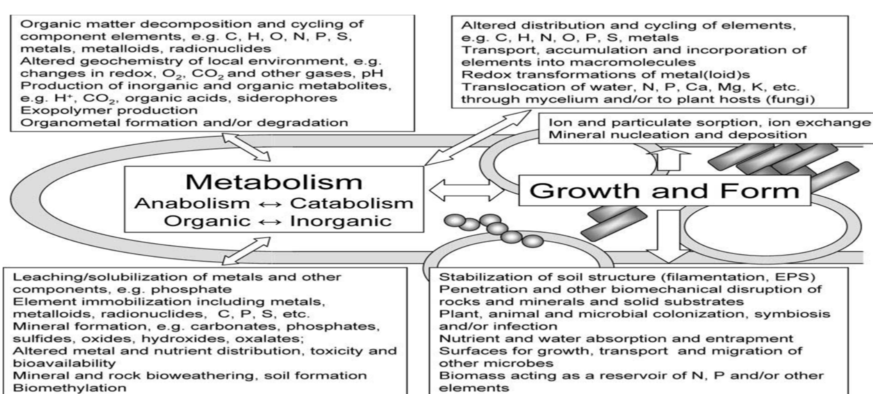


Figure 2.2 Some of the important microbial roles and activities in biogeochemical processes leading to altered metal distributions and speciation (Gadd, 2008a).

Some of the major or representative microbial roles in metal and other elemental cycles are indicated without reference to their relative global significance. Microbes possess transport systems for essential metals; inessential metal species

can also be taken up (Table 2.9). Microbes are also capable of mediating metal and mineral bioprecipitation, e.g. by metabolite production, by changing the physioco-chemical microenvironmental conditions around the biomass, and also by the indirect release of metal-precipitating substances from other activities, e.g. phosphate from organic decomposition or phosphate mineral solubilization. Many different metal-containing minerals formed as a direct or indirect result of microbial activity, e.g. various carbonates, phosphates, etc., are omitted from the table. Microbial cell walls, outer layers, and exopolymers can sorb, bind or entrap many soluble and insoluble metal species as well as e.g. clay minerals, colloids, oxides, etc. which also have significant metal-sorption properties. Redox transformations are also widespread in microbial metabolism, some also mediated by the chemical activity of structural components (Gadd, 2010).

Table 2.9 Microbial roles in key biogeochemical cycles of metals and other elements (Gadd, 2010).

Elements	Microbial roles in elemental cycles
C, H, O	Uptake, assimilation, degradation and metabolism of organic and inorganic compounds; respiration (CO_2 production); photosynthesis; photorespiration; CO_2 fixation; biosynthesis of polymers, organic and inorganic metabolite excretion; humus formation; CN_2 production; carbonate formation; oxalate formation; oxalate–carbonate cycle; dissolution of carbonates; methanotrophy; methanogenesis (archaea); hydrocarbon degraders; organometal(loid) degradation; metal(loid) biomethylation and demethylation; xenobiotic oxidation; CO utilization; water uptake; water transport, translocation and conduction (fungal mycelium); hydrogen oxidation and production
N	Decomposition of nitrogenous compounds; assimilation and transformations of organic and inorganic N compounds; N_2 fixation (prokaryotes only); nitrification and denitrification; ammonia and nitrite oxidation; anaerobic nitrification; biosynthesis of N-containing biopolymers, e.g. chitin; production of N-containing metabolites and gases, e.g. N_2O; ammonia fermentation under anaerobic conditions; *mycorrhizal* N transfer to plants (fungi); fixed N transfer to plants (symbiotic N_2 fixers)

(Table 2.12 continued)

P	Dissolution of inorganic phosphates and P-containing minerals in soils and rocks; decomposition of P-containing organic compounds; formation of insoluble P, e.g. polyphosphate, secondary phosphate minerals; release of organically bound P by phosphatases; assimilation and transformation of inorganic P species; oxidation of reduced forms of phosphate, e.g. phosphate; transformations of soil organic P; production of diphosphates and phosphonates; P transfer to plants (*mycorrhizas)*
Cr	Cr(VI) reduction to Cr(III); Cr(III) oxidation; accumulation of Cr oxyanions
Cu	Mobilization from Cu-containing minerals in rocks and soils; CuS formation; biosorption; uptake and accumulation; bioprecipitation, e.g. oxalates
Pb	Biosorption; lead oxalate formation; biomethylation
As	Biomethylation of As species, e.g. arsenite to trimethylarsine; reduction of As oxyanions, e.g. arsenate to arsenite; oxidation of As oxyanions, e.g. arsenite to arsenate
Hg	Hg biomethylation; reduction of Hg(II) to Hg(0); oxidation of Hg(0) to Hg(II); Hg volatilization as Hg(0); degradation of organomercurials; biosorption; accumulation
Al	Al mobilization from Al-containing minerals in soils and rocks; aluminosilicate dissolution; Al precipitation as oxides (early stage of bauxitization); biosorption
Mg, Ca, Co, Ni, Zn, Cd, Sr	Bioweathering of minerals in rocks and soil; biosorption; uptake and accumulation; bioprecipitation, e.g. oxalates, sulfides, phosphates, carbonate; Co(III) reduction
Fe	Bioweathering of Fe-containing minerals in rocks and soils; Fe solubilization by siderophores, organic acids, metabolites etc.; Fe(III) reduction to Fe(II); Fe(II) oxidation to Fe(III); Fe biomineralization, e.g. oxides, hydroxides, carbonates, sulfides; metal sorption to Fe oxides
Mn	Mn(II) oxidation and immobilization as Mn(IV) oxides; Mn(IV) reduction; indirect Mn(IV)O2 reduction by metabolites, e.g. oxalate; bioaccumulation of Mn oxides to surfaces and exopolymers; contribution to desert varnish formation; biosorption; accumulation; intracellular precipitation; Mn biomineralization, e.g. oxides, carbonates, sulfides, oxalates; metal sorption to Mn oxides

2.9 The Effects of Heavy Metals on Soil Microorganisms

Metals without biological function are generally tolerated only in minute concentrations, whereas essential metals with biological functions are usually tolerated in higher concentrations (Hafeburg and Kothe, 2007). They have either metabolic functions as constituents of enzymes or meet structural demands, e.g. by supporting the cell envelope. Frequently the concentration and the speciation of metal determine whether it is useful or harmful to microbial cells (Hafeburg and Kothe, 2007).

Microorganisms are the first biota that undergoes direct and indirect impacts of heavy metals. Some metals (e.g. Fe, Zn, Cu, Ni, Co) are of vital importance for many microbial activities when occur at low concentrations. These metals are often involved in the metabolism and redox processes. Metals facilitate secondary metabolism in bacteria, actinomycetes and fungi (Hafeburg and Kothe, 2007; Weinberg E.D., 1990). E.g. chromium is known to have stimulatory effect on both actinorhodin production and growth yield of the model actinomycete *S. coelicolor* (Abbas and Edwards, 1990). However, high concentrations of heavy metals may have inhibitory or even toxic effects on living organisms (Bruins *et al.,* 2000). Adverse effects of metals on soil microbes result in decreased decomposition of organic matter, reduced soil respiration, decreased diversity and declined activity of several soil enzymes (Tyler, 1974). Some of the general changes in morphology, the disruption of the life cycle and the increase or decrease of pigmentation are easy to observe and evaluate (Hafeburg and Kothe, 2007). Rajapaksha *et al.,* (2004) compared the reactions of bacteria and fungi to toxic metals in soils (Zn and Cu). They concluded that bacterial community is more sensitive to increased concentrations of heavy metals in soils than the fungal community. The relative fungal/bacterial ratio increased with increasing metal levels. Those authors also noticed the varying effect of soil pH on the microbial reaction to soil pollution, i.e. that lower pH in contaminated soils enhanced the negative effect on bacteria, but not on fungi.

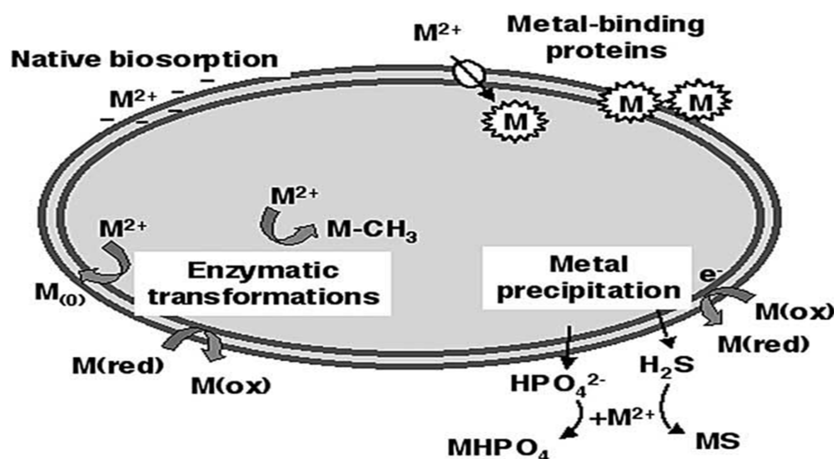


Figure 2.3 How bacteria cope with toxic concentrations of heavy ions (Pazirandeh *et al.,* 1995).

Metal exposure may lead to the establishment of tolerant microbial populations that are often represented by several Grampositive genera such as *Bacillus*, *Arthrobacter* and *Corynebacterium* or Gram-negatives, e.g. *Pseudomonas*, *Alcaligenes*, *Ralstonia* or *Burkholderia* (Piotrowska *et al*., 2005). It was shown that the impact of heavy metals on the bacterial metabolism depends on the growth form. The resistance towards metals seems higher in consortia than in pure cultures (Sprocati *et al.,* 2006). A great number of heavy metal-resistant bacteria, such as e.g. *Cupriavidus metallidurans* possess efflux transporters that excrete toxic or overconcentrated metals outside the cell (Nies, 2003).

The toxic concentration of heavy metals may cause enzyme damage and consequently their inactivation, as the enzymes-associated metals can be displaced by toxic metals with similar structure (Bruins *et al.,* 2000). Moreover, heavy metals alter the conformational structures of nucleic acids and proteins, and consequently form complexes with protein molecules which render them inactive. Those effects result in disruption of microbial cell membrane integrity or destruction of entire cell (Bong, 2010). Heavy metals also form precipitates or chelates with essential metabolites (Sobolev and Begonia, 2008).

2.9.1 Cell Damage

The concentration of a toxic metal that affects the growth and survival of different microorganisms as most heavy-metal ions must first enter the cell (Babich and Stotzky, 1977) and the toxicity of heavy metal is also time dependent with the varying concentrations. At higher concentrations, however, heavy-metal ions form unspecific complex compounds in the cell, which leads to toxic effects. Some heavy-metal cations, e.g. Hg^{2+} ,Cd^{2+} and Ag^{+}, form strong toxic complexes, which makes them too dangerous for any physiological function. Even highly reputable trace elements like Zn^{2+} or Ni^{2+} and especially Cu^{2+} are toxic at higher concentrations (Nies, 1999).

To have any physiological or toxic effect, most heavy-metal ions have to enter the cell. At first glance, divalent heavy-metal cations are structurally very similar; the divalent cations Mn^{2+} ,Fe^{2+} ,Co^{2+} ,Ni^{2+} ,Cu^{2+} and Zn^{2+} have ionic diameters between 138 pm and 160 pm (Weast 1984), a diference of 14%, and all, of course, carry a double positive charge. Oxyanions like chromate, with four tetrahedrally arranged oxygen atoms and two negative charges, differ mostly in the size of the central ion, so the structure of chromate resembles that of sulfate. The same is true for arsenate and phosphate. Thus, uptake systems for heavy-metal ions have to bind those ions tightly if they want to differentiate between a couple of structurally very similar ions.

Most metal toxicity arises from the reaction of metal ions with proteins. Inside the cell, toxicity occurs through the displacement of essential metals from their native binding sites or through ligand interactions. For example, the toxicity of mercuric ions arises from the aggressive binding of the ions to sulfhydryl groups in organic molecules. This action can inhibit macromolecular synthesis and enzyme activity. Proteins often contain R-SH groups that control the tertiary and quaternary structure of the molecule. Other heavy-metal cations may interact with physiological ions, Cd^{2+} with Zn^{2+} or Ca^{2+}, Ni^{2+} and Co^{2+} with Fe^{2+}, Zn^{2+} with

Mg^{2+} thereby inhibiting the function of the respective physiological cation. Finally, heavy-metal oxyanions interfere with the metabolism of the structurally related non-metal (chromate with sulfate, arsenate with phosphate) and reduction of the heavy-metal oxyanion leads to the production of radicals, e.g., in case of chromate.

2.10 Heavy Metal Toxicity to Microbes

Most heavy metals are transition elements with incompletely filled'd' orbital. These'd' orbital provides heavy-metal cations with the ability to form complex compounds which may or may not be redox-active. Thus, heavy-metal cations play an important role as ``trace elements" in sophisticated biochemical reactions. When a cell faces a high concentration of any heavy metal that is accumulated by an unspecific system, the specific heavy-metal ion is transported into the cytoplasm in spite of its high concentration, because these unspecific transporters are constitutively expressed. Thus, the gate cannot be closed. This ``open gate" is the first reason why heavy-metal ions are toxic (Nies and Silver, 1995).
Although some heavy metals are required for life's physiological processes (e.g., components of metalloenzymes), their excessive accumulation in living organisms is always detrimental. In high concentrations, heavy metal ions react to form toxic compounds in cells (Nies, 1999). To have any physiological or toxic effect, most heavy-metal ions must first enter the cell. Because some heavy metals are necessary for enzymatic functions and bacterial growth, uptake mechanisms exist that allow for the entrance of metal ions into the cell (Figure 2.4).

One of the major mechanisms behind heavy metal toxicity has been attributed to oxidative stress. The metals are capable of interacting with nuclear proteins and DNA causing oxidative deterioration of biological macromolecules (Leonard, 2004). Studies show that heavy metal possesses the ability to generate reactive radicals resulting in cellular damage to lipid bilayer and DNA. These reactive

radicals' species include a wide variety of radicals and chelate of amino acid, peptides and proteins complexed with toxic metals (Stohs and Bagchi, 1995).

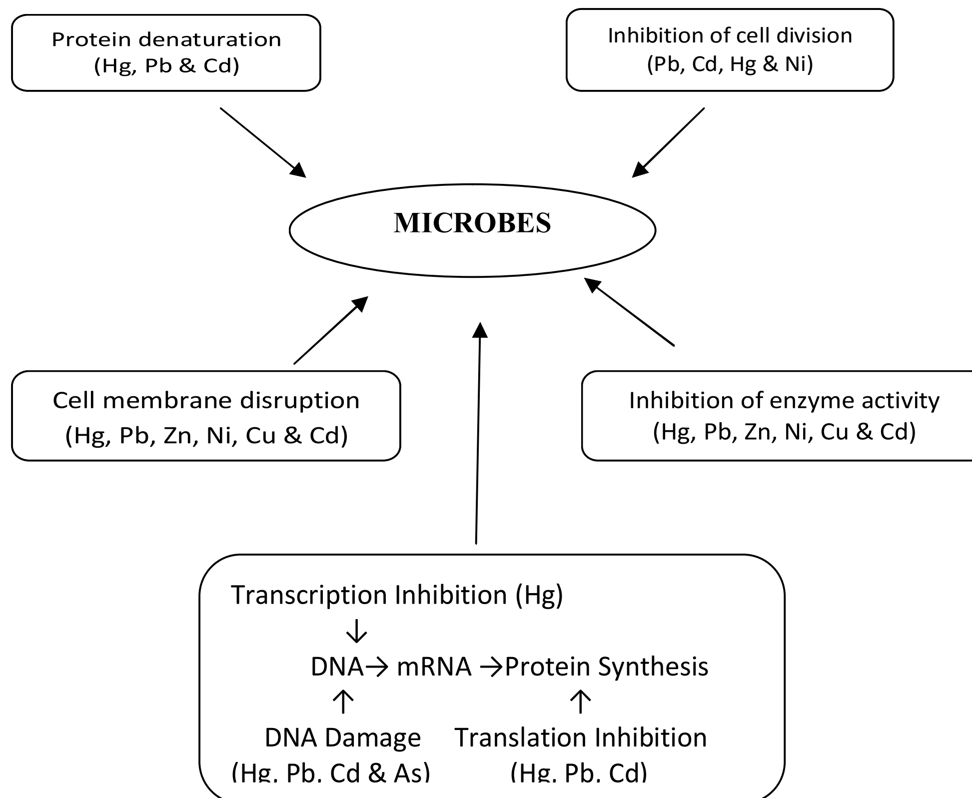


Figure 2.4 Heavy metal toxicity mechanism to microbes (Rajendran *et al.,* 2003)

2.11 Microbial Metal Tolerance and Resistance Mechanisms

Organisms respond to heavy metal stress using different defense system such as exclusion, compartmentalization, formation of complexes and synthesis of binding proteins like metallothioneins (MTs) and phytochelatins (PCs). Ochari, (1997) has divided general toxicity mechanism for metal ions into three categories:

1. blocking the essential biological functional groups of biomolecules especially proteins and enzymes,
2. displacing the essential metal ion in biomolecules and
3. modifying the active conformation of biomolecules resulting the loss of specific activity.

Microorganisms can affect heavy metal concentrations in the environment because they exhibit a strong ability for metal removal from solution; this can be achieved through either enzymatic or non-enzymatic mechanisms (Nealson *et al.,* 1992). Avoidance, restriction of metal entry into the cell, either by reduced uptake/active efflux or by the formation of complexes outside the cell and sequestration, reduction of free ions in the cytosol either by synthesis of ligands to achieve intercellular chelation or by compartmentalization are the two major strategies of organisms to protect themselves against heavy metal toxicity (Tomsett *et al.*, 1992). The general mechanisms of metal tolerance in microbes are shown in Table 2.10.

Table 2.10 Mechanism of metal tolerance in microorganisms (Rajendran *et al.,* 2003)

Metal	Tolerance mechanism
AsO^{2-}, AsO_4^{3-} & Sb^{3+}	Anion efflux (ATPase)
Cd^{2+} & Zn^{2+}	Efflux (ATPase)
Hg^{2+}	Reduction
Co^{2+} & Ni^{2+}	Efflux
CrO_4^{2-}	Decreased uptake
Cd^{2+} & Zn^{2+}	Cation efflux
CrO^{2-}	Decreased uptake
Cu^{2+}	DNA damage

Microorganisms are characterized by high adaptability to undesirable environmental conditions. Tolerant species demonstrate higher resistance to stress factors than sensitive ones (Rensing *et al.*, 2002). Their tolerance is associated with such metabolic functions as:

1) Specific transport of metal ions which involves permeases localized in the cytoplasmic membrane (Chmielowski and K£apciñska, 1984; Binet *et al.,* 2003);

2) Synthesis and excretion to the environment chelating compounds, which bind and transport ions dissolved in the environment (Chmielowski and K£Apciñska 1984; Renella *et al.,* 2006; Paul *et al.,* 2007);

3) Non-specific accumulation of metals: sorption of ions onto mucosal surfaces and the binding by bio-polymers of the wall and membrane complex (Chmie-lowski and K£Apciñska, 1984; Ledin 2000)

4) Presence of plasmids in a bacterial cell, which enable it to acquire resistance to toxic elements: Ag, As, Cd, Cr, Hg, Ni, Sb, Te (Zhang *et al.,* 2001; Meguro *et al.,* 2005). The *Rhizobium* bacteria possessing more plasmids are more tolerant to heavy metals than cells of the same species with fewer plasmids (Lakzian *et al.,* 2002).

Microbial survival in polluted soils depends on intrinsic biochemical and structural properties, physiological, and/or genetic adaptation including morphological changes of cells, as well as environmental modifications of metal speciation (Wuertz and Mergeay, 1997). Bacteria develop heavy-metal resistance mostly for their survivals, especially a significant portion of the resistant phenomena was found in the environmental strains (with or without the presence of heavy metals). Earlier observations revealed that heavy metal tolerance by a particular group of bacteria or an individual isolate in artificial media supplemented with heavy metal showed high tolerance level (Hayat *et al.,* 2002) where, conditions are totally different with natural condition of soil.

Research carried out since the early 1970s identified several microorganisms as being resistant to certain metals. These reports included mostly aerobic microorganisms, with prominent examples of *Staphylococcaus* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus* sp. (Wang and Shen, 1995).

Heavy metals such as cadmium and lead are not readily absorbed or captured by microorganisms. Heavy metals can damage the cell membranes, alter enzymes specificity, disrupt cellular functions and damage the structure of the DNA. Toxicity of these heavy metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Bruins *et al.,* 2000). Also, toxicity can occur as a result of alterations in the conformational structure of the nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance (Poole *et al.,* 1989). Metals can replace essential metals in pigments or enzymes disrupting their function (Henry, 2000) (Figure 2.5). Thus, metals render the land unsuitable for plant growth and destroy the biodiversity.

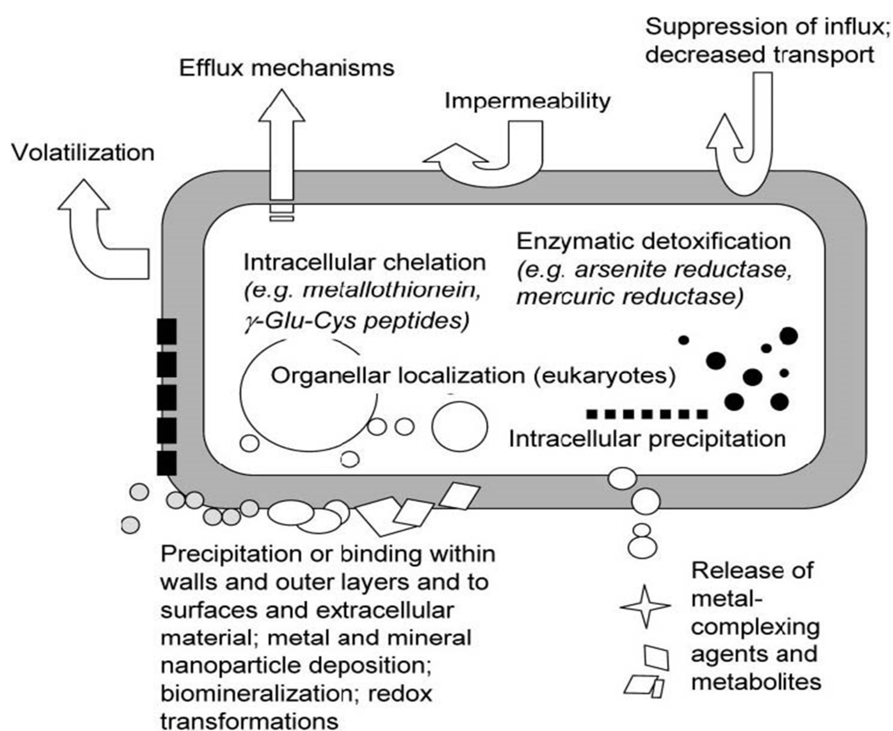


Figure 2.5 Mechanisms involved in the detoxification and transformation of metals, including mechanisms that restrict entry into the cell and intracellular detoxification and organellar compartmentation, the letter occur in some eukaryotes, e.g algae and fungi (Gadd, 2010).

To survive under metal-stressed conditions, microorganisms have evolved several types of mechanisms to tolerate the uptake of heavy metal ions and resist the heavy metal stress. Exposure to heavy metals selects for resistance to heavy metals in the surviving microorganisms (Coyne, 1999). However, prior exposure to one heavy metal does not mean better survival when a different heavy metal is present. Selective pressures from a metal-containing environment have led to the development of resistance systems to virtually all toxic metals (Rouch *et al.*, 1995). Bacteria have adapted to metals through a variety of chromosomal, transposon, and plasmid-mediated resistance systems. Bacterial plasmids have genes for resistances to many toxic ions of heavy metal elements, including Ag^{+}, AsO_2^{-}, AsO_4^{3-} , Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, TeO_3^{2-}, Tl^{+}, and Zn^{2+}. It was predicated that bacterial resistance would be found in almost every toxic ions, if enough efforts could be made.

Bacterial resistance mechanisms generally involve efflux or enzymic detoxification, which can also result in release from cells, e.g. Hg(II) reduction to Hg(0) (Nies and Silver, 1995; Nies, 1992a, 1999 and 2003; Silver and Phung, 1996 and 2009; Rosen, 2002; Osman and Cavet, 2008). Bacterial plasmids have resistance genes to many toxic metals and metalloids, e.g. Ag^{+} , AsO^{-} , AsO^{3-} ,Cd^{2+},Co^{2+} , CrO^{2-},Cu^{2+} , Hg^{2+},Ni^{2+},Sb^{3+} , TeO^{2-},Tl^{+} and Zn^{2+} . Related systems are also frequently located on bacterial chromosomes, e.g. Hg^{2+} resistance in Bacillus,Cd^{2+} efflux in Bacillus and arsenic efflux in Escherichia coli (Silver and Phung, 1996; Rosen, 2002).

Many micro-organisms synthesize extracellular polymers (EPs) that bind cations of toxic metals, thus protecting metal-sensitive and essential cellular components (Bruins *et al.,* 2000). The composition of extracellular polymers (EPs) is very complex, including proteins, humic acids, polysaccharides and nucleic acids, which chelate metals with different specificity and affinity (Pal and Paul, 2008; Guibaud *et al.,* 2003; Roane, 1999). Pb(II) binding by extracellular polymers has

been reported for *Bacillus firmus*, *Pseudomonas* sp. (Salehizadeh and Shojaosadati, 2003), *Cyanobacteria* (Freire-Nordi *et al.,* 2005; Paperi *et al.,* 2006), *Halomonas* sp. (Amoozegar *et al.,* 2012) and *Paenibacillus jamilae* (Perez *et al*., 2008). EP produced by the latter strain is highly Pb(II) specific, and adsorbs ten times more Pb(II) than other metals such as Cd(II), Co(II), Ni(II), Zn(II) and Cu(II) (Morillo *et al.,* 2006; Aguilera *et al.,* 2008). This EP exhibits a characteristic feature, namely a high content of uronic acids (28.29 %), which are considered to play an important role in its Pb(II) binding specificity.

The most detailed research has been done for As, Hg, Cd, Cu, Co, Zn, Pb, Ag, Ni and Te, for which resistance genes have been sequenced and mechanisms proposed (Osman and Cavet, 2008; Silver and Phung, 2009). As with bacteria, intracellular metal concentrations in fungi may be regulated by transport, including efflux mechanisms and internal compartmentation (Gadd, 1993a; Macreadie *et al*., 1994; Blaudez *et al*., 2000; Eide, 2000; Van Ho *et al*., 2002) as well as the direct and indirect mechanisms listed above. Microbes may also synthesize a variety of metal-binding peptides and proteins, e.g. metallothioneins and phytochelatins, which regulate metal ion homeostasis and affect toxic responses (Eide, 2000; Avery, 2001). In eukaryotes, intracellular compart-mentation may also be significant in tolerance (Gadd, 1993a; Eide, 2000; Avery, 2001) (Figure 2.5).

2.12 Cadmium in Relation to Soil Microbes

Cadmium (Cd) is one of the most toxic pollutants of the environment (Das *et al*., 1997). It is widely used by industry for electroplating, stabilization of plastics, corrosion protection, pesticides, and fertilizers. Cadmium (Cd) concentrations in soil from unpolluted areas have been reported to be less than 1ppb (Bhamra and Costa, 1992). Both air and water borne Cd can cause an increase in Cd concentrations normally found in soil. Cadmium (Cd) is highly labile on the soil plant systems.

Adsorption to mineral surfaces and organic matter is thought to be an important process regulating the levels of soluble and plant available Cd in soils. Most of this form is thought to be adsorbed in a more or less exchangeable form but a significant non-exchangeable specific adsorption also exists (Eriksson, 1988). Cadmium is a nonessential metal that is toxic at very low concentrations and it accumulates in organisms. Tanaskovic (1987) showed that in the presence of Lead (Pb), the toxic effects of Cd increases.

Cadmium enters bacteria and other cells through divalent ion transport systems and can be co-transported with manganese Mn(II) in *Lactobacilli plantarum* (Archibald and Duong, 1984). Divalent ion transport systems are normally required to transport essential metals such as magnesium, phosphate, and sulfate (Laddaga *et al*., 1985). Nutrient metal transport systems are often up-regulated in times of need or starvation. An adverse consequence of this is the co-transport of other cations that may be toxic to the organism. Sensitive bacteria can accumulate 3 to 15 times more Cd(II) than resistant bacteria (Trevors *et al*., 1986). Inside the cell, Cd(II) can bind to sulfhydryl groups on essential proteins interfering with important cellular functions (Lebrun *et al*., 1994) and can also cause single-strand breakage of DNA in *E. coli* (Trevors *et al*., 1986).

2.12.1 Microbial resistance to cadmium

Soil contamination with high rates of cadmium (30 and 40 mg Cd kg^{-1} of soil) caused a significant decrease in the number of oligotrophic bacteria, oligotrophic sporulating bacteria, and copiotrophic and copiotrophic sporulating bacteria in soil (Wyszkowska and Wyszkowski, 2002).

Several bacteria demonstrate resistance to Cd(II) such as *Saccharomyces aureus*, *Bacillus subtillis*, and *E. coli* (Cohen *et al*., 1991). The best characterized Cd(II) resistance efflux system are the plasmid-encoded *cad* system in *S. aureus* (Smith

and Novick, 1972) and *Czc* system in *Alcaligenes eutrophus* (Nies, 1992). A third system exists that is chromosomally encoded.

Cadmium resistance occurs through all of the biochemical resistance mechanisms with the exception of enzymatic detoxification. Unlike Hg(II), Cd(II) does not undergo enzymatic detoxification. Enzymatic resistance through NAD(P)H-dependent reaction is not energetically favored for metals like Cd(II), Zn(II), Ni(II), and Co(II) (Nies, 1992). Covalent modification of the divalent form of these metals is also not biologically favorable because this form is more unstable and toxic. Unlike Hg(II), these metals will not move out of the cell; instead they will remain to undergo oxidation back to their original form (Nies, 1992). Resistance to Cd(II) and Zn(II) can be mediated by chromosomes, plasmids, or transposons (Lebrun *et al*., 1994). The most prominent metal resistance system for Cd(II) is by efflux pumps.

2.12.2 Cadmium toxicity to microbes

Cadmium introduced to soil at a higher dose (30 and 40 mg Cd kg^{-1}) depressed the number of soil microorganisms, which is consistent with the results reported by Jiang - Xianjun *et al.,* (2000). Also in the research done by Jiang - Xianjun *et al.,* (2000), the number of bacteria in soil contaminated with cadmium applied with or without other heavy metals was significantly smaller than in the control objects (without Cd). Some authors (Dias-Junior *et al.,* 1998) tried to arrive at a systematic description of the effect of cadmium on every group of soil microorganisms. Hiroki (1992) pointed to a large decrease in the amount *of Actinomyces,* a slightly lower decrease in bacteria and the smallest decrease in fungi. According to Dias-Junior *et al.,* (1998) the tolerance of soil microorganisms to cadmium decreases in the order: fungi > *Actinomyces* > bacteria.

Microorganisms, like strains of *A. eutrophus*, are being studied for their ability to survive in extremely high levels of Cd(II), in some cases up to 50,000 ppm (Mergeay, 1991). These bacteria have been isolated from mines and mine waste, and during later growth phases cellular pH changes allow sequestration of significant amounts of Cd(II), Zn(II), or Ni(II). These features make the organism a very attractive candidate for decontamination of metal polluted soil and effluents. The understanding of the genetic basis of metal resistance is rapidly increasing the ability to use these systems for environmental applications. Improvements in genetic manipulation techniques make it possible to create super accumulator bacteria or plants that can help decontaminate polluted soils, water, and sewage. While much still remains to be learned about the metal resistance mechanisms of microorganism, the future applications of these processes have exciting environmental remediation potential.

2.13 Lead in Relation to Soil Microbes

Lead is neither an essential nor a beneficial element for plants or animals. Heavy metal resistance in bacteria results from their primary contact with metals that appear naturally in the environment. However, intensive human activity and exploitation of natural deposits has led to the expansion of metal-resistant micro-organisms (Bruins *et al.,* 2000). A part from 13 other metals and metalloids, lead (Pb) is considered to be one of the major pollutants (Sparks, 2005). Industrial activities, such as production of batteries and pigments and metal smelting, as well as manufacture of products such as lead arsenate insecticides or lead water pipes are the main sources of Pb in the environment. Natural processes including soil erosion, volcanic emission and mobilization of Pb from minerals contribute only to a minor degree to Pb pollution of the environment (Gerba, 1996; Siegel, 2002; Spiro and Stigliani, 2002; Yokel and Delistraty, 2003; Gadd, 2010). The total Pb concentration in industrial areas can reach up to 10,000 mg kg^{-1}, while the average value in soils ranges from 10 to 100 mg kg^{-1} (Schwab *et al.,* 2005; Akmal

and Jianming, 2009). Additionally, the level of Pb(II) in industrial wastewaters reaches 200–250 mgl^{-1}, whereas, according to accepted quality standards, it should not exceed 0.05–0.10 mgl^{-1} (Sag *et al.,* 1995). Zimdahl and Skogerboe (1977) demonstrated that soils have rather large capacities for the immobilization of Pb, that the organic fraction was largely responsible for the observed fixation of the metal. The main compartments for Pb in the soil are soil solution, the adsorption surfaces of the clay-humus exchange complex, precipitated forms, secondary Fe and Mn oxides and alkaline earth carbonates, the soil humus and silicate lattices. pH and CEC are the main soil properties involved in the immobilization of Pb and that soil organic matter are more important in this process than precipitation as the carbonate or sorption by hydrous oxides.

Nriagu (1979) noted that Pb concentrations increases with decreasing particle sizes and lead is largely adsorbed by silt and clay. Gregsons and Alloway (1984) extracted soil solution from contaminated soils by a centrifugation technique and fractionated the solution by chromatography with Sephadex gels. Table 2.11 provides selected data from the author's results.

Increased concentrations of lead in surface soil layers negatively affect soil microflora. Processes of organic matter decomposition, particularly cellulose, are inhibited as a result of decreased enzymatic activity of microorganisms. This results in soil degradation. Biosorption of lead by soil microorganisms reaches on average 0.2% of this metal, but in some cases it may reach even 40% of biomass and may be used for biological remediation (Kabata and Pendias, 1999).

Table 2.11 Lead in soils and soil solutions (Gregsons and Alloway, 1984).

Total Lead (mg/kg)	Soil solution lead (mg/l)	Solution lead as % total
49900	112	0.05
2820	18	0.13
45800	11	0.005
1890	4	0.04
3830	4	0.02

2.13.1 Lead toxicity to microbes

Pb(II) enters bacterial cells through the uptake pathways for essential divalent metals such as Mn(II) (Laddaga *et al.,* 1985; Tynecka *et al.,* 1981) and Zn(II) (Laddaga and Silver, 1985; Bruins *et al.,* 2000; Makui *et al.,* 2000; Grass *et al.,* 2002). Pb is not known to be of any biological importance and is toxic at very low concentrations (Bruins *et al.,* 2000). The toxicity of Pb depends upon its bioavailability. Since most Pb(II) is bound to clay minerals and complex organic molecules, the bioavailable fraction of Pb(II) to which microbes are exposed may be rather low (Kotuby-Amacher *et al.,* 1992; Singh *et al.,* 1996). Pb(II) toxicity occurs as a result of changes in the conformation of nucleic acids and proteins, inhibition of enzyme activity, disruption of membrane functions and oxidative phosphorylation, as well as alterations of the osmotic balance (Bruins *et al.,* 2000; Vallee and Ulmer, 1972). Pb(II) also shows a stronger affinity for thiol and oxygen groups than essential metals such as calcium and zinc (Bruins *et al.,* 2000). Despite the high toxicity of Pb, many micro-organisms have evolved mechanisms that enable them to survive Pb exposure (Figure 2.9).

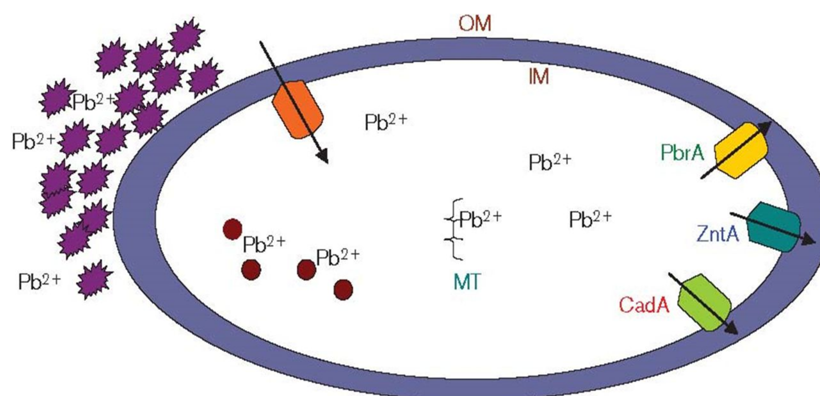


Figure 2.6 Selected mechanisms of cell protection against lead toxicity (based on Dopson *et al.,* 2003, modified).

One of the mechanisms that micro-organisms use to avoid the toxicity of metals of no biological function is to limit their movement across the cell envelope (Bruins *et al.,* 2000). Early studies initiated on the interactions between Pb(II) and the cell envelope in *Micrococcus luteus* and *Azotobacter* sp. revealed that these ions are mostly present in the cell wall and cell membrane, with a minor portion present in the cytoplasmic fraction (Tornabene and Edwards, 1972; Tornabene and Peterson, 1975). The cell wall is a natural barrier for Pb(II), since the functional groups of several macromolecules are involved in binding this metal. In Gram-negative bacteria, this role is played mainly by lipopolysaccharide, a significant component of the outer membrane. In Gram-positive bacteria, peptidoglycan together with teichoic and teichuronic acids are responsible for Pb binding (Figure 2.6) (Beveridge and Fyfe, 1985). This protection mechanism is an uncontrolled process, and specific resistance mechanisms become operative when the cell envelope reaches its saturation point (Beveridge and Fyfe, 1985).

2.13.2 Microbial resistance to lead

Micro-organisms resistant to Pb(II) have been isolated from metal contaminated soils, industrial wastes, and from plants growing on metal contaminated soil. Among these isolates the following examples have been identified: the Gram-positive bacteria *Bacillus cereus*, *Arthrobacter* sp. and *Corynebacterium* sp.; the Gram-negative bacteria *Pseudomonas marginalis*, *Pseudomonas vesicularis* and *Enterobacter* sp.; and fungi *Saccharomyces cerevisiae* and *Penicillium* sp. (Chen and Wang, 2007; Hasnain *et al.,* 1993; Sun and Shao, 2007; Roane and Kellogg, 1996; Trajanovska *et al.,* 1997; Zanardini *et al.,* 1997). Some lead-resistant bacteria have been found to play a specific role in the growth of lead-exposed plants. For example, the endophyte *Bacillus* sp. MN3-4 increases Pb(II) accumulation in Alnus firma, and *Pseudomonas fluorescens* G10 and *Mycobacterium* sp. G16 promote plant growth and reduce Pb toxicity in Brassica napus (Sheng *et al.,* 2008; Shin *et al.,* 2012). Complexes of lead ion (Pb^{2+}) with

DL-cysteine are much more toxic to *Aspergillus niger* than are those of aspartate and citrate, whose accumulation in the media detoxifies Pb^{2+}.

Asparagine, cysteine, aspartate, and citrate form soluble complexes with Pb which remain soluble in the presence of phosphate. Lead ion (Pb^{2+}) stimulates growth of *Sarcinaflava*; this is accompanied by an increase in peptone degradation and in a number of biosynthetic reactions. Lead sulfide (PbS) appears as the final metabolites. Additionally, the activity of the lead-resistant bacteria *Streptomyces* sp. and *Ps. vesicularis* has been noticed on the marble facade of a cathedral, where it was manifested in the form of red stains. These bacteria were capable of producing a red and red–brown pigment, identified as the lead tetroxide, minium (red lead, Pb_3O_4) (Zanardini *et al.,* 1997).

2.14 Metal speciation in soil

Within the terrestrial ecosystem, soils play a major role in element cycling and accumulate heavy metals in concentration orders of magnitude higher than in water and air. Meanwhile, soils are the reservoir for many harmful constituents, elemental and biological, including heavy metals and trace metals, henceforth referred to as just metals (Cottenie and Verloo, 1984). Total metal content of soils is useful for many geochemical applications but often the speciation (bioavailability) of these metals is more of an interest agriculturally in terms of what is biologically extractable (Cottenie *et al.,* 1980).

Speciation of heavy metals in soils can be defined as the extraction and quantification of a soil phase which is functionally designated in that its element content is, for example, the plant-available content. Speciation is defined as the identification and quantification of the different, defined species, forms, or phases in which an element occurs (Tack and Verloo, 1995) and is essentially a function of the mineralogy and chemistry of the soil sample examined (Tessier, *et al.,* 1979).

Quantification is typically done using chemical solutions of varying but specific strengths and reactivity to release metals from the different fractions of the examined soil (Ryan *et al*., 2008). In terms of bioavailability, various species of metals are more biologically available in the ecosystem (Nelson and Donkin, 1985). Bioavailability and the mobility of metals are also related to each other, then higher the concentration of mobile toxic metals (Cu, Pb, Cd, and Al) in the soil column which increases the potential for plant uptake, and animal/human consumption (Lund, 1990; Tack and Verloo, 1995; Ratuzny *et al.,* 2009).

Heavy metals take part in biogeochemical cycles and are not permanently fixed in soils; therefore, assessment of their distribution in soils is a key issue in many environmental studies (Salim *et al.,* 1993). Heavy metals are included in soil minerals as well as bound to different phases of soil particles by a variety of mechanisms, mainly absorption, ion exchange, co-precipitation, and complexation. Moreover, soil properties such as contents of organic matter, carbonates, oxides as well as soil structure and profile development influence the heavy metal mobility (Kabata-Pendias and Pendias, 2001). The knowledge of the binding of metals with the different soil phases and components is of major interest to assess the connections with other biotic and abiotic elements of the environment (Hirner, 1992). Nevertheless, as Cabral and Lefebvre indicate, the metal speciation is a more complex task that determination of total metal contents (Cabral and Lefebvre, 1998).

It is widely recognized that to assess the environmental impact of soil pollution, the determination of the metal speciation will give more information about the potential for release of contaminants and further derived processes of migration and toxicity (Rauret *et al.,* 1988; Usero *et al*., 1998). Therefore, in geo-environmental studies of risk assessment, chemical partitioning among the various geochemical phases is more useful than measurements of total heavy metals contents (Campanella *et al*., 1995; Quevauviller *et al.,* 1996).

It has been demonstrated that metal speciation and solubility affect the mobility, bioavailability and toxicity of metals significantly (Griscom *et al.,* 2000; Yap *et al.,* 2002; Amiard *et al.,* 2007). Although the sequential extraction method elaborated by Tessier *et al.* (1979) and modified by other researchers (Ure *et al.,* 1993; Mossop and Davidson, 2003) has some shortcomings for identifying metal speciation (Martin *et al.,* 1987; Zhang *et al.,* 2001), it has been widely employed to study the geochemical occurrence of metals in suspended and deposited sediments (Pueyo *et al.,* 2001; Svete *et al.,* 2001; Martinez *et al.,* 2002; Peng *et al.,* 2005). To access the mobility and bioavailability of Cd, Cu and Pb, five-step sequential extraction method was performed to determine the geochemical distribution of Cd, Cu, Pb, Fe and Mn in sediments according to the modified Tessier method (Ure *et al.,* 1993).

2.14.1 Sequential extraction

Soil, which comprised of detritus, inorganic or organic particles, is relatively heterogeneous in terms of its physical, chemical and biological characteristics (Hakanson, 1992). Heavy metals are associated with various soil components in different ways, and these associations determine their mobility and availability (Ahumuda *et al*., 1999). Water soluble and exchangeable forms are considered readily mobile and available to plants, while metals incorporated into crystalline lattices of clays appear relatively inactive. The other forms like carbonate bound, occlusion in Fe, Mn and Al oxides, or complexes with organic matter and Fe–Mn oxides have been found to be the most important in soil and might be the components, which influence the medium to long term effect on lability and bioavailability of metals (Iyenger *et al*., 1981; Karczewska *et al*., 1998).

In recent years studies on the speciation or chemical forms of heavy metals in polluted soils using sequential techniques have increased, because these provide knowledge on metal affinity to soil components and the strength with which they

are bound to the matrix (Narwal *et al.*, 1999). Numerous extraction schemes have been described in the literature (Chao 1972; Tessier *et al.*, 1979; Sposito *et al.*, 1982; Welte *et al.*, 1983; Clevenger 1990; Ure *et al.*, 1993; Howard and Vandenbrink, 1999). The procedure of Tessier *et al.* (1979) is one of the most thoroughly researched and widely used procedures to evaluate the possible chemical associations of metals in sediments and soils. In this method, metal distribution is studied through five major geochemical forms: (i) exchangeable phase; (ii) bound to carbonate phase; (iii) bound to Fe–Mn oxides; (iv) bound to organic matter, and (v) residual metal phase.

2.14.2 Sequential extraction procedures

The theory behind sequential extraction procedure is that the most mobile metals are removed in the first fraction and continue in order of decreasing of mobility. All sequential extraction procedures facilitate fractionation. Tessier *et al*. (1979) named these fractions exchangeable, carbonate bound, Fe and Mn oxide bound, organic matter bound, and residual. These are also often referred to in the literature as exchangeable, weakly absorbed, hydrous-oxide bound, organic bound, and lattice material components, respectively (Maiz *et al.,* 2000). Typically metals of anthropogenic inputs tend to reside in the first four fractions and metals found in the residual fraction are of natural occurrence in the parent rock (Ratuzny *et al.,* 2009).

The exchangeable fraction is removed by changing the ionic composition of water allowing metals sorbed to the exposed surfaces of sediment to be removed easily. A salt solution is commonly used to remove the exchangeable fraction. The carbonate-bound fraction is susceptible to changes in pH; an acid solution is used second. Metals bound to Fe and Mn oxides are particularly susceptible to anoxic (reducing) conditions so a solution capable of dissolving insoluble sulfide salts is used third. To remove metals bound in the organic phase, the organic material must be oxidized. The residual fraction consists of metals incorporated into the

crystal structures of primary and secondary minerals. This fraction is the hardest to remove and requires the use of strong acids to break down silicate structures (Tessier *et al.,* 1979).

Most sequential extraction procedures follow similar fractional degradation with little variation. Ure *et al.* (1993) extracted the exchangeable and carbonate-bound fractions in a single step versus the two steps used in the Tessier procedure. The sequential extraction procedure used by the Geological Survey of Canada (GSC) divides the Fe and Mn oxide fractions into the amorphous oxyhydroxides and crystalline oxides, thereby increasing sequential fractionation from five to six steps (Hall *et al.,* 1996). Other sequential extraction procedures with greater fractions include the procedure developed by Zeien and Brummer (1989) which included EDTA extractable, moderately reducible, and strongly reducible fractions for a total of seven; and that by Miller *et al.* (1986) which consisted of nine fractions designed to test waste amended and agriculturally polluted sediments.

The information needed from the sequential extraction procedures determines, to some extent, how the extraction is performed with respect to the final fraction, the residual. From a geochemical standpoint, total metal concentration is desired requiring the use of often dangerous reagents. From a biological or agricultural standpoint, less dangerous reagents may be utilized in lieu. The extraction conditions and reagents are listed in Table (2.12, 2.13, 2.14, 2.15, and 2.16) for the five discussed sequential extraction procedures.

2.14.2.1 Tessier procedure

In the extraction procedure by Tessier *et al.* (1979), 1 g of sample is placed in a 50mL tube. The sample is exposed to reagents and shaken (Table 2.12). Each fraction is separated from the supernatant by centrifugation at 10,000 rpm, (~12,000 gravity) for 30 min.

Table 2.12 Sequential extraction procedures by Tessier *et al.* (1979).

	Time	Temperature	Quantity	Tessier
				1 g
Exchangeable	1 hr	continuous agitation	8 mL	1mol$MgCl_2$ pH 7.0
			8 mL	or 1mol NaOAc pH 8.2
Bound to Carbonates	5 hr	Continuous agitation-leached at rm temp.	8 mL	1mol NaOAc pH 5.0 w/acetic acid
Bound to Iron and Manganese Oxides	6 hr		20 mL	0.3mol $Na_2S_2O_4$+ 0.175 mol Na-citrate + 0.025 mol H-citrate
		or $96^0 \pm 3$ occasional agitation	20 mL	0.04 mol $NH_2OH*HCl$ in 25% (v/v) HOAc
Bound to Organic Mater	1 hr	$85^0 \pm 2$ with occasional agitation	3 mL	0.02mol HNO_3
			5 mL	30% H_2O_2 pH 2 with HNO_3
	1 hr	$85^0 \pm 2$ with intermittent agitation	3 mL	30% H_2O_2 pH 2 with HNO_3
	30 min	continuous agitation	5 mL	3.2mol NH_4OAc in 20% (v/v) HNO_3-dilute to 20mL
Residual			1mL Unk	HF-$HClO_4$ 5 : 1 HF-$HClO_4$ 10 :1 $HClO_4$ 12N HCl

2.14.2.2 Community bureau of reference (BCR) procedure

This procedure is largely similar to that produced by Tessier *et al.* (1979) with the chief difference in the first fraction of the procedure. Instead of evaluating the exchangeable and carbonate bound separately, the community bureau of reference procedure combines both in the first fraction (Ure *et al.,* 1993). In the community bureau of reference procedure, 1 g of sample is placed into a 100mL tube, exposed to reagents and shaken (Table 2.13). After each fraction, the solution is centrifuged at ~5000 rpm (3000 gravity) for 20min and the supernatant is collected.

Table 2.13 Sequential extraction of the community bureau of reference procedure (BCR)

	Time	Temperature	Quantity	BCR
				1 g
Exchangeable	16 hr	22∘C ± 5∘ w/constant agitation	40 mL	0.11 mol CH_3COOH
Bound to Iron and Manganese Oxides	16 hr	22∘C + 5∘ w/constant agitation	40 mL	0.1mol $NH_2OH*HCl$ pH 2 with HNO_3
Bound to Organic Mater	1hr	Room temp. w/occ. Agitation	10 mL	8.8mol H_2O_2 pH 2-3
	1hr 1hr	85∘ degrees C	10 mL	reduce vol. to less than 3mL H_2O_2 pH 2-3 reduce vol. to 1mL
	16 hr	22∘C+5∘ w/constant agitation	50 mL	1mol NH_4OAc pH 2 w/HNO_3
Residual				HF, HNO_3, $HClO_4$

2.14.2.3 Short extraction procedure by Maiz *et al.* (2000).

This procedure is conducted a comparison between the Short and Tessier procedures and found that the Short procedure produced strong correlation data for many metals tested. Three grams of residue are placed in a 50mL tube, exposed to reagents and shaken (Table 2.14). After the first extraction, the solution is centrifuged at 3000 rpm (~1000 gravity) for 10min, the supernatant removed, and analyzed.

Table 2.14 Short sequential extraction procedures by Maiz *et al*., (2000).

	Time	Temperature	Quantity	Maiz-Short
				3 g
Exchangeable	2 hr	rm temp. suspend under agitation	10mL	0.01 mol CaCl2
Bound to Carbonates	4 hr			0.005 mol DTPA +
Bound to Iron and Manganese Oxides		rm temp.	2mL	0.01mol CaCl2 + 0.1mol TEA pH7.3
Bound to Organic Mater				
Residual				aqua regia-HF acid

2.14.2.4 Gal´An procedure

This procedure is also similar in structure to the Tessier and the community bureau of reference procedures. However, this procedure was used in extracting metals from soils severely affected by acid mine drainage in Spain such as those seen along the Rio Tinto (Gal´an *et al.,* 1999). Initial use of the Gal´an *et al.* (1999) method showed increased accuracy of metals extracted in these soils than the Tessier and the community bureau of reference methods. One-half a gram of soil sample is placed into tubes and exposed to reagents (Table 2.15).

Table 2.15 Sequential extraction procedures by Gal'an.

	Time	Temperature	Quantity	Gal'an
				0.5 g
Exchangeable	1hr	20°C w/continuous agitation	35mL	1M NH_4OAc, 2 hr pH 5
Bound to Carbonates				
Bound to Iron and Manganese Oxides	6 hr	96°C manual agitation every 30 min	20mL	0.4 M $NH_2OH*HCl$ in CH_3COOH 25%
	2 hr	85°C w/manual agitation every 30 min	3mL	0.2M HNO_3
			5mL	30% H_2O_2, pH 2
Bound to Organic Mater	3 hr	Continuous agitation	3mL	30% H_2O_2
	30 min		5mL	30% H_2O_2
Residual	2 hr		10mL	HF,HNO_3,HCl 10 : 3 : 1

2.14.2.5 Geological Society of Canada (GCS) procedure

One gram of sample is placed in a 50mL tube and exposed to reagents and shaken (Table 2.16). In between each fraction samples are centrifuged for 10 min at ~1000 g (2800 rpm). The supernatant is collected and the samples are washed in 5mL of water, centrifuged, adding the wash water to the previous supernatant. Repeat the washing procedure. Prior to performing the fourth fraction (Table

2.16). The amount of time needed to complete the fraction is proportional to the time for the reduction of sample to an appropriate volume. The modified geological society of Canada procedure (GCS) is the most modified of the sequential extraction procedures. That experiment was later adapted by Doelsch *et al.* (2008) into the modified geological society of Canada procedure (GCS).

Table 2.16 Sequential extraction procedures by Canada.

			Time	Temperature	Quantity	Canada
						1 g
Exchangeable	mobile	AEC	6 hr		20mL	1.0mol CH_3CO_2Na pH 5
			6 hr		20mL	1.0 mol CH_3CO_2Na pH 5
Bound to Carbonates		Am Fe ox	2 hr	60°C vortex every 30min	20mL	20mL 0.25 mol NH_2OH*HCl in 0.05 mol HCl
Bound to Iron and Manganese Oxides	mobilisible	Cry Fe ox	30 min	60°C	20mL	20mL 0.25 mol NH_2OH*HCl in 0.05 mol HCl
			3hr	90°C vortex every 20min	30mL	1.0mol NH_2OH*HCl in 25% CH_3CO_2H
Bound to Organic Matter		Org/Sulf	1.5 hr 90°	90°C	30mL	1.0mol NH_2OH*HCl in 25% CH_3CO_2H
			30 min			750 mg $KClO_3$ and 5mL 12 mol HCl vortex and add 10mL HCl more 15mL H_2O
			20 min	90°C	10mL	4mol HNO_3
Residual		silicates and residual	Unk	200°C	2mL	16 mol HNO_3 ~reduce to 0.5mL
			20 min	90°C	2mL	12 mol HCl
			1hr	90°C	10mL	acid mix H
			overnight	Evap 70°C		
			last bit	Rai 120°C		
			5–10 min		1mL	12molHCl
					3mL	16mol HNO_3
					3mL	3 mL H_2O and warm then bring up to 20mL

2.15 Future Prospectus of Researchers on Phosphate-Solubilizing bacteria

Phosphate-solubilizing bacteria's are an integral component of soil microbial community and play an important role in P cycle in soil rendering the unavailable P to plants. These have enormous potential for making use of fixed P in the soil particularly in soils with low P availability. However, despite considerable promise microbial products for P mobilization have not had major application to broad-acre farming systems. Because phosphate solubilization by bacteria is a complex phenomenon affected by many factors, such as phosphate-solubilizing bacteria used, nutritional status of soil and environmental factors. The success of bacterization program ultimately depends on aspects such as cost effective ratio, widespread applicability of specific strain, development of practical delivery systems and sustained positive results. Although potential clearly exists for developing such inoculants, but their widespread application remains limited by a poor understanding of microbial ecology and population dynamics in soil, and by inconsistent performance over a range of environments. Hence the formulation of phosphate-solubilizing bacteria inoculation with a reliable and consistent effect, under field conditions is still a bottleneck for their wider use.

Therefore, more research is needed to explore the impact of phosphate-solubilizing bacteria in affecting the various physiological, biochemical and molecular events governing the stimulation of growth by these microbes in the plants. Hence, it needs further studies to understand the characteristics and mechanisms of phosphate solubilization by phosphate-solubilizing bacteria. To conclude, the efforts should be made to identify, screen and characterize more phosphate-solubilizing bacteria for their ultimate application under field conditions. So that, the successful implementation of phosphate-solubilizing bacteria to better exploit soil P resources can be an alternative sustainable strategy for management of soil to optimize P bioavailability. The use of phosphate-solubilizing bacteria has considerable promise for the future as a best management practice (BMP) for soil to optimize P fertilization to meet the

demands of crop production with minimal soil impacts. The use of phosphate-solubilizing bacteria as an inoculants becomes important to the sustainable management of soil and likewise contributes to environmental and economic stability (Dhankhar *et al.,* 2013).

3. MATERIALS AND METHODS

The most important role of soil microorganism is their function as biogeochemical agents for the mineralization of organic phosphorus, nitrogen, carbon and other compounds (Pelczar *et al.*, 1977). The investigators also showed the metabolic activity of microorganisms (production of acids) solubilizes phosphates from insoluble Ca-, Fe- and Al-phosphates. Soil contains larger numbers of phosphate-dissolving microorganisms and the population is especially abundant in the rhizosphere region (Alexander, 1961).

The experiments of this study includes isolation and identification of some soil bacteria from metal contaminated sites and agricultural field followed by phenotypic and genotypic characterization and determination of minimum inhibitory concentration (MIC) and maximum tolerable concentration (MTC) of heavy metal in response to identified soil bacteria.

The present investigation was carried out in the Department of Soil, Water and Environment and Centre for Advanced Research in Sciences (CARS), University of Dhaka in order to investigate the metal toxicity tolerance of some bacteria present in agricultural and metal contaminated soils.

All media compositions, chemicals, and reagents used in this study are presented in the appendices (Appendix A and B). Aseptic condition in every work was maintained wherever it was necessary. Sterilization of media and all necessary equipment were done by autoclaving (CL–32L) at 121°C temperature under 15 lbs per square inch pressure for 20 minutes in autoclave machine. Laminar flow cabinet was used to prevent contamination of biological samples.

The materials used and methods followed in these experiments are described in the following sub-sections.

3.1 Sampling Sites

Soils of agricultural lands beside Bongshi River at Dhamrai and Savar EPZ (Export Processing Zone) area were collected for isolation of bacteria. Agricultural land of Dhamrai and Pabna area, cultivated with different types of vegetables were selected for collection of soils for incubation studies and isolation of bacteria.

3.2 Collection of Soil Samples

Surface soil samples (0-15 cm depth) were collected from the selected sites. During sample collection, plastic bags, disposal gloves, alcohol (70%), marker, and field notebook were taken to the sampling sites. Samples were collected in plastic bags aseptically. After collection, the samples were labeled. Samples were well sealed to prevent them from drying and to maintain proper moisture status at field moisture condition and transferred to the laboratory as soon as possible.

3.3 Preservation of the Samples

Collected samples for microbiological analysis were kept in cool and dark place before and after the analysis so that the sample does not deteriorate and the analytical results are representative.

3.4 Preparation of Soil Sample for Analysis

The soil samples were air dried, crushed and screened through 2 mm sieve and then preserved in plastic bottle for physical and chemical analysis.

3.5 Measurement of Physical and Chemical Parameters of the Samples

3.5.1 Soil pH

The pH of field moist soil was determined electrochemically by using a glass electrode pH meter (Jenway 3305). The soil to water ratio was 1:2.5 and time of shaking was 30 min as outlined by Jackson (1973).

3.5.2 Measurement of Soil Moisture

Soil moisture was determined by heating weighed 2 g soil sample in an oven at 105^0C for 24 hours. The result was expressed as a percentage of oven dry weight (Gupta, 2000).

3.5.3 Mechanical Analysis

The particle size analysis of soil was done by hydrometer method as described by Gee and Bauder (1986). Textural class was determined by Marshall's triangular coordinate.

3.5.4 Cation Exchange Capacity

Cation Exchange Capacity (CEC) was determined by using neutral 1 N NH_4OAc extractant as described by Schollenbergen and Simon (1945).

3.5.5 Soil Organic Carbon

Soil organic carbon was determined by Walkley and Black's (1934) wet oxidation method as outlined by Jackson (1973). Soil organic matter was calculated by multiplying the value of organic carbon with conversion factor 1.724 (van Bemmelon).

3.5.6 Total Nitrogen

Total soil nitrogen was determined by micro Kjeldahl's method as described by Jackson (1973).

3.5.7 Nitric-Perchloric Acid Digestible Nutrients

Soil samples were digested with HNO_3-$HClO_4$ (2:1) mixture as described by Jackson (1973) for the determination of total P, K and S. Total soil P was determined by vanadomolybdate yellow colour method (Jackson, 1973). Total soil K was measured by Flame photometer (JENWAY PFP 7). Total soil S was measured by turbidimetric method (Hunt, 1980).

3.5.8 Determination of Lead and Cadmium of Soil

Content of lead and cadmium were determined directly by Atomic Absorption Spectrophotometer (Varian AA240) after digestion of soil with HNO_3 acid (Jackson, 1967).

3.6 Media and Techniques for Isolation and Purification of Bacteria

3.6.1 Culture Media Used

A. Nutrient agar (NA) (Appendix A5) and Tryptic Soya Agar (TSA) media (Appendix A6) were used for short term preservation of bacteria.

B. Nutrient broth (NB) media (Appendix A7) was used for subculture preparation

C. NaCl Glycine Kim Goepfert (NGKG) agar with 20% egg yolk (Appendix A15) was used for the isolation of *Bacillus* sp.

D. Potato Dextrose Agar (PDA) media (Appendix A16) was used for the isolation of Fungi.

E. MacConkey's Agar (MAC) (Appendix A17) is a differentially-selective media, was used to isolate and differentiate members of the *Enterobacteriaceae*.

F. Mannitol salt agar (Appendix A18), Cetrimide agar (Appendix A19) was used to isolate *Staphyloccocus* spp. and *Pseudomonas* spp. respectively.

G. Eosin Methylene Blue (EMB) agar(Appendix A20) was used for the isolation of gram-negative bacteria such as *E. coli* and *Serratia* spp.

H. Pikovskayas agar (PVK) (Appendix A21) (Pikovskaya, 1948) and National Botanical Research Institute's phosphate growth medium (NBRIP) (Appendix A22) (Nautiyal, 1999) was used for the detection of phosphate-solubilizing microorganism and used for heavy metal treatment comparison, respectively.

3.6.2 Techniques Employed

Soil samples were diluted by serial dilution technique followed by inoculation using pour plate, spread plate, and streak plate techniques.

3.6.2.1 Dilution plate technique

For the isolation of bacteria serial dilution plate technique was carried out (Greenberg *et al*., 1980).

3.6.2.2 Pour plate technique

For the isolation of bacteria pour plate technique was carried out (Greenberg *et al*., 1980).

3.6.2.3 Spread plate technique

Spread plate technique was also used for the isolation and enumeration of bacteria (Greenberg *et al*., 1980).

3.6.2.4 Streak plate technique

For the preparation of pure culture of isolated bacteria streak plate technique was used (Greenberg *et al*., 1980).

3.7 Isolation of Different Organisms

3.7.1 *Bacillus* spp., *Staphylococcus* spp. and *Pseudomonas* spp.

To isolates *Bacillus*, *Staphylococcus* and *Pseudomonas*, isolates were streaked on NaCl Glycine Kim Goepfert (NGKG) agar (Nissui Co., Ltd., Tokyo, Japan) with 20% egg yolk, mannitol salt agar (Nissui Co., Ltd., Tokyo, Japan) and Cetrimide agar (Oxoid Ltd., Hampshire, England) and incubated at 30^0C for 24 hours.

For *Bacillus* spp., typical colonies from NGKG plates, for *Staphylococcus* spp., typical yellow colonies from MSA plates and for *Pseudomonas* spp., typical colonies from Cetrimide agar plates were then grown on Tryptic Soy Agar (Oxoid Ltd., Hampshire, England), Gram stained and then were characterized biochemically by testing for starch utilization by using starch agar plates, indole production using peptone water, nitrate reduction using nitrate broth and catalase test.

The biochemically confirmed isolates were further characterized by using API 50CHB (BioMérieux, Marcy I'Etoile, France) along with API 20E kits (BioMérieux, Marcy I'Etoile, France) for *Bacillus* spp., API Staph kits (BioMérieux, Marcy I'Etoile, France) for *Staphylococcus* spp. and API 20NE (BioMérieux, Marcy I'Etoile, France) diagnostic kits for *Pseudomonas* spp.

3.7.2 *Proteus* spp. and *Serratia* spp.

To isolates *Proteus* spp. and *Serratia* spp., isolates were streaked on MacConkey's agar (Nissui Co., Ltd., Tokyo, Japan) and Eosin Methylene Blue (EMB) agar (Nissui Co., Ltd., Tokyo, Japan), respectively, and incubated at 30^0C for 48 hours.

For *Proteus* spp. and *Serratia* spp. typical colonies from MacConkey's and EMB plates were then grown on Tryptic Soy Agar (Oxoid Ltd., Hampshire, England), Gram stained and then were characterized biochemically by testing for starch utilization by using starch agar plates, indole production using peptone water, nitrate reduction using nitrate broth and catalase test.

The biochemically confirmed isolates were further characterized by using API 20E kits (BioMérieux, Marcy I'Etoile, France) for *Proteus* spp. and *Serratia* spp. respectively.

3.7.3 *Fungal* spp.

To isolates *Fungal* spp. isolates were grown on Potato Dextrose Agar (PDA) medium and incubated at 30^0C for 5 days.

3.8 Isolation of Colonies

Isolation of selected well discrete aerobic heterotrophic bacterial colonies was carried out on the basis of their colony morphology. The selected colonies were marked and studied for various characters *viz*. color, form, elevation, margin, surface, opacity etc. (Eklund and Lankford, 1967). Then the marked and observed bacterial colonies were transferred on other selective agar plate for further study.

3.9 Isolation of Phosphate Solubilizing Microorganisms

Phosphate-solubilizing bacteria are routinely screened by a plate assay method using PVK agar and NBRIP agar media. The test of the relative efficiency of isolated strains is carried out by selecting the microorganisms that are capable of producing a halo/clear zone on a plate owing to the production of organic acids into the surrounding medium (Katznelson *et al*., 1962). However, many isolates that did not produce any visible halo/zone on agar plates could solubilize various types of insoluble inorganic phosphates in liquid medium (Louw and Webley,

1959; Gupta *et al.*, 1994). Phosphate solubilizing microorganisms are bacteria, mobilizing inorganic P through production of organic acids (Whitelaw, 2000).

All the isolates might not be phosphate dissolving micro-organisms (Sperber, 1958; Taha *et al.*, 1969). So, preliminary selection of the isolates was considered necessary. Preliminary selection for phosphate dissolving bacteria was done by growing the isolate on PVK and NBRIP agar medium. The plates were inoculated using point inoculation and incubated at 30^0 C for 2-3 days and 5 days for bacteria and fungi, respectively (Vikram, 2007). On the presence of growth, the colonies of the organism are an indication of the presence of phosphate-solubilizing organisms. Such cultures were isolated and identified by biochemical methods (Subba, 1993; Tilak, 1993). The biochemically confirmed isolates were further characterized by using API kits.

3.10 Purification of Isolates

The selected isolates were purified through repeated plating (by streak plate method). When a plate yielded only one type of colony the organism was considered as pure.

3.11 Maintenance and Preservation of Isolates

The purified isolates were then transferred on nutrient agar slant. The slants were kept in a refrigerator at 4ºC for further study. Periodic transfer of isolates on agar slants was done for maintaining viability of the organisms after each week (Cappuccino and Sherman, 2005).

3.12 Morphological Observation of Isolated Strains

For the identification of selected isolated strains, following morphological characters were studied and recorded according to Cappuccino and Sherman (2005).

3.12.1 Colonial Morphology

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

3.12.2 Growth on Agar Slants

The selected bacteria were transferred to nutrient agar (NA) slants. The appearances of their growth on slants such as filiform, echinulate, beaded, effuse, arborescence and rhizoidal, spreading, adherent or slimy etc. were studied.

3.12.3 Growth in Broth Media

The isolated bacteria were grown in nutrient broth, PVK broth and NBRIP broth media and the different appearance of growth were observed *viz*. uniform fine turbidity, flocculant, pellicle, sediment and membranous etc.

3.12.4 Motility Test

Motility test was carried out by the method of Semi-solid Medium (Appendix A6) (Eklund and Lankford, 1967).

3.13 Preparation of Bacterial Cells for Microscopic Examination

Bacterial cell suspension was made by using fresh culture with physiological saline water (0.85%). 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 free slide. The smear was allowed to dry in air and was fixed by passing the slide over the flame of Bunsen burner. The following two different staining methods (Cappuccino and Sherman, 2005) were employed to stain the fixed smears.

1. Simple staining method, and
2. Differential staining methods.

3.13.1 Simple Staining

To identify the shape and arrangement of cells of bacteria Crystal violet (Appendix B) was used as simple staining reagent. Each fixed smear was flooded with dye solution for one minute. The flooded smear was washed off with water and dried in air and observed under microscope.

3.13.2 Differential Staining

Staining procedure that makes visible differences between microbial cells or parts of the cells, are termed as differential staining (Pelczar *et al*., 1993). For this purpose fixed smear was exposed to more than one dye solution. In this study a differential technique such as Gram staining was done.

3.13.2.1 Gram staining

For Gram staining, the procedure as described by Claus (1995) was followed. The fixed smear was treated with the following solutions and after application of each solution slide was gently washed off with water.

Crystal violet for 30 sec., Lugol's iodine solution for 60 sec., 95% Ethyl alcohol for 15-25 sec., and safranine solution for 60 sec. were used. The slide was blot dried and observed under microscope. The results were recorded as Gram positive (blue-violet) and Gram negative (light red).

3.14 Microscopic Observation of Bacteria

The shape of vegetative cells of selected strains was observed under microscope. The arrangement of cells whether single or in chains or clusters were carefully recorded.

3.15 Biochemical Test of Isolates

Following Bergey's Manual (Sneath *et al*., 1986), the physiological and biochemical tests of the isolated bacteria were carried out. Along with Bergey's Manual several other manuals such as Manuals of Microbiological Methods (SAB, 1957), Microbiology Laboratory Mannual (Cappuccino and Sherman, 2005), Microbiological Methods (Collins and Lyne, 1984) and Understanding Microbes (Claus, 1995) were also consulted.

3.15.1 Catalase Test

To demonstrate catalase activity, test organisms were taken by a sterilized loop on microscope slide and a drop of hydrogen peroxide was added to each of them. Production of bubbles indicated the positive result i.e. production of catalase (Claus, 1995).

3.15.2 Acid and Gas Production from D-Glucose

The selected strains were tested for their ability to produce acid from carbon source. Acid production from carbohydrate was determined by the change of the blue color to yellow of the indicator in the tube and the production of carbon dioxide was indicated as a gas bubble in the Durham's tube (Gordon, 1966).

3.15.3 Deep Glucose Agar Test

Microorganisms vary widely in their requirements for oxygen. In relation to free oxygen, organisms are generally classified as strict aerobes, microaerophiles, facultative anaerobes and strict anaerobes. Deep glucose agar medium (Appendix) was used in this test followed by SAB (1957).

3.15.4 Liquefaction of Gelatine

For this test, nutrient gelatin medium was used followed by Cowan, (1974).

3.15.5 Hydrolysis of Starch

For this test, starch-agar plates were inoculated with test organisms and the plates were incubated at 37ºC for 48 h. After incubation, the surface of these plates was flooded with iodine solution. Development of a clear zone around the growth indicated starch hydrolysis (Claus, 1995).

3.15.6 Production of Indole

In this method 1% tryptone broth medium and the test reagent (Kovac's reagent) were used (Sneath *et al.,* 1986).

3.15.7 Methyle Red Test

For this test VP/MR broth (Appendix) was used (Bryan, 1950).

3.15.8 Voges Proskaur Test

For this test Voges proskaur broth was used. Development of crimson to ruby red color indicates a positive reaction (Bryan, 1950).

3.15.9 Utilization of Citrate

For this test, tubes of citrate (Simmon's citrate agar) medium were used. The positive test was indicated by changing the colour from green to blue (Claus, 1995).

3.15.10 Oxidase Test

For oxidase test, 1% aquesous tetramethyl-phynylenediamine dihydrochloride solution was used. Blue color indicated a positive result (Claus, 1995).

3.15.11 Identification of the Isolates

Following Bergey's Manual of Systematic Bacteriology Vol. 2 (Sneath *et al.,* 1986) Gram positive aerobic heterotrophic bacteria were identified and following Vol. 1 of Bergey's Manual of Systematic Bacteriology (Kreig and Holt, 1984) Gram negative aerobic bacteria were identified.

3.16 Analytical Profile Index (API)

Analytical Profile Index is a quite easy system to identify microorganisms. The API's comprise plastic strips that generally contain many miniature tubes. The number of tubes varies according to different API kits. The plastic strip is inoculated with an isolated pure culture of microorganism suspension. The results are read after incubation (24 hours or more- depending on which API test is used) in a humidity chamber. The colour reactions are read (some of the tubes will have colour changes due to pH differences and some with the aid of added reagent to detect end metabolic products) and the reactions (oxidase reaction done separately) are generally converted to a seven-digit code. The code is input into the manufacturer's database, which gives the identification, usually as genus and species of microorganisms.

3.16.1 API 20 E

The API 20E system facilitates the 24-hour identification of *Enterobacteriaceae* as well as 24 or 48-hour identification of other Gram negative bacteria. The API 20E strip consists of micro tubes containing dehydrated substrates for the demonstration of enzymatic activity and carbohydrate (CH_2O) fermentation. The substrates are reconstituted by adding a bacterial suspension. After incubation, the metabolic end products are detected by indicator systems or the addition of reagents. CH_2O fermentation is detected by colour change in the pH indicator (Smith, 1972).

3.16.2 API 50 CHB

The API 50CHB/E Medium was used for the identification of Bacillus and related genera, as well as Gram-negative rods belonging to the *Enterobacteriaceae* and *Vibrionaceae* families. It is a ready-to-use medium which allows the fermentation of the 49 carbohydrates on the API 50CH strip to be studied. The API 20E strip may be used in association with the API 50CH strip to provide supplementary tests (optional for Bacillus and related genera but essential for *Enterobacteriaceae* and *Vibrionaceae*) (Logan and Berkeley, 1984).

For the API 20 E strip: The reagents were added just before the last reading. The results of the first 11 tests and the NIT reaction in the GLU test (by adding 1 drop of reagent NIT1 and NIT2) were recorded for final interpretation.

3.16.3 API 20 NE

The API 20NE is a standardized system for the identification of non-fastidious, non-enteric Gram-negative rods (e.g. *Pseudomonas, Acinetobacter, Flavobacterium*, *Moraxella, Vibrio, Aeromonas*, etc.), combining 8 conventional tests, 12 assimilation tests and a database. The API 20NE strip consists of 20 micro tubes containing dehydrated substrates. The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software (Dance *et al*, 1989).

NO_3 test: 1 drop of NIT 1 and 1 drop of NIT 2 reagents were added to the NO_3 cupule. After 5 minutes, a red color would indicate a positive reaction. For negative reaction (may be due to the production of nitrogen indicated by the presence of tiny bubbles): 2-3 mg of Zn reagent was added to the NO_3cupule. After 5 minutes, a cupule remaining colorless indicated a positive reaction. If the cupule turns pink-red, the reaction was negative as nitrates were present in the tube and were reduced to nitrite by the zinc.

TRP test: 1 drop of JAMES reagent was added. The reaction takes place immediately: a pink colour which developed in the whole cupule indicated a positive reaction

Assimilation tests: The bacterial growth was observed. An opaque cupule indicated a positive reaction.

3.16.4 API Staph

The API staph is a standardized system for identifying genera *Staphylococcus, Micrococcus* and *Kocuria* including miniaturized biochemical tests and a database. The API staph strip consists of 20 micro tubes containing dehydrated substrates. The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software (Gemmell and Dawson, 1982).

3.16.5 API 20 Strep

The API 20 Strep is a standardized method combining 20 biochemical tests that offer widespread capabilities. It enables group or species identification of most streptococci encountered in medical bacteriology (Appelbaum *et al.,* 1984).

3.17 Determination of Efficiency of Isolates in Dissolving Insoluble Phosphate in Liquid Culture

Pikovskaya and NBRIP broth medium, before autoclaving the pH was adjusted to 7. 20 mL of the medium was distributed in each of the 50 ml test tube and tubes containing the medium were sterilized at 121^0 C under 15 lbs pressure per square inch for 15 minutes. Then the media were inoculated with the organisms. Each flask was inoculated by only one organism and incubated at 30^0C for 3, 7, 11 and

15 days. They were tested for their change in pH and the amount of phosphate made soluble by the organisms was determined after 3, 5, 7, 11 and 15 day from the date of incubation. Soluble phosphate in the medium was determined colorimetrically by Ascorbic acid blue colour method as out lined by Murphy and Riley (1962).

3.18 Determination of Efficiency of Isolates in Mineralizing of Organic Phosphate in Liquid Medium

In modified PVK, NBRIP broth medium for this research, rock phosphate (RP) used in the previous experiment was replaced either by compost or oil cake (as organic source of phosphorus) equivalent to 5 g RP and the pH was adjusted to 7 before autoclaving. Twenty mL of the medium was distributed in each of the 50 mL test tube and tubes containing the medium were sterilized at 121^0 C under 15 lbs pressure per square inch for 15 minutes. Then the media were inoculated with the organisms. Each flask was inoculated by only one organism and incubated at 30^0C for 3, 7, 11 and 15 days. They were tested for their change in pH and the amount of phosphate made soluble by the organisms. Soluble phosphate in the medium was determined colorimetrically by Ascorbic acid blue colour method as out lined by Murphy and Riley (1962).

3.19 Screening of Lead and Cadmium Tolerant Bacteria

Lead and Cadmium tolerant bacterial isolates were selected by using Pb and Cd containing growth medium for the isolates. These isolates were further used for the application of metal treatment to determine Minimum Inhibitory Concentration (MIC) and Maximum Tolerable Concentration (MTC).

3.19.1 Isolation of Lead Tolerant Bacteria

One gram of each soil sample was suspended in 99 ml of sterile sodium chloride solution 0.85% (w/v) and mixed thoroughly. The mixture was serially diluted with sterile sodium chloride solution (0.85%). Aliquots of 1 ml from each dilutions were spreaded onto Pikovskayas (PVK) agar (Pikovskayas, 1948), National Botanical Research Institute's phosphate growth medium (NBRIP) (Natiyal, 1999) and Nutrient Agar (NA) plates with 0, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450 and 500 μgmL^{-1} of Pb. The pH of the PVK agar and NBRIP agar were adjusted to 7 before autoclaving. Bacteria, resistant/tolerant to Pb, were evaluated by growing on these agar medium without and with added lead.

All plates were incubated at 37°C for 48 h according to Chowdhury *et al.* (2008). Isolated colonies grown in plates containing high concentrations of Pb were picked up and were streaked onto PVK agar, NBRIP agar and nutrient agar plates of media plus Pb concentrations of 0, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450 and 500 μgmL^{-1}. The plates were again incubated at 37°C for 3-5 days to confirm their abilities to grow at high concentrations of Pb containing media.

3.19.2 Isolation of Cadmium Tolerant Bacteria

To isolate Cd tolerant strains same procedure was followed as described in section 3.18.1. Soil aliquots of 1 ml from each dilutions were spreaded onto PVK agar, NBRIP agar and nutrient agar plates with 0, 30, 50, 75,100, 150 and 200 μgmL^{-1} of Cd. All plates were incubated at 37°C for 24 h according to Chowdhury *et al.* (2008), and isolated colonies were picked and streaked onto plates of PVK agar, NBRIP agar and nutrient agar media plus Cd concentrations of 0, 30, 50, 75, 100, 150 and 200 μgmL^{-1} again. The plates were again incubated at 37°C for 3 days to confirm their abilities to grow at high concentrations of Cd containing media.

3.20 Determination of Bacterial Response to Metal Tolerance

The MIC (Minimum inhibitory concentration) was defined as the minimum concentration of the metal, which inhibits the bacterial growth in the agar plate or broth containing tube after overnight incubation (Roane and Kellogg, 1996). Another related term MTC (Maximum tolerable concentration) is defined as the maximum tolerable concentration of heavy metal to which bacteria show highest tolerance and after which growth is totally being inhibited (Affan *et al*., 2009).

Analytical grades of metal salts of $Pb(NO_3)_2$ and $Cd(NO_3)_2$ were used to prepare stock solutions of Pb and Cd, respectively. Each stock solution was filter-sterilized and added to growth media *viz*. nutrient broth, PVK broth and NBRIP broth at varying concentrations of metals to determine the MIC and MTC (Mergeay *et al.,* 1985) at each medium. To determine MIC and MTC for each isolates concentrations of 10, 20, 30, 50, 75, 100, 150, 200, 250, 300 and 350 μgmL^{-1} of Cd were used in NB medium; concentrations of 30, 50, 75, 100, 120, 150, 200, 250, 300 and 350 μgmL^{-1} of Cd^{2+} were used in PVK broth medium and concentrations of 30, 50, 75, 100, 120, 150, 200, 250, 300 and 350 μgmL^{-1} of Cd were used in NBRIP media. Similarly concentrations of 10, 20, 30, 50, 75, 100, 150, 200, 250, 300 and 350 μgmL^{-1} of Pb were used in NB medium to determine MICs and MTCs for each isolate. Concentrations of 10, 20, 30, 50, 75, 100, 120, 150, 200, 250, 300 and 350 μgmL^{-1} of Pb were used in PVK broth medium and concentrations of 10, 20, 30, 50, 75, 100, 120, 150, 200, 250, 300 and 350 μgmL^{-1} of Pb were used in NBRIP broth medium.

Identified isolates were inoculated in duplicate tubes containing heavy metals of different concentrations added with previously mentioned three liquid growth media and incubated for 24-48 h at 37°C. After incubation growth of bacteria in liquid media containing heavy metal was confirmed by spreading of liquid sample of isolates from each tube on agar plate to test the appearance of growth. The plates were also incubated for 24-72 h and observed for the presence of growth.

Minimum growth observed in agar plate indicates the tolerance capacity of each isolate to respective metal concentration (Maximum tolerable concentration) whereas the absence of growth after 72 h indicates that the respective metal concentration inhibits the growth of bacteria (Minimum inhibitory concentration). The agar plate method was used since this is an accepted approach that has been used in many reported studies (Taghavi *et al.,* 1997).

3.21 Determination of Tolerance of Indigenous Soil Bacteria Amended With Heavy Metal

Metals, most commonly associated with pollution include Cadmium (Cd), Copper (Cu), Chromium (Cr), Mercury (Hg), Lead (Pb), zinc (Zn) and metalloid Arsenic (As). At low concentrations, metals can serve as important components in life processes, often serving important functions in enzyme productivity. However, above certain threshold concentrations, metals can become toxic to many species. Toxicity of the heavy metals is concentration and time dependent for each bacterial isolate. However, in general aerobic heterotrophic populations were more sensitive to metal groups like Ni and Cd followed by Cu, Pb, Hg, Mn, Cr and least to Zn (Maier *et al*., 2000).

3.22 Soil Preparation for Incubation

Before incubation, three types of soils (Silt loam, Sandy loam, Silty clay loam) were sieved through a 2 mm pore size screen. Soils were preserved in plastic container for further experiment (Ahmed *et al*., 2005). Two gram soils were taken in each test tube for incubation experiment. Compost, Oil cake and Rock Phosphate as source of phosphorus were added in each test tube at a rate of 40 $kgha^{-1}$ to enhance microbial activity and growth (Taha *et al*., 1969). After

plugging with cottons, incubation tubes were sterilized in autoclave at 121^0C under 15 lbs pressure per square inch for 20 minutes.

3.23 Preparation of Stock Solution of Metals

Stock solution of different concentrations were prepared for Cadmium from $Cd(NO_3)_2$ and lead from $Pb(NO_3)_2$. All of the salt solutions were sterilized in autoclave machine before mixing into soils.

3.24 Inoculation of Phosphate-Solubilizing Microorganisms in Soil

Among the isolated strains, only 8 strains were used as inoculants on the basis of phosphate solubilization in liquid medium. These organisms were used to test against Pb and Cd tolerance in culture medium. All of the bacterial isolates were grown on nutrient broth for 48 h which was used for incubation (Hiroki, 1992). Identified bacterial strains were inoculated in three types of soils at metal concentrations of Cadmium and Lead. After mixing, all tubes were incubated at 30^0C for 24 h, 15 days and 30 days. Moisture level was maintained at 50% maximum water holding capacity during incubation (Patil *et al*., 1979). Every care was taken to maintain the moisture level more or less constant.

3.25 Application of Concentrated Metal Solution in Soil

Metal salt solutions were mixed with three soil samples at the concentrations level 15,000 µg/g; 20,000 µg/g and 30,000 µg/g, and maintaining 50% water content (Patil *et al*., 1979). Control soils were set up without heavy metal and all the set-up were prepared in duplicates. To determine tolerance of isolate to Cd and Pb,

concentrations in the range 15,000 μg/g, 20,000 μg/g and 30,000 μg/g for silt loam, sandy loam and silty clay loam soil were used.

3.26 Determination of Bacterial Growth after Incubation

Bacterial viability in soils was determined at high metal concentration conditions. After 24 h, 15 days and 30 days from the date of incubation, the soils were diluted in sterile distilled water. After dilution, sample was spreaded over the surface of nutrient agar plate (Cappuccino and Sherman, 2005). The plates were then kept for 24-72 h in an incubator at 37^0C. The absence of bacterial growth in certain metal concentration indicated its sensitivity, while the presence of bacterial growth at a certain metal concentration indicated that bacteria were resistant against the metal concentration at that level. Minimum growth was observed in agar plate indicated the tolerance capacity of each isolate to respective metal concentration whereas the absence of growth after 72 h indicated that the respective metal concentration inhibits the growth of bacteria (Ahmad *et al.*, 2005).

3.27 Method of Sequential Extraction Scheme for Lead and Cadmium

Methods for assessing the bioavailability of metals in the field are dependent on chemical extraction techniques (Bryan, 1992). Sequential extraction was used to speciate Pb and Cd from surface soils to assess metal mobility. Numerous extraction schemes have been described in the literature (Chao 1972; Tessier *et al.*, 1979; Sposito *et al.*, 1982; Welte *et al.*, 1983; Clevenger 1990; Ure *et al.*, 1993; Howard and Vandenbrink, 1999). The procedure of Tessier *et al.* (1979) is

one of the most thoroughly researched and widely used procedures to evaluate the possible chemical associations of metals in soils.

The sequential extraction method of Salbu and his coworkers (Salbu *et al.*, 1998), modified from that of Trssier and his coworkers (Tessier *et al.*, 1979), was used to determine the solid-phase speciation of Pb and Cd. The sequences of extractions were six operationally defined groups: water soluble (F1), exchangeable (F2), carbonate (F3), oxide (F4), organic (F5) and residual (F6) (Kashem and Singh, 2001).

Two-gram soil samples were used throughout the experiment. All equipment and containers were soaked in 10% HCl and rinsed thoroughly in deionized water before use. Metals were determined by flame atomic absorption spectrophotometry (AAS). The experimental details of this procedure are presented (Table-3.1). [Here the uses symbol in the table (4.48 to 4.65) indicates that (B = Soil without organic matter), (C = Soil mixed with organic source-Compost), (O = Soil mixed with organic source-Oil cake), (R = Soil mixed with inorganic source-Rock phosphate) and (T1- 15,000 µg/g, T2- 20,000 µg/g and T3-30,000 µg/g)].

Table 3.1 Procedure of sequential extraction scheme (Kashem and Singh, 2001).

Step	Fraction	Extract	Reaction time	Device	Centrifuge/Filtrate
1	F1: Water soluble	20 mL deionized water	1 h in 20^0C	Rolling table	10,000 rpm in 30 min
2	F2: Exchangeable	20 mL 1-M NH_4OAc (pH 7)	2 h in 20^0C	Rolling table	10,000 g in 30 min
3	F3: Carbonate bound	20 mL 1-M NH_4OAc (pH 5)	2 h in 20^0C	Rolling table	10,000 g in 30 min
4	F4: Fe & Mn oxide bound	20ml 0.04-M NH_2OH*HCl in 25% (v/v) Aac (pH 3)	6 h in 80^0C	Shaking water bath	10,000 g in 30 min
5	F5: Organically bound	15 ml 30% H_2O_2 (adj. pH 2), 5 ml 3.2-M NH_4OAc in 20% (v/v) HNO_3	5.5 h in 80^0C, 0.5 h in 20^0C	Shaking water bath and Rolling table	10,000 g in 30 min
6	F6: Residual	7 M HNO_3	6 h in 80^0C	Shaking water bath	Filtrate

4. RESULTS AND DISCUSSION

4.1 PHYSICAL AND CHEMICAL PARAMETERS OF SOILS

The pH value and metal (Pb and Cd) content of the agricultural and contaminated soils from which the bacteria were isolated are presented in Table 4.1. Physical and chemical parameters of the three soil samples used for incubation study are presented in Table 4.2. Physical and chemical parameters of soil have partial effect on the resistances of the microorganisms (Kermanshahi *et al.*, 2007). Since the resistance of the microorganisms would be affected by the variation of pH and the pH of one agricultural soil sample was near about neutral value, there would be little effect on bacterial resistance. The physical, chemical and physico-chemical properties and heavy metal characteristics of the soils are below the permissible limit, recommended for soil as described by Krishna Murti and Vishwanathan (1991). Analytical data of source materials and presented in Table 4.3.

Table 4.1 pH and metal contents of the soil samples used for isolation of bacterial strains

Soil	Sample	Location	pH	Metal content (ppm)	
				Pb	Cd
Agricultural area (Uncontaminated)	S-1	Dhamrai	6.95	0.0582	0.0006
	S-2	Pabna	8.60	BDL	BDL
Industrial area (Contaminated)	S-3	Saver EPZ Zone	7.32	0.5310	0.0064

(BDL→ below demanding level)

Table 4.2 Soil properties and heavy metal status of soil before metal amendments.

Parameters	Soil Sample-1	Soil Sample-2	Soil Sample-3
pH	6.95	5.50	8.60
% Sand	1.76	6.66	52.60
% Silt	78.87	63.76	41.66
% Clay	19.37	29.58	5.74
Texture	Silt loam	Silty clay loam	Sandy loam
Organic Carbon (%)	0.72	0.86	0.18
Organic matter (%)	1.24	1.48	0.32
CEC cmole/kg	22.90	29.80	8.74
Total Nitrogen (%)	0.19	0.20	0.03
Total Phosphorus (%)	0.05	0.03	0.04
Total Potassium (%)	0.03	0.02	0.03
Sulphur (%)	0.11	0.04	0.04
Cadmium (Cd) mg/kg	0.0006	0.0008	BDL
Lead (Pb) mg/kg	0.0582	0.0118	BDL

(BDL→ Below detection level)

Table 4.3 Important chemical properties of the organic residues.

Parameters	Compost	Oil cake	Rock phosphate
Total Phosphorus (%)	0.81	0.74	10
Lead (µg/g)	1.5	2.0	00
Cadmium (µg/g)	0.15	0.21	0.02

4.2 Isolation and Purification of Bacteria

After purification of the colonies, these were streaked on various selective media for isolation of specific microorganisms (Cappuccino and Sherman, 2005) (Figure 4.1). After their respective incubation time, the recorded results are presented in Table 4.4.

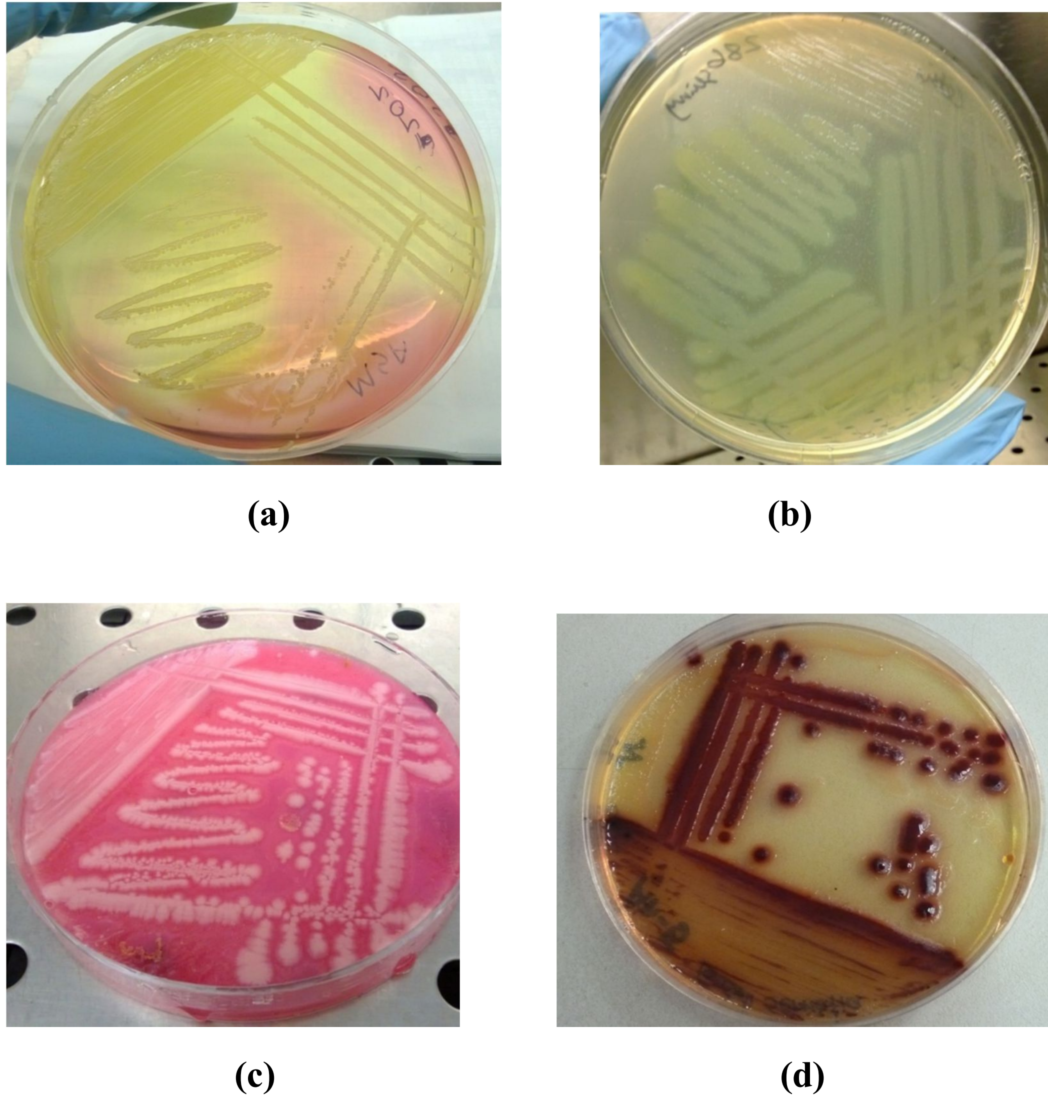


Figure 4.1 Isolation of different bacteria by growing on different selective media. **(a)** Presumptive *Staphylococcus aureus* for yellow colonies on MSA plate. **(b)** Presumptive *Pseudomonas aeruginosa* for fluorescent colonies on cetrimide agar plate. **(c)** Presumptive *Bacillus* sp. for white colonies with the media turning pink from yellow on NGKG agar plate. **(d)** Presumptive *Serratia Marcescens* on TSA agar plate.

Table 4.4 Growth of organisms on selective media for their identification.

Sample ID	NGKG	MSA	Cetrimide	EMB	PDA	PVK	NBRIP	MAC
C1	+	-	-	-	-	-	-	-
C2	-	+	-	-	-	-	-	-
C3	+	-	-	-	-	+	+	-
C4	+	-	-	-	-	+	+	-
C5	+	-	-	-	-	-	-	-
C6	+	-	-	-	-	-	-	-
C7	-	+	-	-	-	-	-	+
C8	-	+	-	-	-	-	-	-
C9	+	-	-	-	-	-	-	+
C10	+	-	+	-	-	-	-	-
A1	+	-	-	-	-	+	+	-
A2	+	-	-	-	-	-	-	-
A3	+	-	-	-	-	+	+	-
A4	-	-	-	+	-	+	+	-
A5	+	-	-	-	-	+	+	-
A6	-	-	+	-	-	-	-	-
A7	-	+	-	-	-	-	-	+
A8	-	+	-	-	-	-	-	-
A9	+	-	+	-	-	-	-	-
A10	+	-	-	-	-	-	-	+
A11	+	-	-	-	-	-	-	+
A12	-	+	-	-	-	-	-	+
A13	-	-	-	+	-	-	-	-
A14	+	-	+	-	-	-	-	-

During this study, a total of 24 discrete colonies in the plates were primarily selected from different samples (Table 4.2). From 3 different soil samples a total 13 isolated bacterial colonies were finally selected (Table 4.5). No fungal colony was found to grow. These 13 isolates were selected for morphological observation, Gram reaction, biochemical test and API test. The isolates and the source sample from which they were isolated are mentioned in Table 4.5.

Table 4.5 Bacterial samples isolated from contaminated and agricultural soils

Bacterial samples	Soil sample
C1	Contaminated soil
C2	
C3	
C4	
C5	
C6	
A1	Agricultural soil
A2	
A3	
A4	
A5	
A6	
A7	

4.3 Morphological Characteristics of Isolates

For the identification of selected strains, following morphological characters were studied and recorded (Cappuccino and Sherman, 2005).

4.3.1 Study of colonial morphology

Colonial characteristics of purified bacterial strains grown in solidified nutrient agar plates were observed after 24, 48 and 72 hours of incubation and data were recorded regarding the color, size, shape, elevation, margin, surface and optical features. The results are presented in Table 4.6.

4.3.2 Study of cell morphology

All isolates were observed morphological characteristics under microscope. Most of the aerobic heterotrophic bacterial isolates were rod shaped. According to Gram reaction, 11 isolates were Gram positive and the remaining 2 isolates were Gram negative (Table 4.7).

Table 4.6 Morphological characteristics of the bacterial cell and colonies.

Bacterial strains	Colonial Morphology					Cell morphology	
Strain No.	Form	Elevation	Margin	Surface	Color	Shape	Motility
C1	Circular	Effuse	Entire	Smooth	Off white	Rod, rounded end	-
C2	Circular	Convex	Regular	Smooth	Yellow	Cocci	-
C3	Circular to irregular	Low convex	Entire	Smooth	White or grays	Rod, rounded end	+
C4	Circular	Effuse	Undulated	Smooth	White	Rod, rounded end	+
C5	Circular	Effuse	Entire	Smooth	Brown	Rod, rounded end	-
C6	Circular	Convex	Regular	Smooth	Yellow	Cocci	-
A1	Circular	Convex	Entire	Smooth	Light pink	Rod, rounded end	+
A2	Circular to irregular	Low convex	Entire	Smooth	White or grays	Rod, rounded end	+
A3	Circular	Effuse	Entire	Smooth	Off white	Rod, rounded end	-
A4	Circular	Convex	Erose	Concen-tric	White	Rod, rounded end	+
A5	Circular	Convex	Undulated	Smooth	Off white	Rod, rounded end	-
A6	Circular	Convex	Entire	Smooth	Brown	Short Rod, rounded end	+
A7	Circular	Effuse	Erose	Smooth	Orange	Rod, rounded end	+

4.4 Physiological and Biochemical Characteristics of Isolates

A large number of biochemical and physiological characteristics have been used in bacterial identification of selected strains. Results of the test are used in bacterial identification on the basis of Numerical Taxonomy (Sneath *et al.*, 1986).

Some important physiological and biochemical tests such as catalase, oxidase, hydrolysis of starch, liquefaction of gelatin, VP (Voges-Proskauer), MR (Methyl Red) test, indole formation, utilization of citrate, acid and gas production from glucose by the isolated strains were performed and the isolates were grouped into two categories: (a) Aerobic Gram-positive heterotrophic bacteria and (b) Aerobic Gram-negative heterotrophic bacteria. All the results of physiological and biochemical tests are presented in Table 4.7.

Table 4.7 Biochemical characteristics of the selected bacterial isolates.

Biochemical Characteri-stics	**Strain No**												
	C1	**C2**	**C3**	**C4**	**C5**	**C6**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**
Gram reaction	+	+	+	+	+	+	+	+	+	-	+	-	+
Oxygen require-ment	Faculta-tive Anaero-bes	Strictly aerobes	Faculta-tive Anaero-bes	Faculta-tive Anaero-bes	Faculta-tive Anaero-bes	Strictly aerobes	Faculta-tive Anaero-bes	Faculta-tive Anaero-bes	Faculta-tive Anaero-bes	Strictly aerobes	Faculta-tive Anaero-bes	Strictly aerobes	Facultative Anaerobes
Oxidase test	-	+	+	+	+	+	+	+	-	-	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole formation	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	+	+	+	+	+	+	-	-	-	-	-
VP test	-	-	+	+	+	+	-	+	-	+	-	-	-
MR test	-	-	-	-	-	-	+	-	-	-	+	-	+
Gelatin Liquefa-ction	+	+	-	+	+	+	+	-	+	+	+	+	+
Utilization of Citrate	+	+	-	+	-	+	+	-	+	+	+	+	+
Nitrate reduction	+	-	-	+	+	+	+	-	+	+	+	+	+
Acid production from D-Glucose	+	-	+	+	-	+	+	+	+	+	+	+	+
Gas production from D-Glucose	-	-	-	-	-	-	-	-	-	+	-	+	+

4.4.1 Motility test

Out of 13 isolates 6 isolates C3, C4, A1, A2, A4, A6 and A7 were motile and remaining were non-motile (Table 4.6) (Figure 4.2) found in semisolid medium.

4.4.2 Oxygen requirement

On the basis of their oxygen requirements 4 strains (C2, C6, A4, and A6) were found to be aerobic and 09 strains were facultative anaerobes (Table 4.7) (Figure 4.2).

4.4.3 Catalase test

All the isolates showed positive result for catalase test (Table 4.7) (Figure 4.2).

4.4.4 Oxidase test

Out of the 13 isolates 3 strains C1, A3 and A4 were unable to produce oxidase and remaining isolates were oxidase positive (Table 4.7) (Figure 4.2).

4.4.5 Hydrolysis of starch

Out of 13 isolates 6 strains C3, C4, C5, C6, A1 and A2 were capable of hydrolyzing starch, remaining isolates showed negative results (Table 4.7) (Figure 4.3).

4.4.6 Liquefaction of gelatin

Among the isolates 11 strains positive result i.e. capable of liquefying gelatin except C3 and A2 strains (Table 4.7).

4.4.7 Voges proskaur (VP) test

In case of VP test out of 13 isolates 7 strains were negative and 6 strains C3, C4, C5, C6, A2 and A4 were positive (Table 4.7).

4.4.8 Methyl red (MR) test

Out of 13 strains, 4 strains C4, A1, A5 and A7 showed positive result in methyl red reaction (Table 4.7) (Figure 4.4).

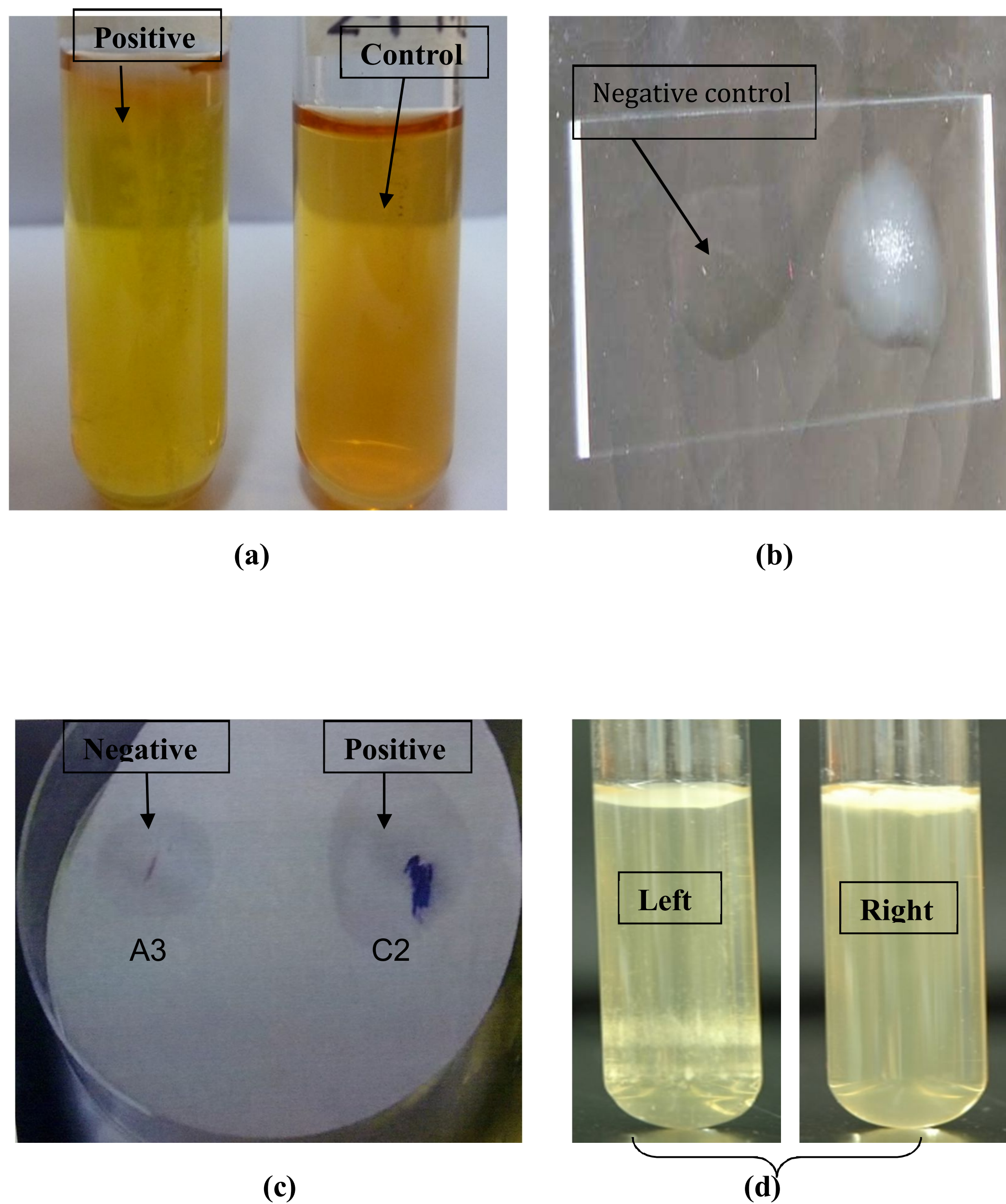


Figure 4.2 (a) Motility test of isolates C4; **(b)** Catalase test of all isolates; **(c)** Oxidase test of isolates (A3 and A4) (-ve); **(d)** Oxygen requirement test are shown here, aerobic (C2, A4 and A6 isolates) right and the rest of the isolates were facultative anaerobes (left).

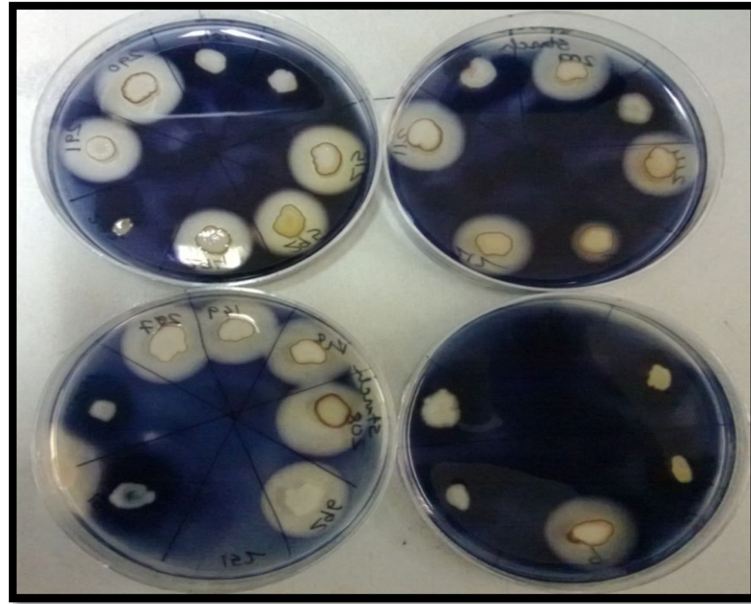


Figure 4.3 Hydrolysis of starch by *Bacillus* sp.

(e)

Figure 4.4 (e) MR tests of isolates (A1, A5 and A7) were (+ve).

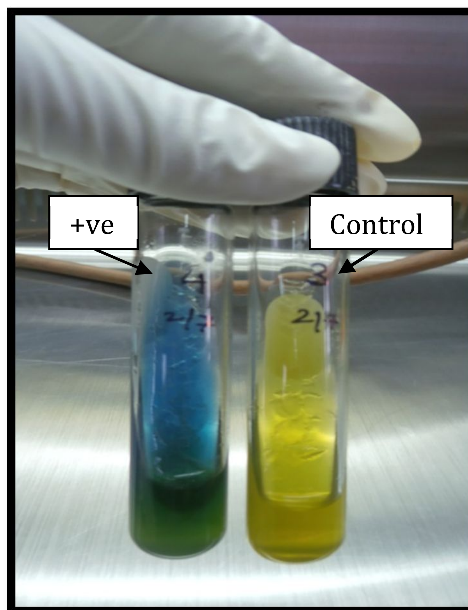


Figure 4.5 Utilization of citrate (+ve) by isolates (C1, C2, C4, C6, A1, A3, A4, A5, A6, and A7).

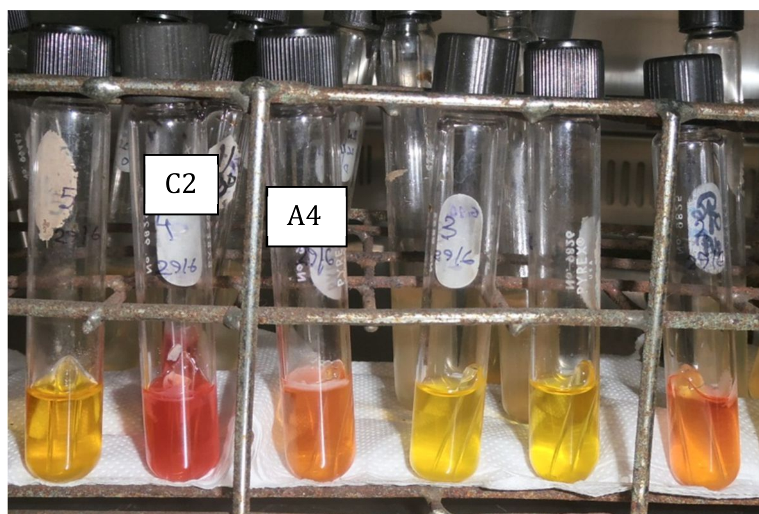


Figure 4.6 Acid production by two isolates C2 and C5 (-ve), and gas production by isolates (A4, A6 and A7) (+ve).

4.4.9 Indole production test

All strains showed negative result in indole production (Table 4.7).

4.4.10 Utilization of citrate

Out of 13 strains, 5 isolates (C3, C5, A2, A5, and A7) were unable to utilize citrate and remaining 8 isolates were able to utilize citrate (Table 4.7) (Figure 4.5).

4.4.11 Acid and gas production test

Most of the strains showed positive result in acid production from D-glucose except C2 and C5. Among 13 strains only 2 strains A4 and A6 were able to produce gas (Figure 4.6) during glucose fermentation (Table 4.7).

4.5 Results of API Test

4.5.1 *Bacillus* sp.

Bacillus sp. grew on NGKG agar plate gave characteristic white colonies along with the change in media from pink to yellow and 8 strains were selected for API test for the identification of *Bacillus* sp. The results are presented in Table 4.8, 4.9 and Figure 4.7.

Table 4.8 API 50 CHB result for identification of *Bacillus* sp.

Sl. No.	Test	C1	C3	C4	C5	A1	A2	A3	A5
0	**0**	-	-	-	-	-	-	-	-
1	**GLY**	+	+	+	+	+	+	+	+
2	**ERY**	-	-	-	-	-	-	-	-
3	**DARA**	-	-	-	-	-	-	-	-
4	**LARA**	+	+	-	+	+	+	+	+
5	**RIB**	+	+	+	+	+	+	+	+
6	**DXYL**	-	+	-	+	+	+	+	+
7	**LXYL**	-	-	-	-	-	-	-	-
8	**ADO**	-	-	-	-	-	-	-	-
9	**MDX**	-	-	-	-	-	+	-	-
10	**GAL**	+	+	-	+	-	+	+	-
11	**GLU**	+	+	+	+	+	+	+	+
12	**FRU**	+	+	+	+	+	+	+	-
13	**MNE**	+	+	-	+	+	+	+	-
14	**SBE**	-	-	-	-	-	-	-	-
15	**RHA**	-	-	-	+	-	-	-	-
16	**DUL**	-	-	-	+	-	-	-	-
17	**INO**	-	-	-	+	+	-	-	+
18	**MAN**	+	+	-	+	+	+	+	+
19	**SOR**	-	-	-	+	+	-	-	+
20	**MDM**	-	-	-	-	-	-	+	-
21	**MDG**	-	+	-	+	-	-	-	+
22	**NAG**	+	+	+	+	-	-	+	-
23	**AMY**	+	+	-	-	+	+	+	+
24	**ARB**	+	+	+	+	-	+	+	-

(Table 4.8 continued)

25	**ESC**	+	+	+	+	+	+	+	+
26	**SAL**	+	+	+	+	-	+	+	-
27	**CEL**	+	+	+	+	+	+	+	+
28	**MAL**	+	+	+	+	+	+	-	+
29	**LAC**	+	+	-	+	+	+	+	+
30	**MEL**	+	+	-	+	+	+	-	+
31	**SAC**	+	+	-	+	+	+	+	+
32	**TRE**	+	+	+	+	+	+	+	+
33	**INU**	-	-	-	-	+	+	-	-
34	**MLZ**	+	-	-	-	-	-	-	-
35	**RAF**	+	+	-	+	+	+	-	+
36	**AMD**	+	+	+	+	-	+	-	-
37	**GLYG**	+	+	+	+	+	+	-	+
38	**XLT**	-	-	-	-	-	-	-	-
39	**GEN**	+	+	-	+	-	+	-	-
40	**TUR**	-	+	-	-	+	+	-	+
41	**LYX**	-	-	-	-	-	-	-	-
42	**TAG**	-	-	+	-	-	-	+	-
43	**DFUC**	-	-	-	-	-	-	-	-
44	**LFUC**	-	-	-	-	-	-	-	-
45	**DARL**	-	-	-	-	-	-	-	-
46	**LARL**	-	-	-	-	-	-	-	-
47	**GNT**	-	-	+	+	-	-	-	-
48	**2KG**	-	-	-	+	-	-	-	-
49	**5KG**	-	_	-	-	-	-	-	-

Table 4.9 API 20 E result for identification of *Bacillus* sp.

Test	C1	C3	C4	C5	A1	A2	A3	A5
ONPG	+	+	-	-	+	+	+	-
ADH	+	+	+	+	-	-	-	+
LDC	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-
CIT	-	-	+	+	+	-	+	+
H2S	-	-	-	-	-	-	-	-
URE	-	-	+	-	-	-	-	-
TDA	+	+	+	-	-	-	+	-
IND	-	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	+	+
GEL	+	-	+	+	+	+	+	+
GLU (NIT)	-	-	+	+	+	+	-	+

According to the API 50 CHB and API 20 NE tests, 08 isolates were confirmed to be *Bacillus megaterium* (C1), *Paenibacillus lautus* (C3), *Bacillus cereus* (C4), *Bacillus pocheonensis* (C5), *Bacillus subtilis* (A1), *Paenibacillus polymyxa* (A2), *Bacillus pumilus* (A3) and *Bacillus amyloliquefaciens* (A5).

Figure 4.7: API 20 E (top) and API 50 CHB (bottom) test result of *Bacillus cereus* isolate.

4.5.2 *Pseudomonas* sp.

Out of 24 isolates, 4 isolates that grew on Cetrimide agar, only 1 of them gave characteristic colonies and they were subjected to biochemical tests Table 4.7. These isolate was then further characterized using API 20 NE. The results are recorded in Table 4.10 and Figure 4.8.

Table 4.10 Result of API 20 NE for identification of *Pseudomonas* sp.

Isolate	NO3	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC
A6	+	-	+	+	-	-	+	-	+	-	-	+	-	-	+	+	+	+	+	-

According to the API 20 NE tests, one isolate was confirmed to be *Pseudomonas aeruginosa* (A6).

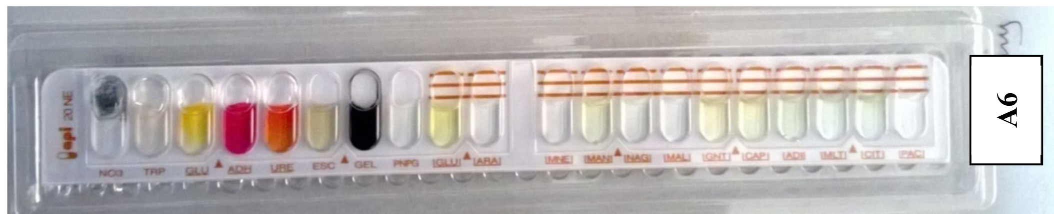


Figure 4.8 Result of API 20 NE obtained for *Pseudomonas aeruginosa* (A6).

4.5.3 *Staphylococcus* sp.

Out of 24 isolates which grew on MSA plate, 6 of them gave characteristic yellow colonies (presumptive of *Staphylococcus* sp.). These were then subjected to biochemical characterization (Table 4.7). Among them only one isolate was confirmed. The results are presented in Table 4.11.

Table 4.11 Result of API Staph for identification of *Staphylococcus* sp.

Isolate ID	O	GLU	FRU	MNE	MAL	LAC	TRE	MAN	XLT	MEL	NIT	PAL	VP	RAF	XYL	SAC	MDG	NAG	ADH	URE
C6	-	+	+	+	+	-	+	+	-	-	+	+	+	-	-	+	-	+	+	+
C2	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-

According to the results presented in Table 4.11, C2 and C6 isolates were confirmed as *Micrococcus luteus* and *Staphylococcus lentus.*

4.5.4 *Proteus* sp.

Six isolates grew on MacConkey agar; one of them gave characteristic colony and subjected to biochemical tests (Table 4.7). The results are shown in Table 4.12 and Figure 4.9.

Table 4.12 Result of API 20 E for identification of *Proteus* sp.

Isolate ID	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU
A7	-	-	-	+	-	+	+	+	-	-	+	+

According to the results in Table 4.12, the isolate was confirmed as *Proteus mirabilis.*

Figure 4.9 Result of API 20 E obtained for *Proteus mirabilis* (A7).

4.5.5 *Serratia* sp.

Two isolates grew on EMB agar plate, one of them gave characteristic colony and subjected to biochemical tests Table 4.7. The results are presented in Table 4.13 and Figure 4.10.

Table 4.13 Result of API 20 E for identification of *Serratia* sp.

Isolates ID	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
A4	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	-	+	+	+	-	-

According to the results in Table 4.13, the isolate A4 was confirmed as *Serratia marcescens.*

Figure 4.10 Result of API 20 E obtained for *Serratia marcescens* (A4).

4.6 Identification of Selected Isolates

Considering all observed characters of the isolated organisms, identification of Gram positive aerobic heterotrophic bacteria was done following Bergey's Manual of Systematic Bacteriology Vol. 2 (Sneath *et al.*, 1986) and Gram negative aerobic bacteria were also identified (Table 4.6). following Vol. 1 of Bergey's Manual of Systematic Bacteriology (Kreig and Holt, 1984). The biochemical tests (Table 4.7) and API test (Table 4.9 to 4.13). The names of the 13 selected isolates are presented in Table 4.14.

Table 4.14 Name of the selected 13 isolates after identification

Strains no.	Name of the isolates
C1	*Bacillus megaterium*
C2	*Micrococcus luteus*
C3	*Paenibacillus lautus*
C4	*Bacillus cereus*
C5	*Bacillus pocheonensis*
C6	*Staphylococcus lentus*
A1	*Bacillus subtilis*
A2	*Paenibacillus polymyxa.*
A3	*Bacillus pumilus*
A4	*Serratia marcescens.*
A5	*Bacillus amyloliquefaciens*
A6	*Pseudomonas aeruginosa*
A7	*Proteus mirabilis*

4.7 Isolation of Phosphate Dissolving Bacteria

Soil contains a diverse group of microorganisms. All the isolates might not be phosphate dissolving microorganisms (Sperber, 1958; Taha *et al.*, 1969). For selection each isolate was transferred to the NBRIP and PVK medium. The growth of bacteria in these media is presented in Table 4.15.

Table 4.15 Growth of phosphate dissolving microorganisms

Isolates ID	NBRIP medium	PVK medium
C1	-	-
C2	+	+
C3	+	+
C4	+	+
C5	-	-
C6	-	-
A1	+	+
A2	-	-
A3	+	+
A4	+	+
A5	+	+
A6	+	+
A7	+	+

"+" indicates growth; "-" indicates absence of growth.

According to the result of Table 4.15, among 13 isolates the isolates C2, C3, C4, A1, A3, A4, A5, A6 and A7 were identified as phosphate dissolving microorganisms.

4.8 Efficiency of Isolates in Dissolving Insoluble Phosphate in Liquid Culture Medium

A total of 9 strains (C2, C3, C4, A1, A3, A4, A5, A6 and A7) from 13 isolates (Table 4.15) were selected for the present investigation to evaluate the efficiency in dissolving insoluble phosphate in culture media.

4.8.1 Efficiency of isolates in dissolving insoluble phosphate in NBRIP and PVK broth medium

Selected phosphate dissolvers were inoculated in NBRIP and PVK broth media. Quantitative determination of soluble phosphate together with pH changes in the culture were investigated during the growth of organisms. In NBRIP and PVK medium the data generally indicated that the soluble phosphate increased progressively till the 11th day of incubation and then decreased. The pH values of the culture generally decreased till the 11th day and then began to rise again thereafter according to Molla *et al.* (1983).

In NBRIP medium the lowest and highest pH values recorded were 6.21 for A4 isolate and the highest value of 6.74 for C4 isolate after 11th day of incubation, respectively (Appendix C 1) (Figure 4.11). And in PVK medium the lowest and highest pH values recorded were 4.80 for A4 isolate and 6.18 for A5 isolate after 11th day of incubation, respectively (Appendix C 2) (Figure 4.12) (Sperber, 1958; Taha *et al*., 1969 and Patil *et al.,* 1979). The release of soluble phosphate and the changes in the pH values of the culture during incubation period of 15 days are given in (Appendix C 1 and 2).

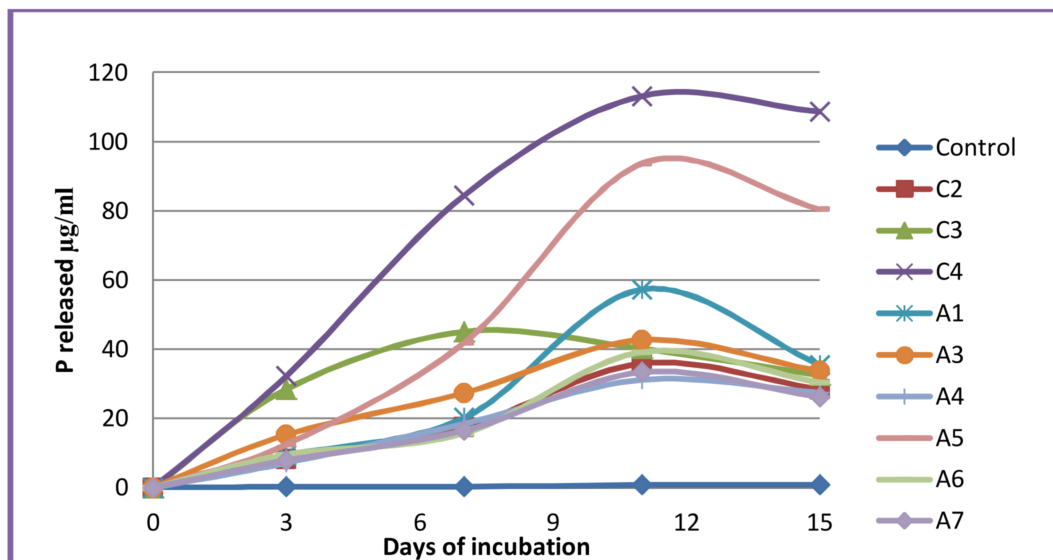


Figure 4.11 Release of soluble phosphate in liquid NBRIP medium.

The increase in the soluble phosphate was parallel with the decrease in the pH of the culture. In NBRIP medium the highest and lowest amount of released phosphate being recorded for C4 and A4 isolates which released 113.2 µg/ml and 31.2 µg/ml after 11 days of incubation, respectively. Other phosphate dissolvers C2, C3, A1, A3, A5, A6 and A7 released phosphate of 35.9, 40.2, 57.3, 42.7, 93.7, 39.2 and 33.4 µg/ml, respectively, after 11 days of incubation (Figure 4.11).

In PVK medium the highest and lowest amount of released phosphate were recorded for A5 and A4 isolates which released 62.4 and 18.2 µg/ml after 11 days of incubation, respectively. Other phosphate dissolvers C2, C3, C4 A1, A3, A6 and A7 released phosphate 23.5, 41.2, 54.4, 43.4, 38.9, 26.4 and 21.2 µg/ml, respectively, after 11 days of incubation (Figure 4.12).

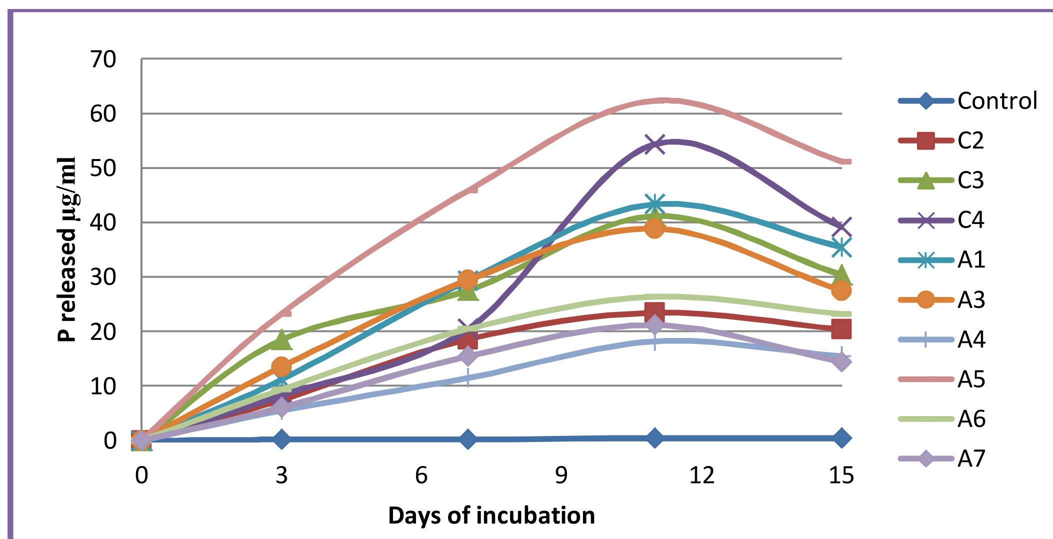


Figure 4.12 Release of soluble phosphate in liquid PVK medium.

The extent of phosphate released by phosphate-dissolvers was found to vary among the isolates. These were attributed to the amount of acid produced which was indicated by the change in pH values of the culture. The amount of soluble phosphate decreased (immobilized) after 11 days of incubation which varied from organism to organism.

The efficiency of bacteria carried out the solubilization of insoluble phosphate were as follows

Bacillus > Pseudomonas > Micrococcus > Proteus >Serratia.

Species of *Bacillus*, *Pseudomonas* and *Micrococcus* were found to be the most efficient organisms in dissolving insoluble phosphate in liquid culture medium. Species of *Proteus* and *Serratia* ranked next in dissolving insoluble phosphate. It was also found that with the increase in soluble phosphate, the pH of the media was decreased by all the species.

Here also showed that the *Bacillus* isolates were more efficient than others Molla *et al.,* (1983) also found that *Bacillus* was the most efficient phosphate dissolvers among the *Pseudomonas; Micrococcus; Proteus; and Serratia* species of soil bacteria.

4.9 Mineralization of Organic Phosphorus in Liquid Culture Medium

A total 9 strains (C2, C3, C4, A1, A3, A4, A5, A6 and A7) were selected from 13 isolates (Table 4.5) for the present investigation to evaluate the efficiency of organic phosphate mineralization in liquid culture media.

4.9.1 Mineralization of organic phosphorus in modified NBRIP*[1] and NBRIP*[2] broth medium

The modified (for this experiment) NBRIP*[1] and NBRIP*[2] broth media with organic phosphate were inoculated with selected phosphate dissolvers. Quantitative determination of mineralization of organic phosphate together with pH changes in the culture were investigated during the growth of organisms. Mineralization of organic phosphate by bacteria with the decrease in the pH of the culture media is presented the (Appendix C 3 and 4).

Data on P concentration obtain in NBRIP*[1] and NBRIP*[2] modified media indicated that the mineralization of organic phosphate increased progressively till the 11th day and then decreased. The pH values of the culture generally decreased till the 11th day and then began to increase again (Figure 4.13 and Figure 4.14).

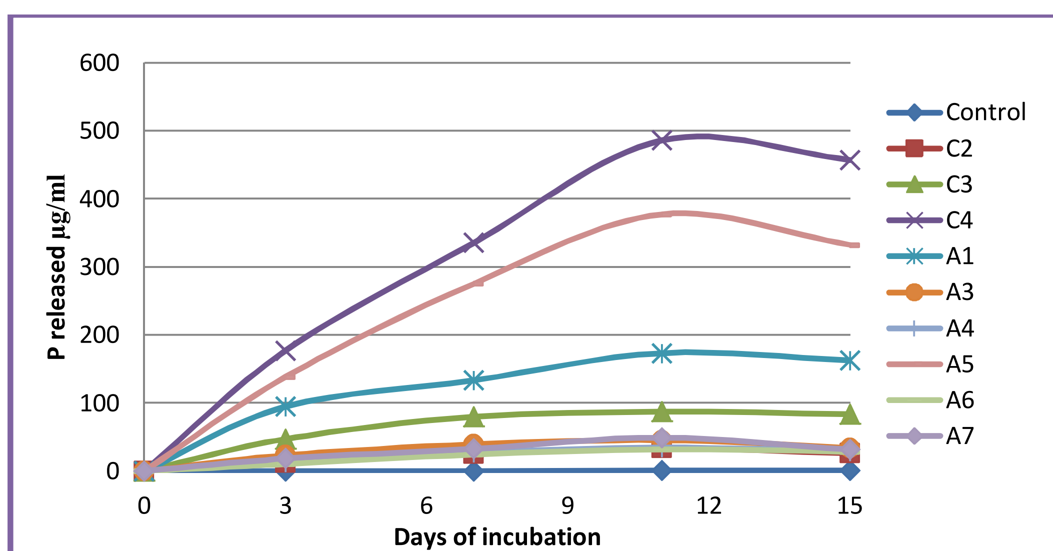


Figure 4.13 Release of soluble phosphate in liquid NBRIP*[1] medium.

In NBRIP*[1] medium the lowest and highest pH values recorded were 4.26 for C4 isolate and 5.11 for A6 isolate after 11th day of incubation respectively (Figure 4.13) and (Appendix C 3). And the highest and lowest amount of released phosphate being recorded for C4 and A6 isolates which released 486.3 µg/ml and 32.2 µg/ml after 11 days of incubation, respectively. Other phosphate dissolvers C2, C3, A1, A3, A4, A5, and A7 released phosphate of 32.5, 87.2, 173.5, 45.5, 34.5, 377.6 and 49.3 µg/ml, respectively, after 11 days of incubation.

In the modified NBRIP*[2] medium the lowest pH value was 3.89 for C4 isolate and highest pH value of 4.50 were recorded for A4 isolate after 11th day of incubation (Figure 4.14) and (Appendix C 4).

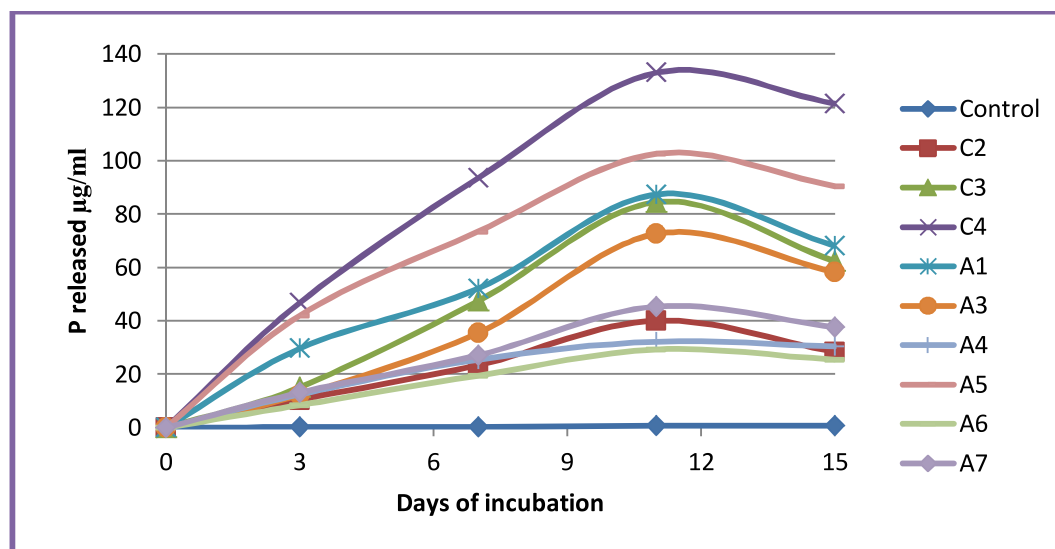


Figure 4.14 Release of soluble phosphate in liquid NBRIP*[2] medium.

In NBRIP*[2] medium the highest and lowest amounts of released phosphate were being recorded with C4 and A6 isolates which released 133.1 µg/ml and 29.4 µg/ml after 11 days of incubation, respectively. Other phosphate dissolvers C2, C3, A1, A3, A4, A5 and A7 released phosphate of 40.2, 84.6, 87.5, 72.7, 32.3, 102.7 and 45.5 µg/ml respectively after 11 days of incubation.

The extent of phosphate released by phosphate-dissolvers was found to vary among the isolates. The amount of soluble phosphate decreased (immobilized) after 11 days of incubation which differed from organism to organism.

The order of sequence on the basis of the efficiency of mineralization of organic phosphate was as follows

Bacillus > *Proteus* > *Serratia* > *Micrococcus* > *Pseudomonas.*

It was also found by Molla *et al.* (1983) that the Bacillus isolates were more efficient in mineralizing organic P than others.

4.9.2 Mineralization of organic phosphorus in modified PVK*[1] and PVK*[2] broth media

Mineralization of organic phosphorus in modified PVK*[1] medium revealed that the lowest and highest pH values were recorded 4.42 with A5 isolate and the highest value of 5.30 with A4 isolate after 11 days of incubation respectively (Figure 4.15). And in PVK*[2] medium the lowest and highest pH values were 4.01 for A5 isolate and 4.44 for A4 isolate after 11 days of incubation respectively (Figure 4.16) (Sperber, 1958; Taha *et al.*, 1969 and Patil *et al.,* 1979). The increase in the mineralization of organic phosphate was positively related to the decrease in the pH of the culture. The mineralization of organic phosphate and the changes in the pH values of the culture medium PVK*[1] and PVK*[2] during incubation period of 15 days are given in (Appendix C 5 and 6), respectively.

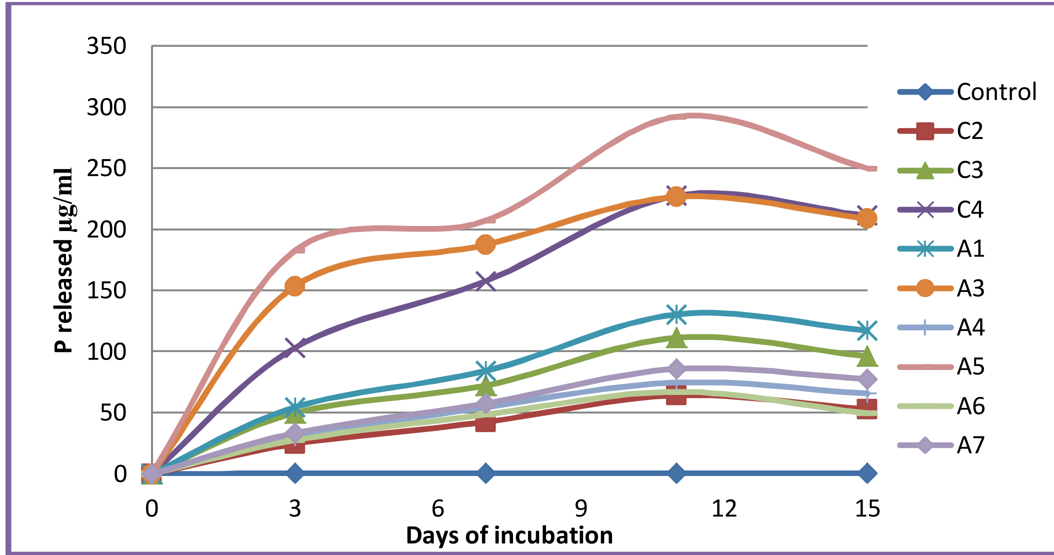


Figure 4.15 Release of soluble phosphate in liquid PVK*[1] medium.

In PVK*[1] medium the highest and lowest amounts of released phosphate being recorded for A5 and C2 isolates which were 292.4 µg/ml and 64.2 µg/ml P after 11 days of incubation, respectively. Other phosphate dissolvers C3, C4, A1, A3, A4, A6 and A7 released phosphate of 111.3, 227.6, 130.5, 226.7, 74.8, 67.2 and 85.9 µg/ml, respectively, after 11 days of incubation (Figure 4.15).

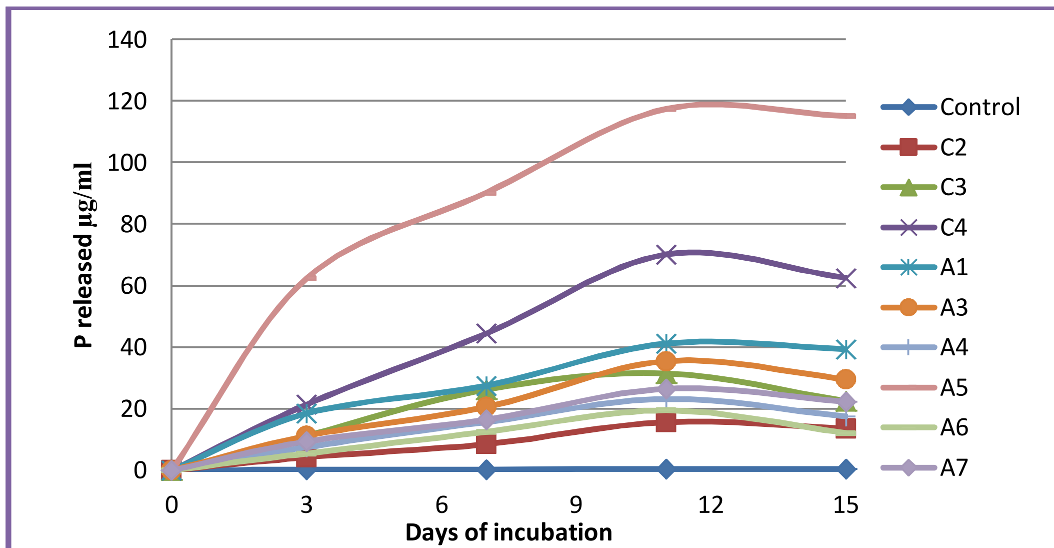


Figure 4.16 Release of soluble phosphate in liquid PVK*[2] medium.

In PVK*[2] medium the highest and lowest amount of released phosphate were recorded with A5 and C2 isolates which released 117.5 µg/ml and 15.7 µg/ml after 11 days of incubation, respectively. Other phosphate dissolvers C3, C4 A1, A3, A4, A6 and A7 were released phosphate of 31.5, 70.2, 41.2, 35.5, 23.2, 19.5 and 26.5 µg/ml, respectively, after 11 days of incubation (Figure 4.16).

The extent of phosphate released by phosphate-dissolvers was found to vary among the isolates. The amount of soluble phosphate decreased (immobilized) after 11 days of incubation which differed from organism to organism.

The order of sequence on the basis of mineralization of organic phosphate was as follows

Bacillus > *Proteus* > *Serratia* > *Pseudomonas* > *Micrococcus.*

Molla *et al*. (1983) also found that *Bacillus* was the most efficient in releasing P from organic source through mineralization.

Species of *Bacillus*, *Proteus* and *Serratia* were found to be the most efficient organisms in mineralizing organic phosphate in both modified NBRIP and PVK liquid culture media. Species of *Micrococcus* and *Pseudomonas* ranked next in mineralizing organic phosphate in modified NBRIP liquid media. It was also found that the mineralizing capability of the species of *Micrococcus* and *Pseudomonas* in modified PVK liquid medium was slightly different from that was found in modified NBRIP medium.

4.10 Preliminary Screening of Lead and Cadmium Tolerant Bacteria

Lead and cadmium tolerant bacterial isolates were selected by using Pb and Cd concentrations at different levels in NB, NBRIP and PVK media as growth medium for the isolates. A total 13 isolates were selected as Pb and Cd tolerant strains. Among 13 isolates, 9 phosphate dissolving isolates (C2, C3, C4, A1, A3,

A4, A5, A6 and A7) were found as Pb tolerant strains based on growth in the concentration range from 30 to 500 µg/ml and Cd tolerant strains based on growth in the concentration range from 15 to 200 µg/ml in NB, NBRIP and PVK media. These isolates were further used to determine the minimum inhibitory concentrations (MICs) and maximum tolerable concentrations (MTCs) to metal treatment.

4.11 Bacterial Growth and Tolerance to Lead and Cadmium Ions in Liquid Media

A total of 13 isolated strains were identified which showed tolerance to Pb and Cd up to certain concentrations. The presence of bacterial growth in Pb and Cd containing three culture media- Nutrient broth (NB), NBRIP broth, and PVK broth was observed and recorded. Results obtained from this study are presented in Table 4.22 to 4.27.

4.11.1 Bacterial growth and tolerance to lead

Table 4.22, 4.23 and 4.24 show the presence or absence of bacterial growth at different concentration of Pb^{2+} ion in NB, NBRIP and PVK media respectively.

4.11.1.1 Bacterial growth and tolerance to lead in nutrient broth (NB) medium

Bacterial growth and tolerance to lead in NB medium was, showed that C4, A1 and A5 were highly capable to grow at higher concentrations of Pb^{2+} up to 150 µg/ml whereas C3 and A7 were sensitive to Pb^{2+} concentration at 75 µg/ml. Remaining isolates were also resistant to Pb^{2+} at variable concentrations (Table 4.16).

Table 4.16 Bacterial growth in presence of lead in Nutrient Broth (NB) medium.

Isolates	Concentration of Pb (µg/ml)								
	15	20	30	50	75	100	120	150	200
C2	+	+	+	+	+	-			
C3	+	+	+	+	-				
C4	+	+	+	+	+	+	+	+	-
A1	+	+	+	+	+	+	+	+	-
A3	+	+	+	+	+	-			
A4	+	+	+	+	+	+	+	-	
A5	+	+	+	+	+	+	+	+	-
A6	+	+	+	+	+	+	-		
A7	+	+	+	+	-				

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.11.1.2 Bacterial growth and tolerance to lead in NBRIP broth medium

Bacterial growth and tolerance to lead in NBRIP liquid medium was studied, isolates showed more tolerance to Pb concentration than NB medium. Isolates A1, A4 and A5 were highly capable to grow at higher concentrations of Pb up to 300 µg/ml whereas isolate A7 was found to grow up to Pb concentration of 100 µg/ml. Isolates A3 and A6 were capable to grow at concentrations of Pb up to 200 µg/ml. Remaining isolates were also resistant to Pb at variable concentrations (Table 4.17).

Table 4.17 Bacterial growth in presence of lead in NBRIP liquid medium.

Isolates	Concentration of Pb (µg/ml)								
	50	75	100	120	150	200	250	300	350
C2	+	+	+	+	+	-			
C3	+	+	+	+	-				
C4	+	+	+	+	+	+	+	-	
A1	+	+	+	+	+	+	+	+	-
A3	+	+	+	+	+	+	-		
A4	+	+	+	+	+	+	+	+	-
A5	+	+	+	+	+	+	+	+	-
A6	+	+	+	+	+	+	-		
A7	+	+	+	-					

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.11.1.3 Bacterial growth and tolerance to lead in PVK broth medium

In PVK liquid medium, isolates A1, A4 and A5 were highly capable to grow at higher concentrations of Pb up to 250 µg/ml whereas A7 was found to grow up to Pb concentration of 100 µg/ml. Isolates C2, C3 and A3 were capable to grow at concentrations of Pb up to 120 µg/ml. Remaining isolates were also resistant to Pb at variable concentrations (Table 4.18).

Table 4.18 Bacterial growth in presence of lead in PVK liquid medium.

Isolates	**Concentration of Pb (µg/ml)**								
	30	50	75	100	120	150	200	250	300
C2	+	+	+	+	+	-			
C3	+	+	+	+	+	-			
C4	+	+	+	+	+	+	+	-	
A1	+	+	+	+	+	+	+	+	-
A3	+	+	+	+	+	-			
A4	+	+	+	+	+	+	+	+	-
A5	+	+	+	+	+	+	+	+	-
A6	+	+	+	+	+	+	-		
A7	+	+	+	+	-				

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.11.2 Bacterial growth and tolerance to cadmium

Table 4.19, 4.20 and 4.21, show the presence or absence of bacterial growth at different concentrations of Cd ion in NB, NBRIP and PVK broth media respectively.

4.11.2.1 Bacterial growth and tolerance to cadmium in nutrient broth (NB) medium

Bacterial growth and tolerance to cadmium in nutrient broth medium, indicated that A1 isolate showed growth at higher concentration of Cd up to 150 µg/ml whereas A5 was sensitive to Cd concentration at 50 µg/ml. Cadmium

concentration of 75 µg/ml was inhibitory for A4 isolate. Remaining isolates were also resistant to Cd concentration at varying concentrations (Table 4.25).

Table 4.19 Bacterial growth in presence of cadmium in NB medium.

Isolates	Concentration of Cd (µg/ml)								
	15	20	30	50	75	100	120	150	200
C2	+	+	+	+	+	+	+	-	
C3	+	+	+	+	+	-			
C4	+	+	+	+	+	-			
A1	+	+	+	+	+	+	+	+	-
A3	+	+	+	+	+	-			
A4	+	+	+	+	-				
A5	+	+	+	-					
A6	+	+	+	+	+	-			
A7	+	+	+	+	+	+	-		

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.11.2.2 Bacterial growth and tolerance to cadmium in liquid NBRIP broth medium

Bacterial growth and tolerance to cadmium in NBRIP medium, revealed that C2, A1 and A3 were more tolerant to Cd concentration of 200 µg/ml whereas A5 was sensitive to Cd concentration at 75 µg/ml. Concentration of 120 µg/ml of Cd was inhibitory for C3, C4 and A6 isolates. Remaining isolates were also resistant to Cd concentration at varying concentrations (Table 4.26).

Table 4.20 Bacterial growth in presence of cadmium in NBRIP medium.

Isolates	Concentration of Cd (µg/ml)								
	20	30	50	75	100	120	150	200	250
C2	+	+	+	+	+	+	+	+	-
C3	+	+	+	+	+	+	-		
C4	+	+	+	+	+	+	-		
A1	+	+	+	+	+	+	+	+	-
A3	+	+	+	+	+	+	+	+	-
A4	+	+	+	+	+	-			
A5	+	+	+	-					
A6	+	+	+	+	+	-			
A7	+	+	+	+	+	+	-		

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.11.2.3 Bacterial growth and tolerance to cadmium in liquid PVK broth medium

In PVK broth medium, concentration of 250 μg/ml of Cd was inhibitory for A1 and A3 isolates. Whereas A4 and A6 isolates were sensitive to Cd concentration at 120 μg/ml. Remaining isolates were also resistant to Cd concentration at varying concentrations (Table 4.27).

Table 4.21 Bacterial growth in presence of cadmium in PVK medium.

Isolates	Concentration of Cd (μg/ml)								
	20	30	50	75	100	120	150	200	250
C2	+	+	+	+	+	+	+	-	
C3	+	+	+	+	+	+	-		
C4	+	+	+	+	+	-			
A1	+	+	+	+	+	+	+	+	-
A3	+	+	+	+	+	+	+	+	-
A4	+	+	+	+	+	+	-		
A5	+	+	+	+	+	-			
A6	+	+	+	+	+	+	-		
A7	+	+	+	+	+	-			

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.12 Determination of Minimum Inhibitory Concentration of Lead and Cadmium in Different Media

Minimum level of heavy metal that inhibits the growth of bacteria is termed as minimum inhibitory concentrations (MICs). The MIC values against Pb and Cd of 9 different isolates in three media are shown in Figure 4.17 and 4.18, respectively and also in Appendix C 7.

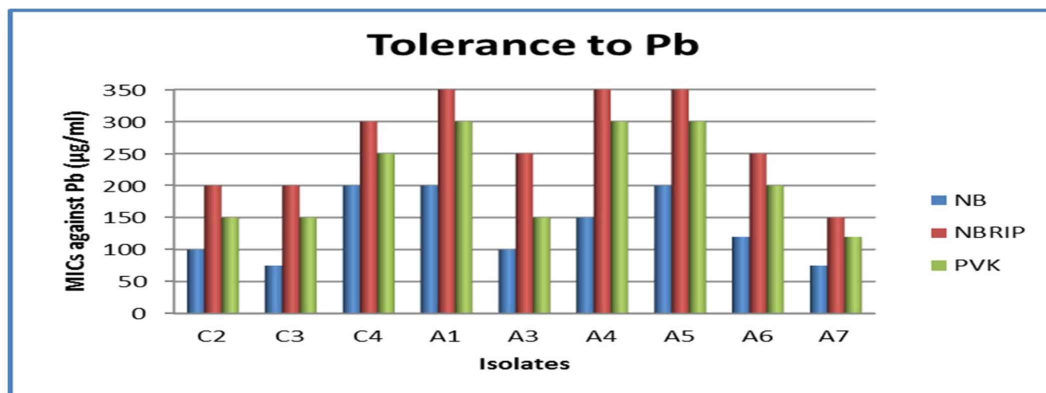


Figure 4.17 Comparison of MICs against Pb in three growth media (NB, NBRIP, and PVK).

Data presented in Figure 4.17 (Appendix C 7) indicated that 4 isolates C4, A1, A4, and A5 showed highest resistant to Pb concentration and grew even above the concentration of 200 µg/ml of Pb in different media. Isolate A7 was the least tolerant to Pb according to above results and could tolerate up to 75, 150 and 120 µg/ml in NB, NBRIP and PVK media, respectively. Isolates C2, C3, A3, A6 and A7 could also tolerate Pb concentrations ranging from 75 to 250 µg/ml in various culture media.

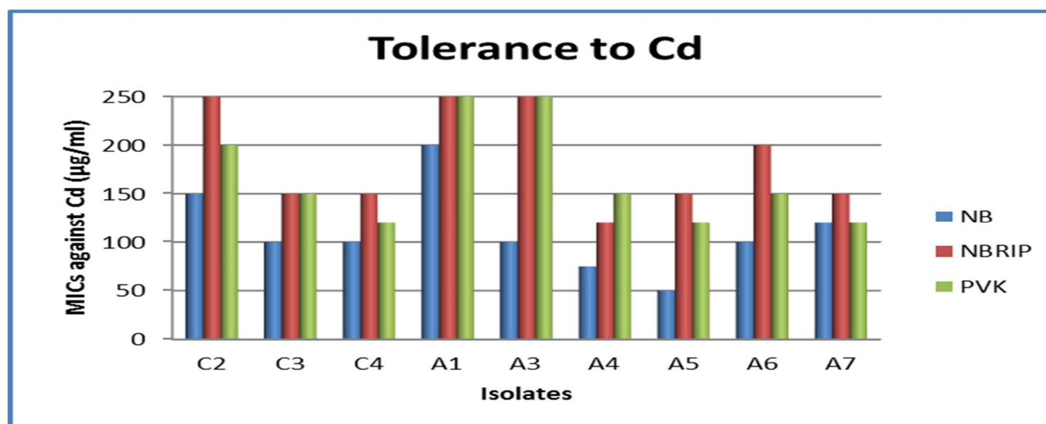


Figure 4.18 Comparison of MICs against Cd in three growth media (NB, NBRIP, and PVK).

Data presented on minimum inhibitory concentrations (MICs) of Cd, in Figure 4.18 and Appendix C 7 also show that C2, A1 and A3 isolates were the most tolerant strains to Cd which could tolerate up to 250 µg/ml. The second most

tolerant strains was A6 that tolerated Cd concentration up to 200 µg/ml. Minimum inhibitory concentration for other isolates ranged from 50 to 150 µg/ml.

In this context, toxicity test in liquid media such as nutrient broth (NB), Pikovskaya broth (PVK) and NBRIP broth media was conducted to determine the optimum concentrations of metals as because the liquid media maintain a high free metal concentration in solution at which the bacterial cultures could grow freely. It was observed that the MIC values were higher in NBRIP broth media than that in nutrient broth (NB) and Pikovskaya broth (PVK) media.

In this study, the metal resistant tests showed that some of the selected isolates had MIC above 300 µg/ml against Pb and above 200 µg/ml against Cd. The MIC of identified strains for Pb^{2+} ranged from 75 to 200 µg/ml in nutrient broth, 150 to 350 µg/ml in NBRIP broth and 120 to 300 µg/ml in PVK broth media. The MIC of identified strains for Cd^{2+} ranged from 50 to 200 µg/ml in nutrient broth, 120 to 250 µg/ml in NBRIP broth and 100 to 250 µg/ml in PVK broth media respectively. Rajbanshi (2008) reported the results of MIC of 150 to 500 µg/ml for chromium and 200 to 300 µg/ml for copper for different bacteria. Brocklehrust and Morby (2000) reported that in response to toxic concentrations of heavy metal ions, *Escherichia coli* strains exhibited varying degrees of tolerance (3 to 14-fold) both to the adaptive metal and its congeners.

4.13 Determination of Maximum Tolerable Concentrations of Lead and Cadmium in Different Growth Media

Maximum tolerable concentrations (MTCs) of all the strains against Pb and Cd revealed that isolated strains were capable of growing at higher concentrations of heavy metal in NB, NBRIP and PVK media. Comparison among MTCs in three culture media (NB, NBRIP and PVK broth media) containing metal ions (Pb and Cd) is given in Figure 4.19 and 4.20, respectively and also in Appendix C 8.

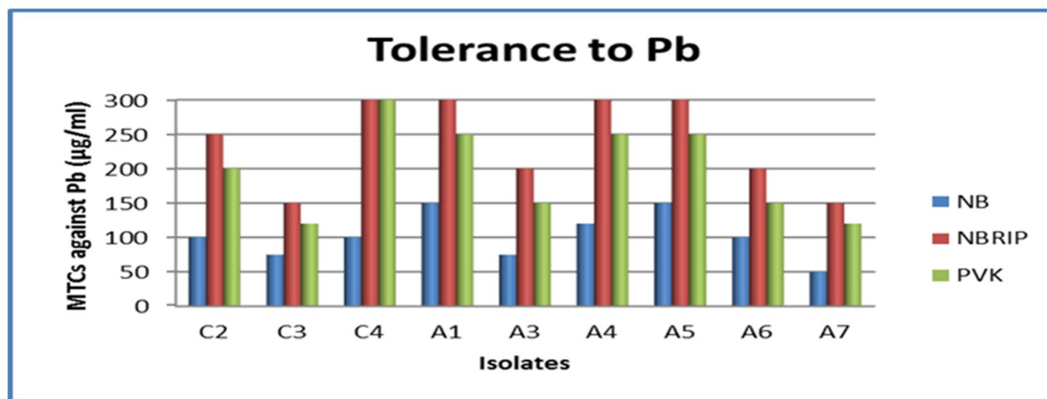


Figure 4.19 Comparison of MTCs against Pb in three growth media (NB, NBRIP, and PVK).

Maximum tolerable concentrations (MTCs) of Pb, presented in Figure 4.19 show that C4, A1, A4 and A5 isolates were the most tolerant strain against Pb in NBRIP medium which could tolerate up to 300 µg/ml. The second most tolerant strain was C2 that tolerated Pb concentration up to 250 µg/ml in NBRIP media. Maximum tolerable concentrations (MTCs) for other isolates ranged from 50 to 200 µg/ml in three media. Maximum tolerable concentrations (MTCs) for other isolates ranged from 30 to 120 µg/ml in three media (Figure 4.19).

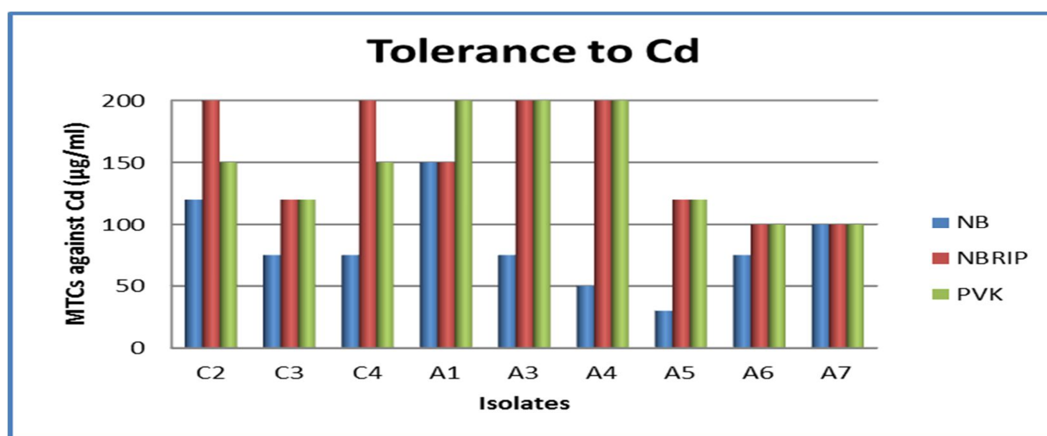


Figure 4.20 Comparison of MTCs against Cd in three growth media (NB, NBRIP, and PVK).

Maximum tolerable concentrations (MTCs) of Cd (Figure 4.20) revealed that C2, C4, A3, and A4 isolates were the most tolerant strain against Cd in NBRIP media which could tolerate up to 200 µg/ml of Cd. The second most tolerant strains were A1 that tolerated Cd concentration up to 150 µg/ml in NBRIP media.

Toxicity testing in liquid media allows a good evaluation of metal toxicity in polluted environments, such as industrial effluents and sewage sludge leachates (Hassen *et al.,* 1998). Hassen *et al.* (1998) also tested the levels of tolerance of environmental bacteria to the different divalent metal ions including Cu^{2+}, Co^{2+}, Cd^{2+} and Zn^{2+} in nutrient broth and reported that the test in liquid media was sensitive at concentrations 10 to 1000 times lower than those obtained in solid media**.**

The results indicated that toxicity to metal concentration of soil bacteria depends on concentration of metals, culture media (NB, NBRIP and PVK) as well as bacterial strains.

The current study revealed that 9 phosphate dissolving isolates showed the metal tolerance against Pb and Cd. Lead was found to be more tolerable metal ion whereas Cd appeared to be toxic to the bacterial strain. Higher MIC or MTC values for Pb than that for Cd for each strain identified indicated that Cd is more toxic than Pb for soil bacteria. So, the order of toxicity of two metals is Cd > Pb.

4.14 Bacterial Growth and Tolerance to Heavy Metal Ions in Soil

Of the 13 isolated strains, only 9 phosphorus dissolving strains were selected to test the tolerance of bacteria against heavy metals (Pb and Cd) and concentrations in different soils. The soils mixed with organic (compost and oil cake) and inorganic (rock phosphate) phosphorus were used as P sources. The presence of bacterial growth in three soils such as silt loam, silty clay loam and sandy loam was observed at metal concentrations (15,000, 20,000 and 30,000 µg/g) of Pb and Cd. The growth appearance was recorded at specified metal concentration.

4.14.1 Bacterial growth and tolerance to lead

The presence or absence of bacterial growth at different concentrations of Pb in soils is presented in Table 4.22 to 4.30.

Table 4.22 Bacterial growth in presence of Pb in silt loam soil after 24 h of incubation.

Isolates	Treatment											
	15000 µg/g of Pb^{2+}				20,000 µg/g of Pb^{2+}				30,000 µg/g of Pb^{2+}			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	+	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.14.1.1 Bacterial growth and tolerance to Pb in silt loam soil

In silt loam soil, some isolates exhibited more tolerance to Pb concentration than sandy loam soil. After one day incubation, all isolates were capable to grow at higher concentration of Pb up to 30,000 µg/g (Table 4.23), which were inhibited after 15 days and 30 days of incubation. After 24 hours of incubation, all isolates grew up to 30,000 µg/g. After 15 days of incubation, isolates C2, C3, and A5 grew up to 20,000 µg/g which were inhibited at 30,000 µg/g. Isolates C4, A3, A4 and A7 grew at 20,000 µg/g and 30,000 µg/g of Pb^{2+} after 15 and 30 days of incubation, respectively. But their growth rate was decreasedwith the passage of time. Remaining isolates were also resistant to Pb concentration at varying concentrations (Table 4.22, 4.23 and 4.24).

Table 4.23 Bacterial growth in presence of Pb in silt loam soil after 15 days of incubation.

Isolates	Treatment											
	15000 µg/g of Pb				20,000 µg/g of Pb				30,000 µg/g of Pb			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	-	-	-	-
C3	+	+	+	+	-	+	+	+	-	-	-	-
C4	+	+	+	+	+	+	+	+	-	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	-	-	-	-
A6	+	+	+	+	+	+	+	+	-	+	+	+
A7	+	+	+	+	+	+	+	+	-	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth.

Table 4.24 Bacterial growth in presence of Pb in silt loam soil after 30 days of incubation.

Isolates	Treatment											
	15000 µg/g of Pb				20,000 µg/g of Pb				30,000 µg/g of Pb			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	-	+	+	+	-	-	-	-
C3	+	+	+	+	-	+	+	+	-	-	-	-
C4	+	+	+	+	+	+	+	+	-	+	+	-
A1	+	+	+	+	+	+	+	+	-	-	-	-
A3	+	+	+	+	+	+	+	+	+	+	+	-
A4	+	+	+	+	+	+	+	+	+	+	+	-
A5	+	+	+	+	+	+	+	+	-	-	-	-
A6	+	+	+	+	+	+	+	+	-	-	-	-
A7	+	+	+	+	+	+	+	+	-	+	+	-

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.14.1.2 Bacterial growth and tolerance to Pb in silty clay loam soil

In silty clay loam soil, some isolates exhibited more tolerance to Pb concentration than silt loam soil. After one day of incubation it was found that all isolates were capable to grow at higher concentration of Pb up to 30,000 µg/g (Table 4.25), which were inhibited after 15 and 30 days of incubation.

Table 4.25 Bacterial growth in presence of Pb in silty clay loam soil after 24 hours of incubation.

Isolates	Treatment											
	15000 µg/g of Pb				20,000 µg/g of Pb				30,000 µg/g of Pb			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	+	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth.

After 15 days of incubation, isolates C3, and A3 grew up to 20,000 µg/g which were inhibited at Pb concentration of 30,000 µg/g. Isolates C2, C4, A1, A4, A5, A6 and A7 grew at 20,000 µg/g and 30,000 µg/g of Pb after 15 and 30 days of incubation respectively (Table 4.25, 4.26 and 4.27).

Table 4.26 Bacterial growth in presence of Pb in silty clay loam soil after 15 days of incubation.

Isolates	Treatment											
	15000 µg/g of Pb				20,000 µg/g of Pb				30,000 µg/g of Pb			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	-	-	-	-
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	-	-	-	-
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	-	+	+	-

"+" indicates the presence of growth; "-" indicates the absence of growth.

Table 4.27 Bacterial growth in presence of Pb in silty clay loam soil after 30 days of incubation.

Isolates	Treatment											
	15000 µg/g of Pb				20,000 µg/g of Pb				30,000 µg/g of Pb			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	-	+	+	+	-	-	-	-
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	-	-	-	-
A4	+	+	+	+	-	+	+	+	-	+	+	-
A5	+	+	+	+	+	+	+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	-	+	+	+
A7	+	+	+	+	+	+	+	+	-	+	+	-

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.14.1.3 Bacterial growth and tolerance to Pb in sandy loam soil

In sandy loam soil, some isolates exhibited considerable tolerance to Pb concentration. After 24 hours of incubation all isolates were capable to grow at higher concentration of Pb up to 30,000 µg/g (Table 4.28), which were inhibited after 15 days and 30 days of incubation. After 15 days of incubation, isolates C4, and A5 grew up to 20,000 µg/g which were inhibited at Pb concentration 30,000 µg/g. After 15 days of incubation isolates C2, C3, A1, A3, A4, A6 and A7 grew up to 30,000 µg/g Pb concentration and among them isolates C2, A3 and A7 were inhibited after 30 days of incubation. Remaining isolates were also resistant to Pb at different concentrations (Table 4.28, 4.29 and 4.30) (Figure 4.21).

Table 4.28 Bacterial growth in presence of Pb in sandy loam soil after 24 hours of incubation.

Isolat-es	Treatment											
	15000 µg/g of Pb				20,000 µg/g of Pb				30,000 µg/g of Pb			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	+	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth.

Table 4.29 Bacterial growth in presence of Pb in sandy loam soil after 15 days of incubation.

Isolat-es	Treatment											
	15000 µg/g of Pb				20,000 µg/g of Pb				30,000 µg/g of Pb			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	+	+	+	+	-	-	-	-
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	-	-	-	-
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	-	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth

Table 4.30 Bacterial growth in presence of Pb in sandy loam soil after 30 days of incubation.

Isolates	Treatment											
	15000 µg/g of Pb				20,000 µg/g of Pb				30,000 µg/g of Pb			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	-	-	-	-
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	-	+	+	-	-	-	-	-
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	-	-	-	-
A4	+	+	+	+	+	+	+	+	-	+	+	-
A5	+	+	+	+	+	+	+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	-	+	+	+
A7	+	+	+	+	+	+	+	+	-	-	-	-

"+" indicates the presence of growth; "-" indicates the absence of growth.

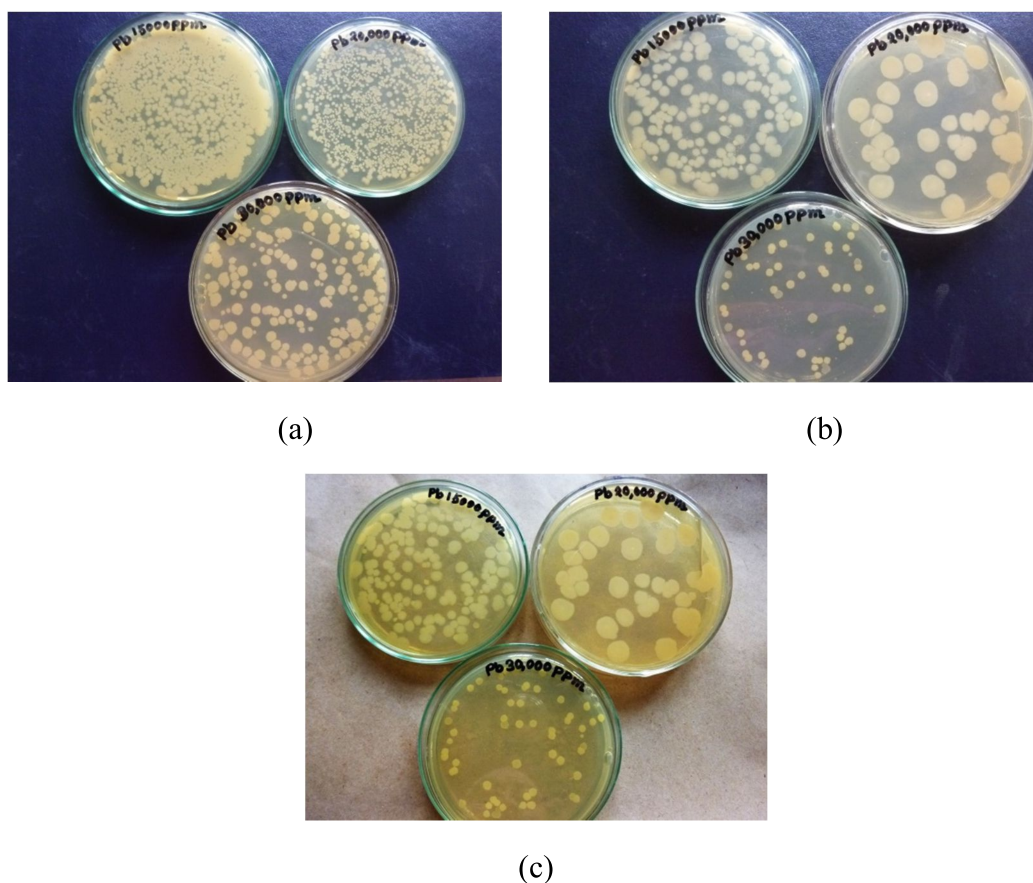


Figure 4.21 Growth of A4 isolates after 24 hours (a); 15 days (b); and 30 days (c) of incubation of Pb in sandy loam soil.

Identified isolates grew up at high concentrations of metals and showed variation varying with soil type and incubation time. Among the three soils, bacterial metal tolerance is high in silty clay loam soil and this might be due to the properties of soil. According to the result shown in Table 4.36, two isolates *Micrococcus luteus* (C2) and *Bacillus cereus* (C4) were isolated from contaminated soil (EPZ area) tolerated Pb up to 30,000 µg/g and 4 isolates *Bacillus subtilis* (A1), *Serratia marcescens* (A4), *Bacillus amyloliquefaciens* (A5), *Pseudomonas aeruginosa* (A6) and *Proteus mirabilis* (A7), isolated from non-contaminated agricultural soil (Dhamrai and Pabna) could tolerate Pb up to 30,000µg/g. Brocklehrust and Morby (2000) reported that in response to toxic concentrations of heavy metal

ions, *Escherichia coli* strains exhibited varying degrees of tolerance (3 to 14-fold) both to the adaptive metal and its congeners.

4.14.2 Bacterial growth and tolerance to cadmium

Results presented in Table 4.31 to Table 4.39 show the appearance or disappearance of bacterial growth in silt loam, silty clay loam and sandy loam soil after adding different concentrations of Cd ion.

4.14.2.1 Bacterial growth and tolerance to Cd in silt loam soil

In silt loam soil, some isolates showed more tolerance to Cd concentration than sandy loam soil. All the isolates were capable to grow at the concentration of Cd up to 30,000 µg/g (Table 4.31) after 24 hours of incubation. After 15 days of incubation, isolates C3, A1, A4 and A6 grew up to 30,000 µg/g which were inhibited after 30 days of incubation. Isolates C3, A1 and A4 grew at 20,000 µg/g and 30,000 µg/g of Cd after 15 days and 30 days of incubation, respectively (Table 4.31, 4.32 and 4.33).

Table 4.31 Bacterial growth in presence of Cd in silt loam soil after 24 hours of incubation.

Isolates	Treatment											
	15000 µg/g of Cd				20,000 µg/g of Cd				30,000 µg/g of Cd			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	+	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth.

Table 4.32 Bacterial growth in presence of Cd in silt loam soil after 15 days of incubation.

Isolates	Treatment											
	15000 µg/g of Cd				20,000 µg/g of Cd				30,000 µg/g of Cd			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	-	-	-	-
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	+	+	+	+	-	-	-	-
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	-	-	-	-
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	-	-	-	-
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	-	-	-	-

"+" indicates the presence of growth; "-" indicates the absence of growth.

Table 4.33 Bacterial growth in presence of Cd in silt loam soil after 30 days of incubation.

Isolat-es	Treatment											
	15000 µg/g of Cd				20,000 µg/g of Cd				30,000 µg/g of Cd			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	-	-	-	-
C3	+	+	+	+	+	+	+	+	-	+	+	-
C4	+	+	+	+	+	+	+	+	-	-	-	-
A1	+	+	+	+	+	+	+	+	-	+	+	-
A3	+	+	+	+	+	+	+	+	-	-	-	-
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	-	-	-	-
A6	+	+	+	+	+	+	+	+	-	-	-	-
A7	+	+	+	+	+	+	+	+	-	-	-	-

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.14.2.2 Bacterial growth and tolerance to Cd in silty clay loam soil

In silty clay loam soil, some isolates exhibited more tolerance to Cd concentration. After 24 hours of incubation, all isolates were capable to grow at higher concentration of Cd up to 30,000 µg/g (Table 4.34), which were inhibited after 15 and 30 days of incubation. After 15 days of incubation, isolates C2, C4, A1, A3, A4 and A7 grew up to 30,000 µg/g of Cd. And isolates C2, C4, A3, A4 and A7 grew up to 30,000 µg/g of Cd after 30 days of incubation. Isolates C3, A1, A5 and A6 were inhibited at 30,000 µg/g concentrations of Cd after 30 days of incubation (Table 4.34, 4.35 and 4.36).

Table 4.34 Bacterial growth in presence of Cd in silty clay loam soil after 24 hours of incubation.

Isolates	Treatment											
	15000 µg/g of Cd				20,000 µg/g of Cd				30,000 µg/g of Cd			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	+	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth.

Table 4.35 Bacterial growth in presence of Cd in silty clay loam soil after 15 days of incubation.

Isolates	Treatment											
	15000 µg/g of Cd				20,000 µg/g of Cd				30,000 µg/g of Cd			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	-	-	-	-
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	-	+	-	+
A3	+	+	+	+	+	+	+	+	+	+	+	-
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	-	-	-	-
A6	+	+	+	+	+	+	+	+	-	-	-	-
A7	+	+	+	+	+	+	+	+	+	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth.

Table 4.36 Bacterial growth in presence of Cd in silty clay loam soil after 30 days of incubation.

Isolates	Treatment											
	15000 µg/g of Cd				20,000 µg/g of Cd				30,000 µg/g of Cd			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	-	+	+	+
C3	+	+	+	+	+	+	+	+	-	-	-	-
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	-	-	-	-
A3	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	-	+	+	+
A5	+	+	+	+	+	+	+	+	-	-	-	-
A6	+	+	+	+	+	+	+	+	-	-	-	-
A7	+	+	+	+	+	+	+	+	+	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth.

4.14.2.3 Bacterial growth and tolerance to Cd in sandy loam soil

In sandy loam soil, after one day of incubation all isolates were capable to grow at higher concentration of Cd up to 30,000 µg/g which were inhibited after 15 and 30 days of incubation. After 15 and 30 days of incubation, isolates C3, A4 and A6 grew up to 20,000 µg/g and 30,000 µg/g of Cd^{2+} concentrations respectively. Other isolates were inhibited at Cd concentration 30,000 µg/g (Table 4.37, 4.38 and 4.39) (Figure 4.22).

Table 4.37 Bacterial growth in presence of Cd in sandy loam soil after 24 hours of incubation.

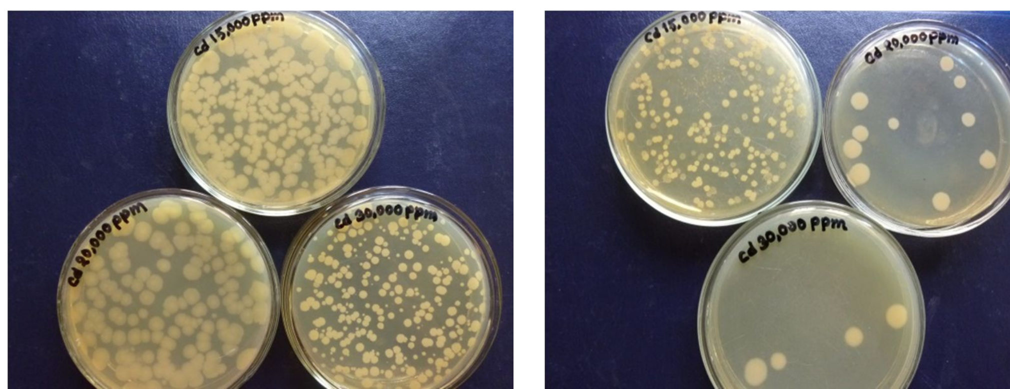
Isolates	Treatment											
	15000 µg/g of Cd				20,000 µg/g of Cd				30,000 µg/g of Cd			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	+	+	+	+	+	+	+	+
A1	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	+	+	+	+

"+" indicates the presence of growth; "-" indicates the absence of growth.

Table 4.38 Bacterial growth in presence of Cd in sandy loam soil after 15 days of incubation.

Isolates	Treatment											
	15000 µg/g of Cd				20,000 µg/g of Cd				30,000 µg/g of Cd			
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
C2	+	+	+	+	+	+	+	+	-	-	-	-
C3	+	+	+	+	+	+	+	+	+	+	+	+
C4	+	+	+	+	+	+	+	+	-	-	-	-
A1	+	+	+	+	+	+	+	+	-	-	-	-
A3	+	+	+	+	+	+	+	+	-	-	-	-
A4	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	-	-	-	-
A6	+	+	+	+	+	+	+	+	+	+	+	+
A7	+	+	+	+	+	+	+	+	-	-	-	-

"+" indicates the presence of growth; "-" indicates the absence of growth.



(a) (b)

(c)

Figure 4.22 Growth of A4 isolates after 24 hours (a); 15 days (b); and 30 days (c) of incubation in sandy loam soil.

Table 4.39 Bacterial growth in presence of Cd in sandy loam soil after 30 days of incubation.

Isolates	**Treatment**											
	15000 µg/g of Cd				**20,000 µg/g of Cd**				**30,000 µg/g of Cd**			
	T1	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**
C2	+	+	+	+	-	+	+	+	-	-	-	-
C3	+	+	+	+	+	+	+	+	-	+	+	-
C4	+	+	+	+	+	+	+	-	-	-	-	-
A1	+	+	+	+	+	+	+	+	-	-	-	-
A3	+	+	+	+	+	+	+	+	-	-	-	-
A4	+	+	+	+	+	+	+	+	-	+	-	+
A5	+	+	+	+	+	+	+	+	-	-	-	-
A6	+	+	+	+	+	+	+	+	-	+	-	-
A7	+	+	+	+	+	+	+	+	-	-	-	-

"+" indicates the presence of growth; "-" indicates the absence of growth.

According to the results presented in Table 4.45, 2 isolates *Micrococcus luteus* (C2) and *Bacillus cereus* (C4) were isolated from contaminated soil (EPZ area) and 3 isolates *Bacillus pumilus* (A3), *Serratia marcescens* (A4) and *Proteus mirabilis* (A7) from non-contaminated agricultural soil (Dhamrai and Pabna) showed tolerance to Cd up to 30,000 µg/g.

These result showed that most of the soil bacteria have the capacity to tolerate a wide range of Pb and Cd concentration in soil system. Metal tolerance of bacteria depends on type of metal, bacterial species as well as bacterial strain. For example, *Micrococcus luteus* (C2) isolated from contaminated soil and *Bacillus pumilus* (A3) isolated from agricultural soil showed different tolerance in case of Pb and Cd in silty clay loam soil after 30 days of incubation.

The metal tolerances to Pb and Cd for bacterial isolates in the contaminated soils as well as in the uncontaminated soils were at the levels regarded as those typical for metal-resistant species. In this study, in case of Pb and Cd the results did not show the similarity in respect of tolerance in three types of soils during incubation study. But in liquid culture medium, the result showed that tolerance limit is lower than that of soil.

4.15 Result of Sequential Extraction of Lead and Cadmium in Soil

The toxicity and mobility of heavy metals mainly depends on their specific chemical forms and binding states (Ma and Rao, 1997; Kabala and Singh, 2001). The fate and transport of a heavy metal in soil depends significantly on the chemical form and speciation of the metal. Sequential extraction procedures indirectly assess the potential mobility and bioavailability of metal in soils. Bioavailability of metals decrease in the order: water soluble > exchangeable > carbonate bound > oxides bound > organic > residual (Lena and Gade, 1997). Results obtained through sequential extraction are presented in (Appendix C 9 to 26) which exhibits the distribution of various fractions of Pb and Cd in the three

soil samples (silt loam, silty clay loam and sandy loam soil) after adding different concentrations of Pb^{2+} and Cd^{2+} ions.

4.15.1 Lead in soil fractions

The distribution of various fractions of Pb in three soils at different time of incubation indicated that on an average, 26 % and 55 % of total Pb was associated with the mobile (F1 and F2) fractions in silt loam soil and in silty clay loam soil respectively. And 23 % of total Pb was associated with the mobile (F1-F3) fractions in sandy loam soil. Of total Pb, 74 %, 45 % and 77 % were immobile (F4-F6) fractions in silt loam soil (Figure 4.23 to 4.25), silty clay loam soil (Figure 4.26 to 4.28) and sandy loam soil (Figure 4.29 to 4.31). Lead extracted with H_2O and NH_4OAc are known as mobile fractions. These fractions ranged from 11 to 58% in silt loam soil, 15 to 80% in silty clay loam soil and 11 to 43% in sandy loam soil.

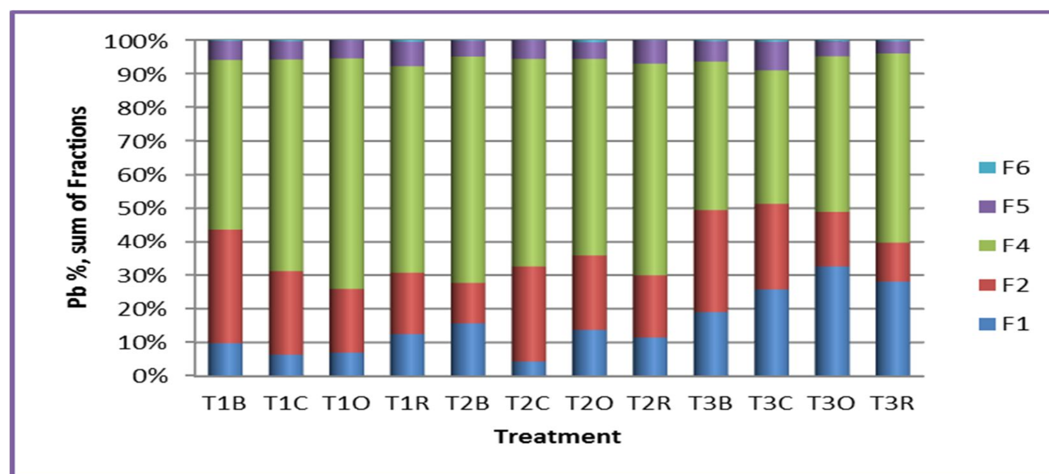


Figure 4.23 Distribution of Pb in various fractions in silt loam soil after 24 hours incubation.

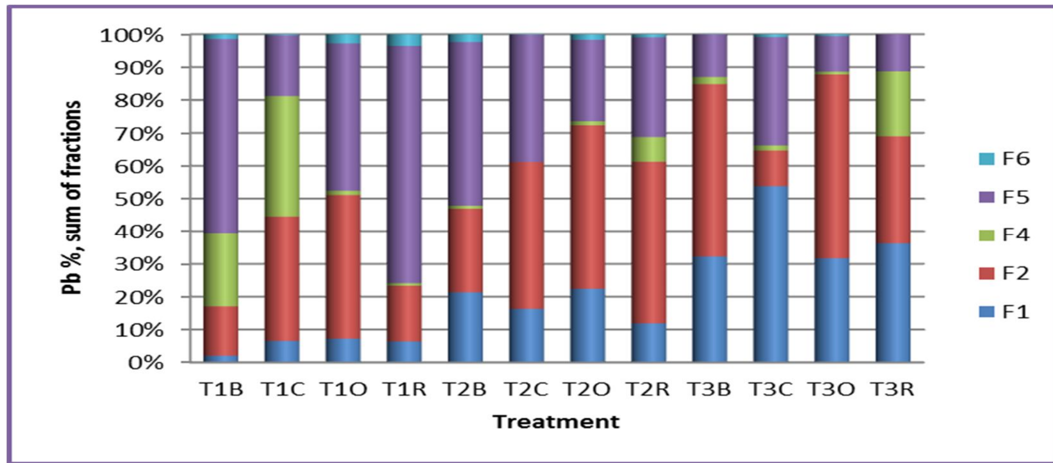


Figure 4.24 Distribution of Pb in various fractions in silt loam soil after 15 days incubation.

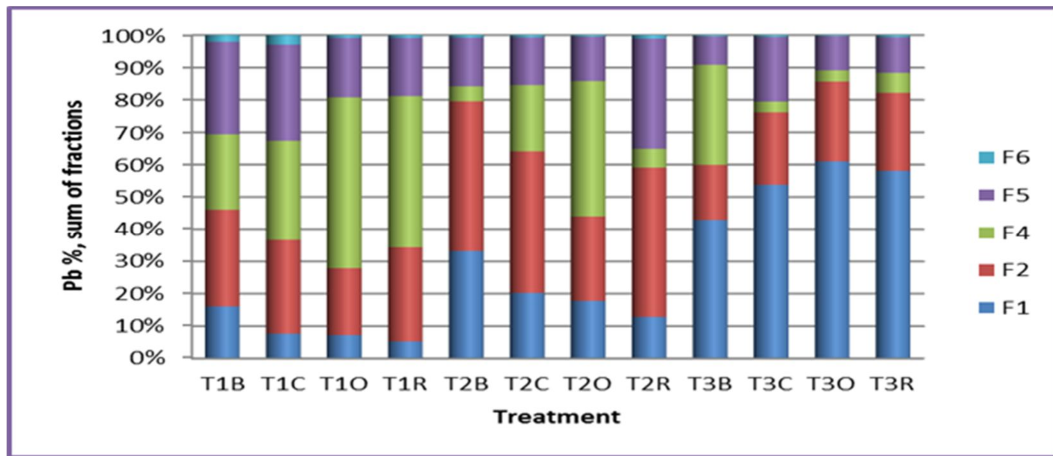


Figure 4.25 Distribution of Pb in various fractions in silt loam soil after 30 days incubation.

In silt loam soil after 24 hours incubation period Pb associated with different fractions was in order: F4 > F2 > F1 > F5 > F6; after 15 days the order of different fractions was: F2 > F5 > F1 > F4 > F6; and after 30 days different fractions was in the order of: F2 > F1 > F5 > F4 > F6 (Figure 4.23 to 4.25).

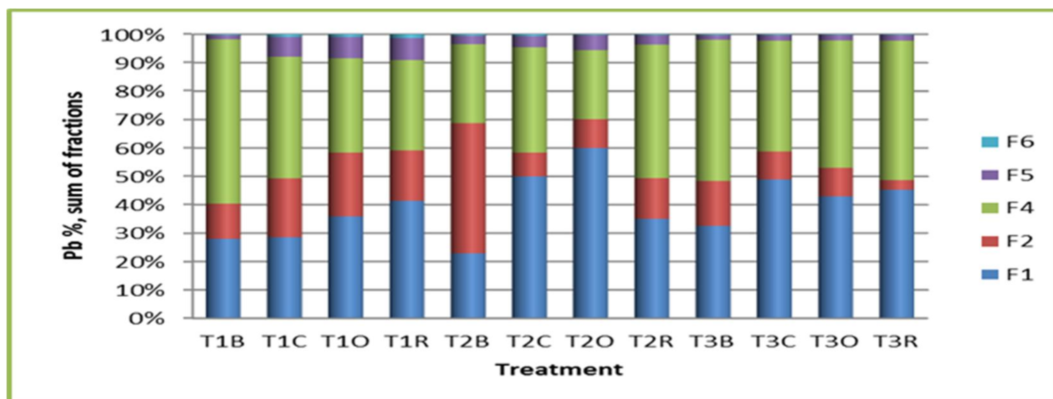


Figure 4.26 Distribution of Pb in various fractions in silty clay loam soil after 24 hours incubation.

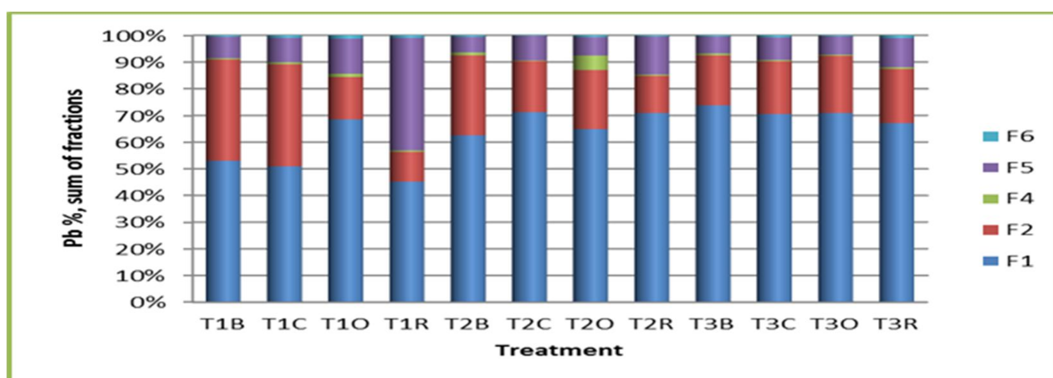


Figure 4.27 Distribution of Pb in various fractions in silty clay loam soil after 15 days incubation.

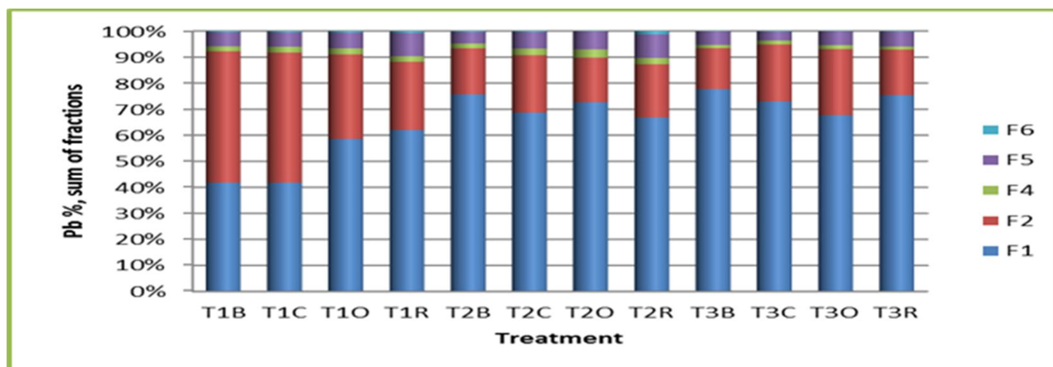


Figure 4.28 Distribution of Pb in various fractions in silty clay loam soil after 30 days incubation.

In silty clay loam soil after 24 hours of incubation period Pb associated with different fractions was in order: F4 > F1 > F2 > F5 > F6; after 15 days different the order was: F1 > F2 > F5 > F4 > F6; and after 30 days different fractions were in order: F1 > F2 > F5 > F4 > F6 (Figure 4.26 to 4.28).

Among the mobile fractions, Pb in the F1 to F2 fractions in two soils (silt loam and silty clay loam soil) indicated that Pb from contaminated sources could account for higher bioavailability and leaching. Moreover, soil properties such as contents of organic matter, carbonates, oxides as well as soil structure and profile development influence the heavy metal mobility (Kabata-Pendias and Pendias, 2001). In silt loam soil and silty clay loam soil, lead percentage in oxide bound fractions (F4) was found to be decreased with the incubation period. Similarly Pb percentage in organically bound fractions (F5) was found to be increased with the incubation period in (silt loam soil and silty clay loam) two soils.

In sandy loam soil, after 24 hours and 30 days of incubation period, Pb associated with different fractions were in the order: F4 > F3 > F5 > F2 > F6 > F1; and after 15 days different order was found: F4 > F3 > F5 > F6 > F2 > F1 (Figure 4.29 to 4.31).

The small proportion of Pb in the water soluble and exchangeable fraction suggests that Pb was relatively less mobile in sandy loam soil. Lead percentage in oxide bound fractions (F4) and carbonate fraction (F3) were also found higher in sandy loam soil. The higher concentrations of Pb in the oxide bound fraction indicated that the amount of lead in this fraction was important for lead immobilization. Oxide bound and residual fraction of Pb was found dominant which highlights that Pb is immobilized with time in these soils. For the immobilization of metal the toxicity will be decreased with the incubation time and it will help to survive the soil organisms in these high concentration in soil.

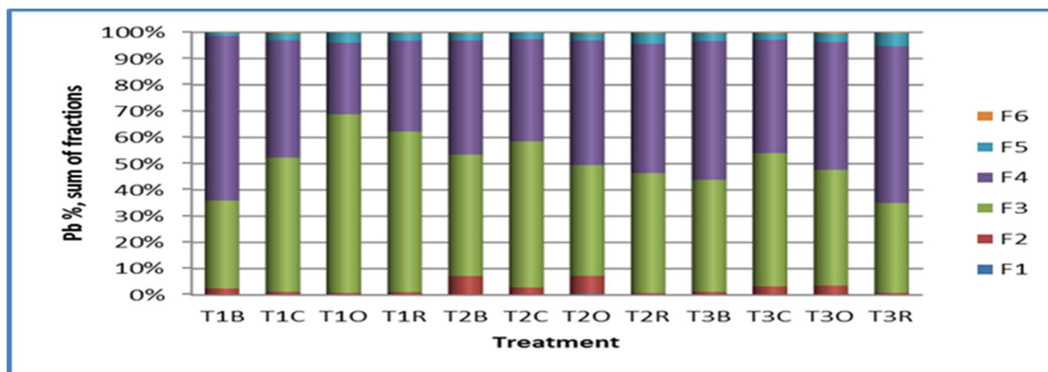


Figure 4.29 Distribution of Pb in various fractions in sandy loam soil after 24 hours incubation.

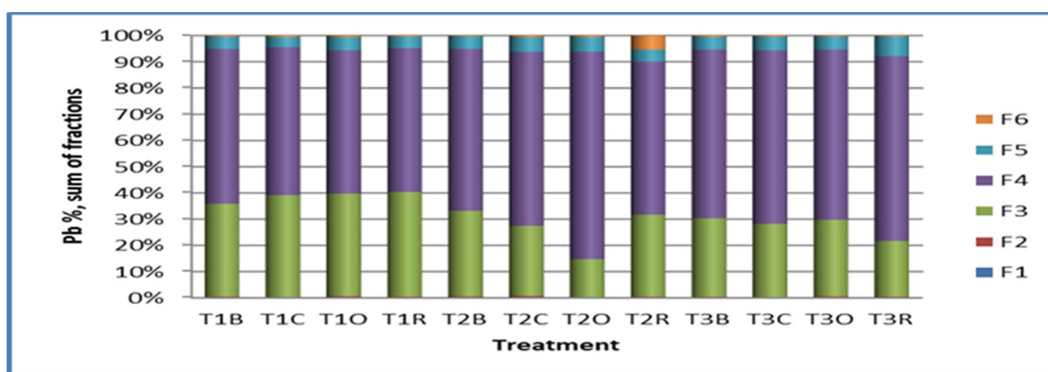


Figure 4.30 Distribution of Pb in various fractions in sandy loam soil after 15 days incubation.

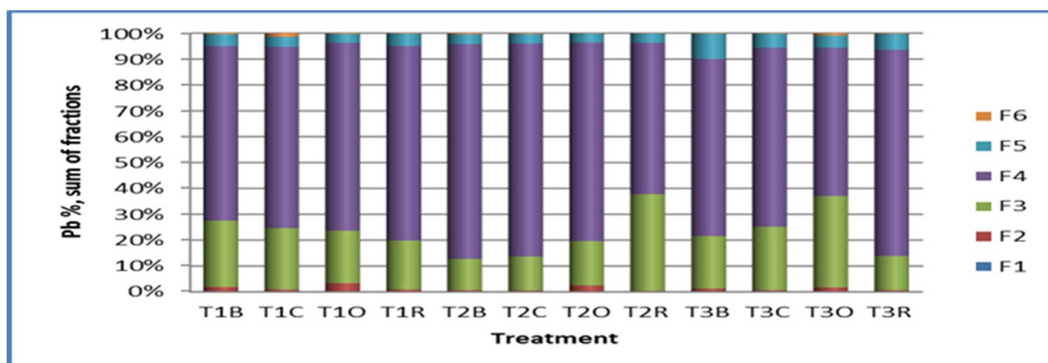


Figure 4.31 Distribution of Pb in various fractions in sandy loam soil after 30 days incubation.

Jalali and Khanlari (2008) observed that the proportions of heavy metals including Pb associated with the most weakly bound fraction were transformed to more stable fractions including carbonate-bound, Fe-Mn oxides-bound and

residual fractions with increasing incubation period due to high sorption capacities of calcareous soils.

The increase in exchangeable Pb might be due to the reason that the influence of organic materials on the chemical forms of metal in soil are pH-dependent and at high pH, soluble organo-metallic complexes may form which can increase metal solubility (Gregson and Alloway, 1984). Moreover, a decrease in organic matter bound Pb is observed indicating the transformation of organic matter bound Pb to exchangeable Pb may be due to formation of soluble organo-metallic complexes.

The immobilization of lead in soil because of the fact that, chemistry of lead in soil is mainly affected by specific adsorption, precipitation of stable compounds, and formation of relatively stable complexes with organic matter (de Santiago-Martin *et al.,* 2013).

4.15.2 Cadmium in soil fraction

The distribution of various fractions of Cd in three soils at different time of incubation indicated that on an average, 58% and 54% of total Cd was associated with the mobile (F1+F2) fractions in silt loam soil and silty clay loam soil and 23% of total Cd was associated with the mobile (F1+F2+F3) fractions in sandy loam soil and 42%, 46% and 77% with the immobile (F4+F5+F6) fractions in silt loam soil, silty clay loam soil and sandy loam soil, respectively (Figure 4.32 to 4.40).

There were variations for all Cd fractions in different soils. Mobile fractions ranged from 14 to 81% in silt loam soil, 14 to 83% in silty clay loam soil and 11 to 42% in sandy loam soil. The higher proportion of mobile Cd was found in silt loam and silty clay loam soil, having low pH values compared to sandy loam soil according to Kashem and Singh (2001). In the floodplain soils, Cd was present in all three mobiles (F1-F3) because of low pH and light texture alluvial soil (Kashem and Singh, 2001).

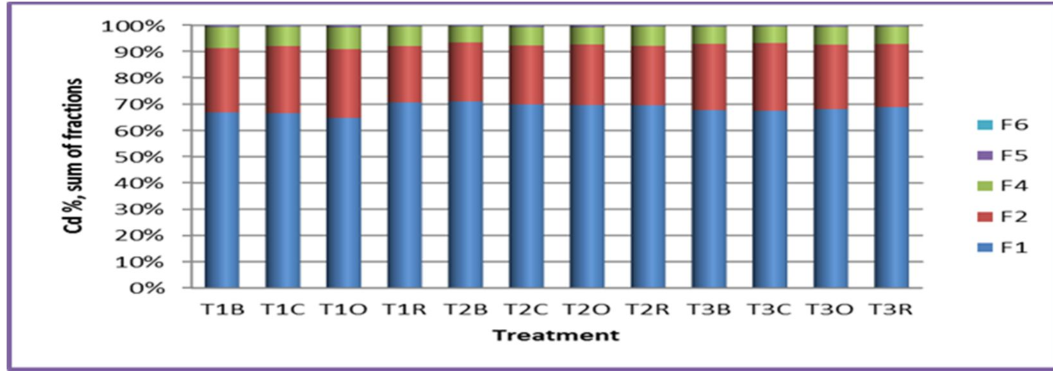


Figure 4.32 Distribution of Cd in various fractions in silt loam soil after 24 hours incubation.

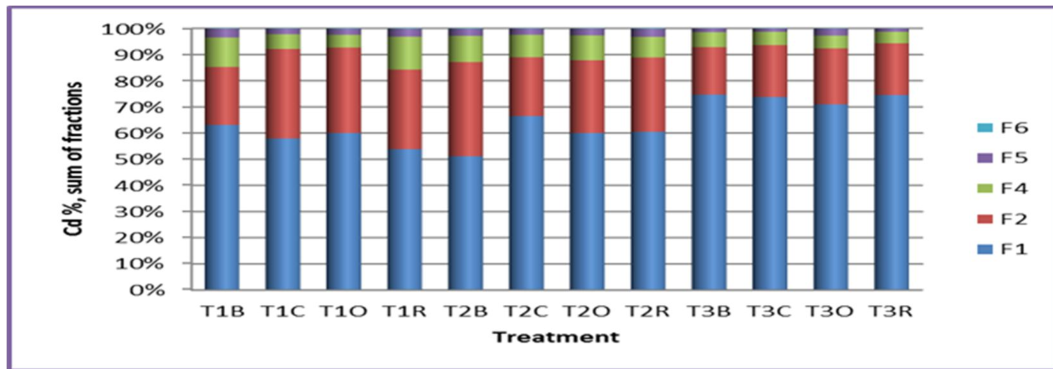


Figure 4.33 Distribution of Cd in various fractions in silt loam soil after 15 days incubation.

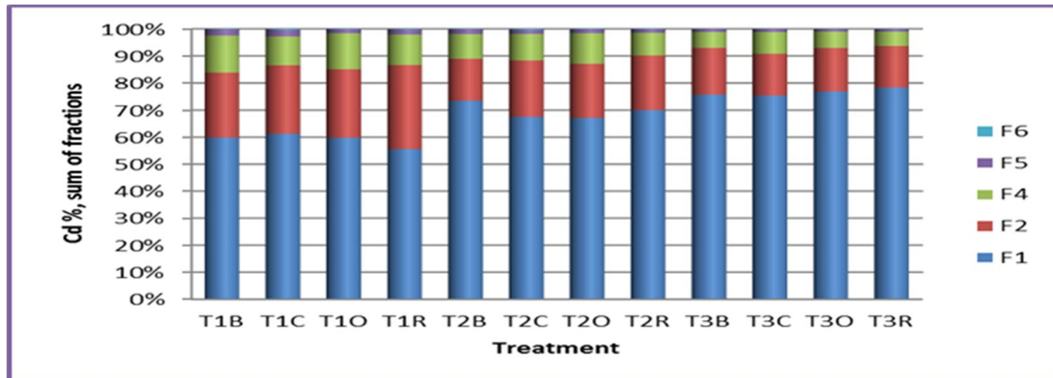


Figure 4.34 Distribution of Cd in various fractions in silt loam soil after 30 days incubation.

In silt loam and silty clay loam soils, after 24 hours, 15 days and 30 days of incubation period Cd associated with different fractions was in the order of: F1 > F2 > F4 > F5 > F6 (Figure 4.32 to 4.37).

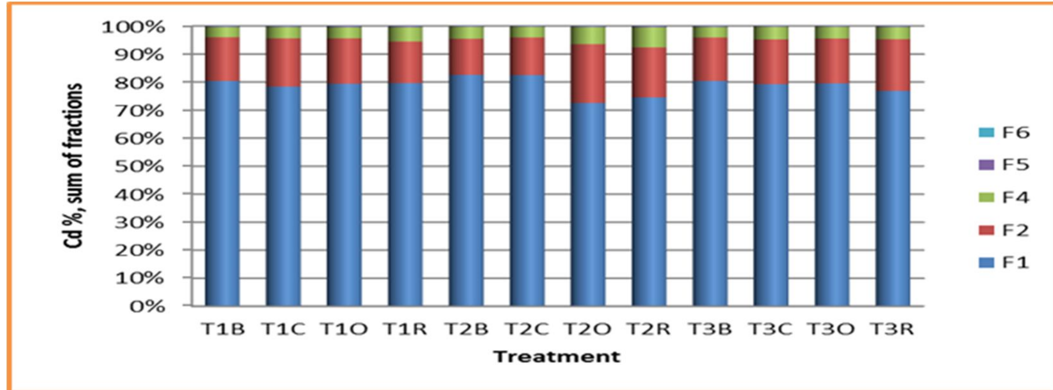


Figure 4.35 Distribution of Cd in various fractions in silty clay loam soil after 24 hours incubation.

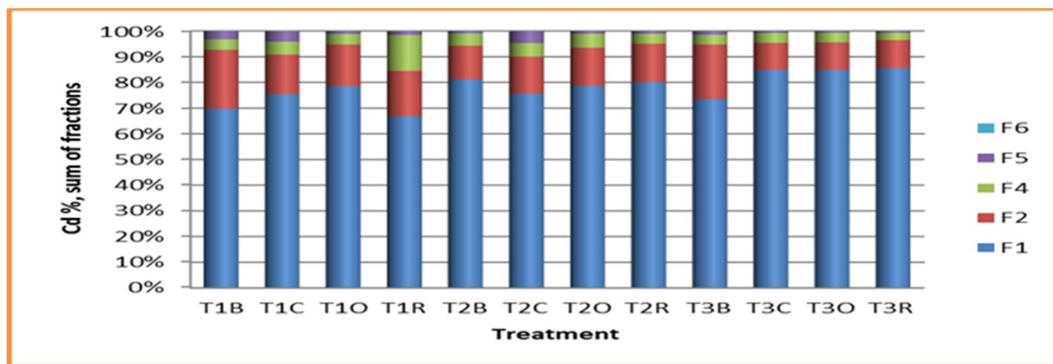


Figure 4.36 Distribution of Cd in various fractions in silty clay loam soil after 15 days incubation.

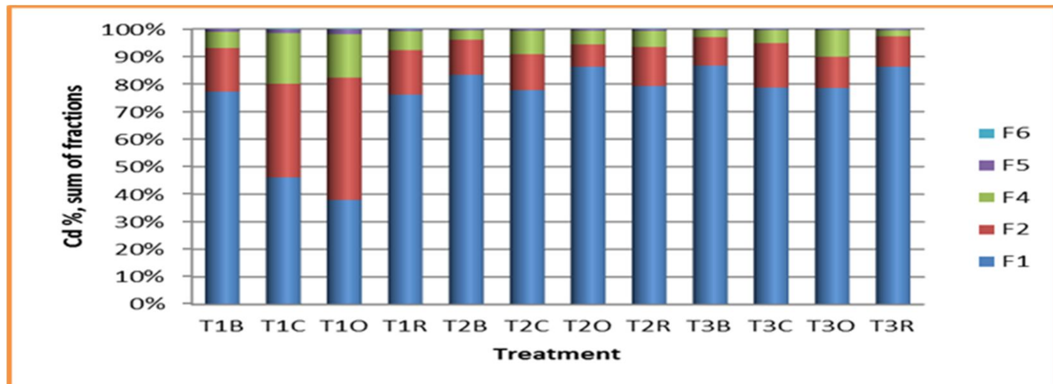


Figure 4.37 Distribution of Cd in various fractions in silty clay loam soil after 30 days incubation.

In sandy loam soil, after 24 hours, 15 days and 30 days of incubation period Cd associated with different fractions followed the order: F1 > F2 > F3 > F4 > F5 > F6 (Figure 4.38 to 4.40). A similar distribution order of Cd was observed by Ramos *et al.* (1994) and Chlopecka *et al.* (1996).

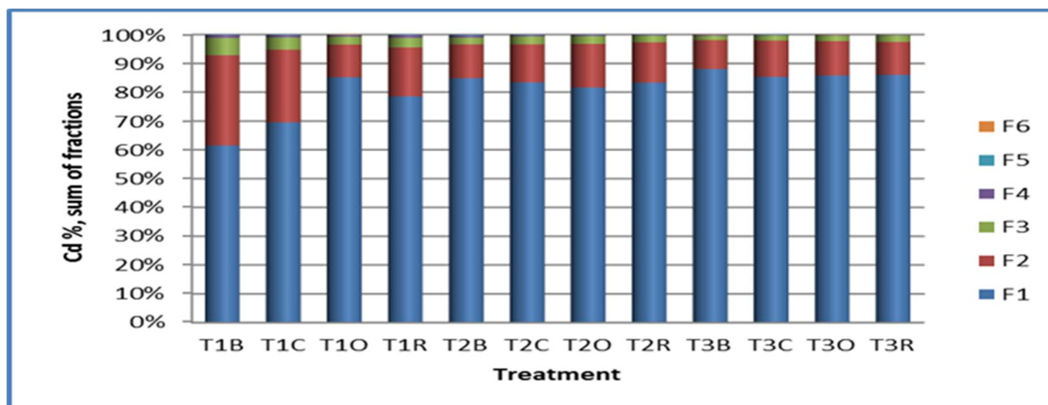


Figure 4.38 Distribution of Cd in various fractions in sandy loam soil after 24 hours incubation.

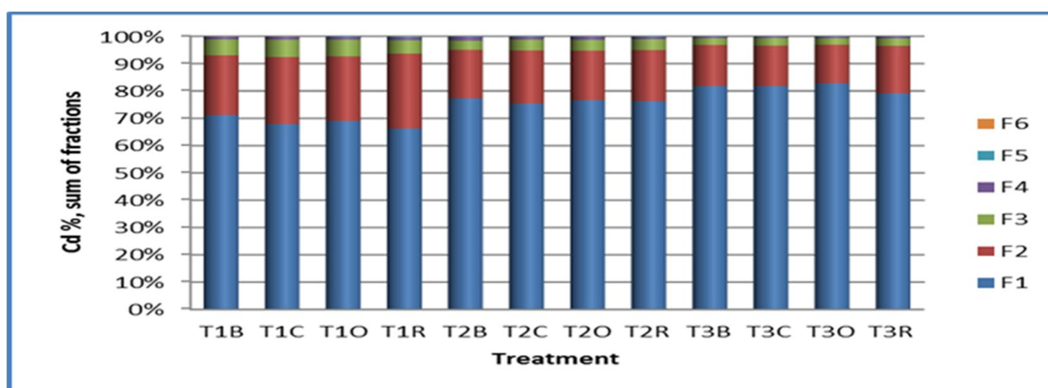


Figure 4.39 Distribution of Cd in various fractions in sandy loam soil after 15 days incubation.

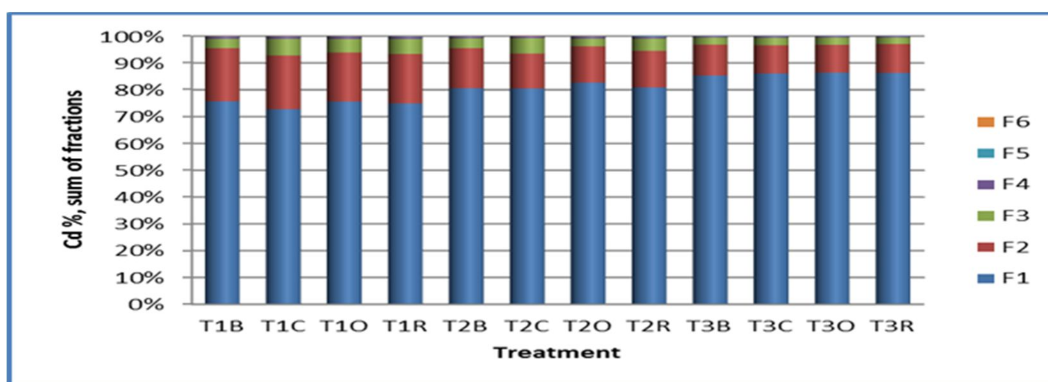


Figure 4.40 Distribution of Cd in various fractions in sandy loam soil after 30 days incubation.

Soil pH is considered the single most important factor controlling mobility and availability of trace metals in soils (Witter, 1989). High pH favors sorption and

precipitation of heavy metals as oxides, hydroxides, and carbonate. Soils that are high in clays and CEC provide sorption sites for metals and strongly retain them in the lattice. It was reported that for soils with the same amount of total Cd, it was more soluble and plant-available in sandy soil than in clay soil (Eriksson, 1989; He and Singh, 1993).

Higher percentages of Cd in the mobile fraction in these three soils (silt loam, silty clay loam soil and sandy loam soil) indicate that the anthropogenically added Cd remains in the mobile fraction and did not incorporated in the crystal lattice of minerals. That means the metal availability in these soils were high. For that reason microorganisms will be affected by Cd toxicity and their growth will be decrease with increasing incubation period (Figure 4.22).

Among the mobile fractions, Cd was found to be higher in carbonate fraction (F3) in sandy loam soil. Cadmium percentage in organic fractions was found to be lower than other fractions because of low organic carbon content but the soils contained lower values in residual fraction. Lower levels in the residual fraction in contaminated soils due to sequential extraction were also reported by other investigators (Zhang *et al.,* 1990; Singh *et al.,* 1998).

Cadmium present in carbonate form is susceptible to pH changes, mainly in the rhizosphere during plant growth, so it may be regarded as potentially phytoavailable. Cadmium added to soil as carbonate is relatively mobile in acidic conditions and within a few years may convert to exchangeable form (Chlopecka, 1993).

The chemistry of Cd in the soil environment is to a great extent, controlled by pH. Under acidic conditions Cd solubility increases and very little adsorption of Cd by soil colloids, hydrous oxides, and organic matter takes place. At pH values greater than 6, cadmium is adsorbed by the soil solid phase or is precipitated, and the concentrations of solution cadmium are greatly reduced (Stietiya and Wang, 2014).

As the behavior and bioavailability of metals in soil is affected by many chemical processes, different mechanisms like adsorption, co-precipitation and organic complexation, transformation, biological methylation and several interaction between metals and other elements present in soil, metal ions may not be available for microbes as well as bacteria. That might be the reason that organisms can survive at high concentration of Pb and Cd contaminated soil.

5. SUMMARY AND CONCLUSION

Agricultural soils of Bangladesh are gradually degrading due to many reasons and industrial pollution is one of them. Effluents discharged from industrial processes contain different chemical compounds some of which also contain heavy metals such as Ni, Cr, Pb, Cd, Hg etc. The discharged effluents are contaminating the agricultural soils through accumulation of heavy metals in soils. The heavy metal create toxicity problem in plants and microorganisms. As microorganisms control transformation of both organic and inorganic compounds leading to the release of available nutrients in soils, the survival of microorganisms in agroecosystem is important for the supply of nutrients to plants.

With the aim of isolation of phosphate-dissolving microorganisms, one soil sample was collected from metal contaminated areas of Saver EPZ area and other two from uncontaminated agricultural fields from Dhamrai and Pabna. Both contaminated and uncontaminated sites were selected due to the fact that tolerance to heavy metals of phosphate-dissolving soil bacteria varied within different soil environment. To determine the metal tolerance of phosphate-dissolving bacteria in different soil systems three soil samples were collected from agricultural soils of Dhamrai, Savar, and Pabna. Initially different types of growth medium were used to isolate the organisms. And for final screening of phosphate-dissolving bacteria were made by using Pikovskayas agar (PVK) and National Botanical Research Institute's phosphate growth medium (NBRIP).

A total of 13 distinct isolates were identified based on morphological, cultural, biochemical characteristics and Analytical Profile Index (API) test. According these tests, among 13 isolates 8 were *Bacillus*, others were species of *Micrococcus, Staphylococcus*, *Serratia*, *Pseudomonas*, and *Proteus* genera.

The isolates of the phosphate-dissolver were studied for their efficiency in dissolving insoluble phosphate in liquid culture medium. To evaluate the efficiency of dissolving insoluble phosphate in culture medium, liquid PVK and NBRIP medium were used. Among 13 isolated strains only 9 strains were identified as phosphate-dissolving bacteria. Among 9 strains 5 were *Bacillus* spp. and others were species of *Micrococcus*, *Serratia*, *Pseudomonas*, and *Proteus*.

The isolates of the phosphate-dissolver were studied for their efficiency in dissolving insoluble phosphate in liquid medium. To evaluate the efficiency of dissolving insoluble phosphate (rock phosphate) in culture medium, NBRIP and PVK media and for studying mineralization of organic phosphate, modified NBRIP and PVK media were used. For this research work, rock phosphate (RP) used in the previous experiment was replaced by each of compost and oil cake (as organic source of phosphorus) equivalent to 5 g rock phosphate (RP) and the pH was adjusted to 7 before autoclaving. Among 13 isolated strains only 9 strains were used as inoculants. Maximum phosphate was mineralized by most of the organisms after 11 days of incubation in sterile liquid media.

Species of *Bacillus*, *Pseudomonas* and *Micrococcus* were found to be the most efficient organisms in dissolving insoluble phosphate in liquid culture medium. Species of *Proteus* and *Serratia* ranked next in dissolving insoluble phosphate. It was also found that the concentration of soluble phosphates increased with the concomitant decreased in pH of the media by all the species.

The order of sequence on the basis of solubilization of insoluble phosphate in both PVK and NBRIP medium was as follows:

Bacillus > *Pseudomonas* > *Micrococcus* > *Proteus* >*Serratia.*

Species of *Bacillus*, *Proteus* and *Serratia* were found to be the most efficient organisms in mineralizing organic phosphate in both modified NBRIP and PVK liquid culture media.

The order of sequence on the basis of mineralization of organic phosphate in both modified NBRIP*^{1} and NBRIP*^{2} media was same:

Bacillus > *Proteus* > *Serratia* > *Micrococcus* > *Pseudomonas.*

Species of *Micrococcus* and *Pseudomonas* ranked next in mineralizing organic phosphate in modified NBRIP liquid medium.

On the basis of mineralization of organic phosphate in modified PVK*^{1} and PVK*^{2} media the order of sequence was as follows:

Bacillus > *Proteus* > *Serratia* > *Pseudomonas* > *Micrococcus.*

It was also found that the mineralizing capability of organic phosphate in modified PVK liquid medium slightly differed with the species of *Micrococcus* and *Pseudomonas* to that of modified NBRIP medium.

Phosphate-dissolving bacterial species showed a wide range of tolerance to metals (Pb and Cd). These bacterial species showed tolerance to Pb and Cd concentrations ranging from 15 to 150 µg/ml in nutrient broth medium. In PVK broth medium, bacterial species showed tolerance to Pb and Cd concentrations ranging from 30 to 250 µg/ml and 20 to 200 µg/ml, respectively. In NBRIP broth medium, bacterial species showed tolerance to Pb and Cd concentrations ranging from 50 to 300 µg/ml and 20 to 200 µg/ml, respectively.

These results showed that tolerance of these bacterial species to metals varied depending on the composition of media. From this study, *Bacillus cereus* strain isolated from the metal contaminated soil of Savar EPZ area and *Serratia marcescens* strain isolated from agricultural soil of Dhamrai were found as the most Pb and Cd tolerant species in liquid culture media, respectively.

Six phosphate-dissolving bacterial species showed a wide range of tolerance to metals (Pb and Cd) in three different soils (silt loam, silty clay loam and sandy soil). Nine phosphate-dissolving bacterial species showed tolerance to Pb and Cd concentrations ranging at 15,000 µg/g, 20,000 µg/g, and 30,000 µg/g depending on soils and duration of incubation.

Maximum tolerable concentrations of metals vary from liquid to liquid media. The results obtained in liquid media in laboratory may not give the similar results in case of soil but give a better understanding about the tolerance of high concentration of metals in soils by bacteria. It is evident from the result that, the maximum tolerable concentration of metals in solid media as soils is higher than that in liquid media.

Soils have the unique system to sequester the chemicals into different fractions. The fate and transport of a heavy metal in soil depend significantly on the chemical forms and speciation of the metal. And the contents varied among the soils with soil properties. The extraction sequence (F1-F6) followed the order of decreasing solubility. The order of Cd association in three soils (silt loam, silty clay loam and sandy loam soil) after 24 hours, 15 days and 30 days of incubation period with different fractions was: F1 > F2 > F3 > F4 > F5 > F6.

The order of Pb association was not similar to that of Cd in these soils (silt loam, silty clay loam, and sandy loam soil) and varied with incubation period. In silt loam soil oxide bound Pb was decreased after 24 hours of incubation period. Exchangeable Pb was increased after 24 hours and organically bound fraction was decreased after 15 days of incubation period. In silty clay loam soil water soluble and organically bound fraction were increased after 24 hours of incubation period. Oxide bound Pb was decreased after 24 hours of incubation period. In sandy loam soil exchangeable fraction of Pb showed slight variation after 15 days of incubation period. It is appeared that with increasing incubation period Pb is transformed from mobile to immobile fraction and the increase in residual Pb fraction resulted a decrease in the availability Pb with time.

Soil parameters that are mentioned to control metal mobility like pH, OM, CEC, clay content etc. So, the metals those are added from different sources are accommodated in soil in different fractions. It may take a long time to exert any disastrous effect of heavy metal on soil bacteria involving in the transformation of phosphorus in soil particularly phosphate solubilizing bacteria.

6. REFERENCES

Abbas, A. S., and C. Edwards. 1990. Effects of metals on *Streptomyces coelicolor* growth and actinorhodin production. Applied Environmental Microbiology. 56: 675-680.

Abd-Alla M. H. 1994. Solubilization of rock phosphate by *Rhizobium* and *Bradyrhizobium*. Folia Microbiological. 39: 53-56.

Abd-Alla, M. H. 1994. Use of organic phosphorus by *Rhizobium leguminosarum* biovar. viceae phosphatases. Biol Fertil Soils. 18:216–8.

Affan, Q. U. A., E. Shoeb, U. Badar, and J. Akhtar. 2009. Isolation and characterization of bacterial isolates having heavy metal tolerance. J. Basic and Appl. Sciences. 5(2): 55 – 60.

Afzal, A., and B. Asghari. 2008. Rhizobium and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum*). *Int* J Agric Biol. 10: 85–88.

Afzal, A., M. Ashraf, S. A. Asad, and M. Farooq. 2005. Effect of phosphate solubilizing microorganisms on phosphorus uptake, yield and yield traits of wheat (*Triticum aestivum L.*) in rain fed area. *Int*. J. Agri. Biol. 7(2): 207-209.

Ahmad, I., S. Hayat, A. Ahmad, A. Inam, and Samiullah. 2001. Metal and antibiotic resistance traits in *Bradyrhizobium* sp. (cajanus) isolated from soil receiving oil refinery wastewater. World J. Microbiol. Biotechnol. 17: 379-384.

Ahmed, M. F., I. R. Kennedy, A. T. M. A. Choudhury, M. L. Kecskes, and R. Deker. 2008. Phosphorus adsorption in some Australian soils and influence of bacteria on the desorption of phosphorus. Commun Soil Sci Plant Anal. 39: 1269-1294.

Ahmed, N. A., Nawaz, and U. Badar. 2005. Screening of copper tolerant bacterial species and their potential to remove copper from the environment. Bulletin of Environmental and Contamination Toxicology 74: 219 – 226.

Ahmuda, I., J. Mendoza, and L. Ascar. 1999. Sequential extraction of heavy metals in soils irrigated with wastewater. Commun. Soil Sci Plant Anat. 30: 1507-1519.

Akmal, M., and X. Jianming. 2009. Microbial biomass and bacterial community changes by Pb contamination in acidic soil. J Agric Biol Sci. 1: 30–37.

Alexander, M. 1961. Introduction to soil Microbiology. John Wiley and Sons, *Inc*. New York.

Alexander, M. 1977. Introduction to Soil Microbiology. Wiley and Sons. *Inc.* New York:

Alexander, M. 1984. Introduction to soil microbiology (4th ed). John Wiley and sons. *Inc*. New York.

Allaway, W. H. 1968. Agronomic controls over environmental cycling of trace elements. Adv. Agron. 20: 235-74.

Amarasiri, S. L. and S. R. Solsen. 1973. Liming as related to solubility of P and plant growth in an acid tropical soil. Soil Sci. Soc. Am. Proc. 37: 716-724.

Anyanwu, C. U., and O. N. Nwachukwu. 2011. Heavy metal resistance in bacteria isolated from contaminated and uncontaminated soils. *Int*. J. of Res. in Chem. and Env. 1: 173 - 178.

Appelbaum, P. C., P. S. Chaurushiya, M. R. Jacobs, and A. Duffett. 1984. Evaluation of the Rapid Strep System for Species Identification of Streptococci. J. Clin. Microbiol. 19: 588-591.

Archibald, F. S., and M. N. Duong. 1984. Manganese acquisition by *Lactobacillus plantarum*. J. Bacteriol. 158: 1 - 8.

Armstrong, D. L. 1988. Role of phosphorus in plants: In Better Crops with Plant Food. pp. 4-5 (ed). D. I. Armstrong. (Atlanta USA: Potash and Phosphate Institute).

Arora, D. and C. Gaur. 1979. Microbial solubilization of different inorganic phosphates. Indian J Exp Biol. 17: 1258–61.

Avery, S. V. 2001. Metal toxicity in yeast and the role of oxidative stress. Adv Appl Microbiol. 49: 111–142.

Avery, S. V., and J. M. Tobin. 1993. Mechanism of adsorption of hard and soft metal ions to *Saccharomyces cerevisiae* and influence of hard and soft anions. Appl. Environ. Microbiol. 59: 2851-2856.

Bååth, E. 1989. Effects of heavy metals in soil on microbial processes and populations. J. Water Air Soil Pollut. 47: 335–379.

Babana, A. H., A. H. Dicko, K. Maïga, and D. Traoré. 2013. Characterization of rock phosphate-solubilizing microorganisms isolated from wheat (*Triticum aestivum* L.) rhizosphere in Mali. Journal of Microbıology and Microbial Research. 1(1): 1-6.

Babenko, Y. S., G. Tyrygina, E. F. Grigoryev. L. M. Dolgikh, and T. I. Borisova. 1984. Biological activity and physiologobiochemical properties of bacteria dissolving phosphates. Microbiologiya. 53: 533–9.

Babich, H., and G. Stotzky. 1977. Sensitivity of various bacteria, including *actinomycetes* and fungi to cadmium and influence of pH on sensitivity. Appl. Environ. Microbiolo. 33: 681 - 695.

Bache, B. W. 1979. The Encyclopedia of Soil Science. Springer. P. 487-492.

Baeuerlein, E. 2000. Biomineralization. Weinheim, Germany: Wiley-VCH.

Bajpai, P. D., and W. V. B. Sundara Rao. 1971. Phosphate solubilizing bacteria III. Soil inoculation with phosphate solubilizing bacteria. Soil Sci Plant Nutr. 17: 46–53.

Banfield, J. F., and K. H. Nealson. (editors) 1997. Geomicrobiology: Interactions between Microbes and Minerals, Reviews in Mineralogy and Geochemistry, Washington, DC: Mineralogical Society of America. vol. 35.

Banik, S., and B. K. Dey. 1983. Phosphate solubilizing potentiality of the microorganisms capable of utilizing aluminium phosphate as a sole phosphate source. Zentralblatt Microbiology. 138: 17–23.

Bardiya, M. C., and A. C. Gaur. 1974. Isolation and screening of microorganisms dissolving low grade rock phosphate. Folia Microbiol. 19: 386–9.

Bazylinski, D. A. 2001. Bacterial mineralization. In Encyclopedia of Materials: Science and Technology. Amsterdam: Elsevier. pp 441–448.

Beever, R. E., and D. J. W. Burns. 1980. Phosphorus uptake, storage and utilization by fungi. Advances in Botanical Research. 8: 121-219.

Belimov, A. A., A. P. Kojemiakov, and C. V. Chuvarliyeva. 1995. Interaction between barley and mixed cultures of nitrogen fixing and phosphate-solubilizing bacteria. Plant Soil. 173: 29–37.

Beveridge, T. J. and W. S. Fyfe. 1985. Metal fixation by bacterial cell walls. Can J Earth Sci. 22: 1892–1898.

Beveridge, T. J., and R. J. Doyle. 1989. Metal ions and Bacteria. Wiley, New York.

Bhamra, R. K., and M. Costa. 1992. Trace elements Aluminium, Arsenic, Cadmium, Mercury, and Nickel. In: *Environmental Toxicants*. Morton Lippman (ed.). Publ. Co. Von Nostrend Reinhold, New York. 19: 606 – 613.

Bhattacharyya, P., and R. K. Jain. 2000. Fert. News. 45: 45-52.

Binet, M. R. B., R. Ma, W. Mcleod, and R.K. Poole. 2003. Detection and characterization of zinc-and cadmium - binding proteins in Escherichia coli by gel electrophoresis and laser ablation – inductively coupled plasma mass spectrometry. Analyt. Biochem. 318: 30-38. DOI: 10.1016/S0003-2697(03)00190-8.

Bishop, M. L., A. C. Chang, and R. W. K. Lee. 1994. Enzymatic mineralization of organic phosphorus in a volcanic soil in Chile. Soil Sci. 157:238–43.

Biswapati, D., S. C. Mandal and L. N. Mandal. 1992. Effect of growth and subsequent decomposition of cyanobacterial on the transformation of phosphorus in submerged soils. Plant and Soil. 143(2): 289-97.

Blaudez, D., C. Jacob, K. Turnau, and J. V. Colpaert, U. Ahonen-Jonnarth, R. Finlay, B. Botton, and M. Chalot. 2000. Differential responses of ectomycorrizal fungi to heavy metals in vitro. Mycol Res. 104: 1366–1371.

Bolan, N. S., and M. J. Hedley. 1990. Dissolution of phosphate rocks in soils. 2. Effect of pH on the dissolution and plant availability of phosphate rock in soil with pH dependent charge. Nutrient Cycling in Agroecosystems. 24: 125-134.

Bong, C.W., f. Malfatti, F. Azam, Y. Obayashi, and S. Suzuki. 2010. The effect of zinc exposure on the bacteria abundance and proteolytic activity in seawater. In: N. Hamamura, S. Suzuki, S. Mendo, C.M. Barroso, H. Iwata, and S. Tanabe. (eds.) Interdisciplinary Studies on Environmental Chemistry – Biological Responses to Contaminants. Tokyo: Terrapub; p. 57-63.

Brady, D., and J. R. Duncan. 1994. Chemical and enzymatic extraction of heavy metal binding polymers from isolated cell walls of *Sccharomyces cerevisiae.* Biotechnol. Bioeng. 44: 297-302.

Brady, N. C. 1990. The nature and properties of soils. 10th ed. Macmillan publishing Company, New York.

Brocklehrust K. R., A. P. Morby. 2000. Metal-ion tolerance in *Escherichia coli*: analysis of transcriptional profiles by gene array technology. Microbiol. 146: 2277 - 2282.

Bruins, M. R., S. Kapil, and F. W. Oehme. 2000. Microbial resistance to metals in the environment. Ecotoxicology and Environmental Safety. 45: 198-207.

Bryan, C. W., and W. J. Langston. 1992. Bioavailability, accumulation and effects of heavy metals an sediments with special reference to United Kingdom estuaries: A review, Environ. Pollut. 76: 89.

Bryan, H. 1950. Manual of Methods for Pure Culture Study Bacteria. 12, Leaflet I-X.

Burdman, S., H. Volpin, J. Kigel, Y. Kapulnik, and Y. Okon. 1996. Promotion of nod gene inducers and nodulation in common bean (*Phaseolus vulgaris*) root inoculated with Azospirillum brasilense. Appl Environ Microbiol 62: 3030–3033.

Burns, R. G. 1983. Extracellular enzyme-substrate interactions in soil. In: J. H. Slater, R. Whittenbury, and J. W. T. Wimpenny. editors. Microbes in their Natural Environment. Cambridge: Cambridge Univ Press. pp. 249–98.

Cappuccino, J. G., and N. Sherman. 2005. Microbiology: A Laboratory manual (7th ed.). Dorling Kindersley (India). Pvt. Ltd. New Delhi, India. P. 1- 453.

Chang, J. S., C. Chou, and S. Y. Chen. 2001. Decolorization of azo dyes with immobilized *Pseudomonas luteola*. Process Biochemistry 36: 757-763.

Chao, T. T. 1972. Selective dissolution of manganese oxides from soils and sediments with acidified hydroxylamine hydrochloride. Proc. Soil Sci. Soc. Am. 36: 746-768.

Chebotar, V. K., C. A. Jr. Asis, and S. Akao. 2001 ability of rhizobacteria enhance the nitrogen fixation of soybean when coinoculated with Bradyrhizobium japonicum. Biol Fertil Soils. 34: 4. Production of growth promoting substances and high colonization 27–432.

Chen, C., and J. Wang. 2007. Response of Saccharomyces cerevisiae to lead ion stress. Appl Microbiol Biotechnol. 74: 683–687.

Chen, Y. P., P. D. Rekha, A. B. Arunshen, W. A. Lai, and C. C. Young. 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl Soil Ecol. 34: 33–41.

Chillar, J. K., B. R. Arora, and B. Singh. 1991. Influence of soil water regime on P nutrition of rice and wheat. J. Indian Soc. Soil Sci. 39: 625-29.

Chlopecka, A., 1993. Forms of trace metals from inorganic sources in soils and amounts found in spring barley. Water Air Soil Pollut. 69: 127.

Chlopecka, A., J. R. Bacon., M. J. Wilson, and J. Kay. 1996. Forms of cadmium, lead and zinc in contaminated soils from southwest Poland. J. Environ. Qual. 25: 69.

Chmielowski, J. and B. K£apciñska. 1984. Mechanism of the uptake of metals by microorganism. Post. Mikrobiol. 23(2): 63-87.

Chuang, C. C., Y. L. Kuo, C. C. Chao, and W. L. Chao. 2007. Solubilization of inorganic phosphates and plant growth promotion by *Aspergillus niger*. Biol Fertil Soils. 43: 575–584.

Claus, G.W. 1995. Understanding Microbes (4th ed.). W. H. Freman and Company. New York. p. 547.

Clevenger, T. E. 1990. Use of sequential extraction to evaluate the heavy metals in mining washes. Water, Air, Soil Pollut. 50: 241-255.

Cohen, I., R. Biton, and N. Yeshagahu. 1991. The effect of zinc and cadmium ions on *Escherchia coli* B. *Microbios*. 68: 157 - 168.

Collins, C. H., and P. M. Lyne. 1984. Microbiological methods (5th ed.). Butterworth and Co. (Publisher) Ltd. London. p. 446.

Condron, L.M., B. L. Turner, B. J. Cade-Menun. 2005. Chemistry and dynamics of soil organic phosphorus. Soil Science Society of America. 87–121.

Cowan, S. T**.** 1974. Cowan and Steel's Manual for the identification of medical bacteria (2nd ed.). Cambridge University Press. Cambridge, London. New York, Melbourne.

Coyne, M. S. 1999. Soil Microbiology: An Exploratory Approach. Delmar Publ. NewYork**.**

Dance, D. A. B., V. Wuthiekanun, P. Naigowit, and N. J. White, 1989. Identification of Pseudomonas pseudomallei in clinical practice: use of simple screening test and API 20NE. J Clin Pathol, 42: 645-648.

Darmwall, N. S.**,** R. B. Singh, and R. Rai. 1989. Isolation of phosphate solubilizers from different sources. Curr Sci. 58: 570–1.

Das, A. C. 1963. Utilization of insoluble phosphates by soil fungi. J Indian Soc Soil Sci. 11: 203–7.

Das, P., S. Samantaray, and G. R. Rout. 1997. Studies on cadmium toxicity in plants, a review. Environ. Pollut. 98(1): 29.

de Santiago-Martín, A., I. Valverde-Asenjo, J. R. Quintana, C. González-Huecas, and A. L. Lafuente. 2013. Soil properties affecting metal extractability patterns in periurban calcareous agricultural soils in the Mediterranean area. IJER. 7(4): 831–840.

Debnath, N. C. and S. K. Mandal. 1982. Inorganic transformation of added phosphorus in soil and the relative availability of the reaction products as influenced by ageing. Agrochimica (Italy). 26(4): 293-304.

Derylo, M, and A. Skorupska. 1993. Enhancement of symbiotic nitrogen fixation by vitamin-secreting fluorescent Pseudomonas. Plant Soil. 154: 211–217.

Dhankhar, R., S. Sheoran, A. Dhaka, and R. Soni. 2013. The role of phosphorus solubilizing bacteria (PSB) in soil management-an overview. Int. J. of Dev. Res. Vol. 3, Issue. 9. pp 031-036.

Dias-Junior H. E, F. M. S. Moreira, J. O. Siqueira, and R. Silva. 1998. Heavy metals, microbial density and activity in a soil contaminated by wastes from the zinc industry. Rev. Brasil. Cienc. do Solo. 22(4): 631.

Diaz, E. 2004. Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. Int. Microbiol. 7: 173 – 180.

Doelman, P., and L. Haanstra. 1979. Effects of lead on the soil bacterial microflora. Soil Biol. Biochem. 1: 487 - 491.

Doelsch, E., G. Moussard, and H. Saint Macary. 2008. Fractionation of tropical soilborne heavy metals-comparison of two sequential extraction procedures. Geoderma. 143(1-2): 168-179.

Dopson, M., C. Baker-Austin, P. R. Koppineedi, and P. L. Bond. 2003. Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic micro-organisms. Microbiology. 149: 1959–1970.

Dove, P. M., J. J. De Yoreo, and S. Weiner. (editors) 2003. Biomineralization. Reviews in Mineralogy and Geochemistry. vol. 54.

Duponnois, R., A. Colombet, V. Hien, and J. Thioulouse. 2005. The mycorrhizal fungus *Glomus intraradices* and rock phosphate amendment influence plant growth and microbial activity in the rhizosphere of *Acacia holosericea*. Soil Biology & Biochemistry. 37: 1460-1468.

Ehrlich, H. L., and D. K. Newman. 2009. Geomicrobiology, 5th edn. Boca Raton, FL: CRC Press/Taylor & Francis.

Eide, D. J. 2000. Metal ion transport in eukaryotic microorganisms: insights from Saccharomyces cerevisiae. Adv Microb Physiol. 43: 1–38.

Eklund, S. C., and C. E. Lankford. 1967. Laboratory Manual for General Microbiology Prentice – Hall. *Inc*. Englewood Cliffs. New Jersery. p. 295.

El-Sawah, M. M. A., F. I. A. Hauka, and H. H. El-Rafey. 1993. Study on some enzymes cleaving phosphorus from organic substrates in soil. J Agric Sci. 18: 2775–85.

Eriksson, J. E., 1989. The influence of pH, soil type and time on adsorption and uptake by plants of Cd added to the soil. Water Air Soil Pollut. 48: 317.

Fankem, H., D. Nwaga, A. Deube, L. Dieng, W. Merbach, and F. X. Etoa. 2006. Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon. Afr J Biotechnol. 5: 2450–2460.

Feller, C., E. Frossard, and M. Brossard. 1994. Phosphatase activity in low activity tropical clay soils. Distribution in the various particle size fractions. Can J Soil Sci. 74: 121–9.

Fox, T. R., and N. B. Comerford. 1992. Rhizosphere phosphatase activity and phosphatase hydrolysable organic phosphorus in two forested spodosols. Soil Biol Biochem. 24: 579–83.

Freire-Nordi, C. S., A. A. H. Vieira, C. R. Nakaie, and O. R. Nascimento. 2005. Effect of polysaccharide capsule of the microalgae Staurastrum iversenii var. americanum on diffusion of charged and uncharged molecules, using EPR technique. Braz J Phys. 36: 75–82.

Frossard, E., B. Truong and F. Jauin. 1986. Effect of organic matter on adsorption and desorption of phosphorous in oxisol. Agronomie (Paris). 6: 66-71.

Gadd, G. M. 1992a. Metals and microorganisms: a problem of definition. FEMS Microbiol Lett. 100: 197–204.

Gadd, G. M. 1993a. Interactions of fungi with toxic metals. New Phytol 124: 25–60.

Gadd, G. M. 1996. Influence of microorganisms on the environmental fate of radionuclides. Endeavour. 20: 150-156.

Gadd, G. M. 2004. Microbial influence on metal mobility and application for bioremediation. Geoderma 122: 109–119.

Gadd, G. M. 2005. Microorganisms in toxic metal polluted soils. In Microorganisms in Soils: Roles in Genesis and Functions, pp. 325–356. Edited by F. Buscot & A. Varma. Berlin: Springer-Verlag.

Gadd, G. M. 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycol Res. 111: 3–49.

Gadd, G. M. 2010. Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology. 156: 609–643.

Gadd, G. M. and A. J. Griffiths. 1978. Microorganisms and heavy metal toxicity. Microb Ecol. 4: 303–317.

Gadd, G. M., 1990. In Microbial Mineral Recovery (Ehrlich, HL and Brierley, CL., Eds.). McGraw-Hill. New York. pp. 249-275.

Galan, E., j. L. Gomez Ariza, I. Gonzalez, J. C. Fernandez Caliani, E. Morales, and I. Giraldez. 1999. Utilidad de las tecnicas de extraction sequential en la mejora de la characterization mineralogical por DRX de suelos y sedimentos con altos contenidos de oxidos de hierro, in Libro de Conferencias y Resumenes de la XV Reunion Cientifica de la Sociedad Espanola de Arcillas. 15: 68-69.

Gao, S., and R. G. Burau. 1997. Environmental factors affecting rates of arsine evolution from and mineralization of arsenicals in soil. J. Environ. Qual. 26: 753-763.

Garcia, C., T. Fernandez, F. Costa, B. Cerranti, and G. Masciandaro. 1992. Kinetics of phosphatase activity in organic wastes. Soil Biol Biochem. 25: 361–5.

Gaur, A. C., M. Madan, and K. P. Ostwal. 1973. Solubilization of phosphatic compounds by native micro flora of rock phosphates. Indian J Expt Biol 11: 427-429.

Gemmell, C. G., and J. E. Dawson. 1982. Identification of Coagulase-Negative Staphylococci with the API STAPH system. J. Clin. Microbiol., 16(5): 874-877.

Gerba, C. P. 1996. Principles of toxicology. In Environmental and Pollution Science. Edited by I. L. Pepper & other authors. San Diego: Academic Press. pp. 322–44.

Gerretson, F. C. 1948. The influence of microorganisms on the phosphorus uptake by plant. Plant Soil. 1: 51–81.

Gihring, T. M., G. K. Druschel, R. J. McCleskey, R. J. Hamers, and J. F. Banfield. 2001. Rapid arsenite oxidation by *Thermus aquaticus* and *Thermus thermophilus:* Field and laboratory investigations.

Giller, K. E., E. Witter, and S. P. McGrath. 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biology and Biochemistry. 30: 1389–1414.

Goldstein, A. H. 1986. Bacterial solubilization of microbial phosphates: Historical perspective and future prospects. Am. J. Alter. Agric. 1: 51-57.

Goldstein, A. H. 1986. Bacterial solubilization of mineral phosphates: historical perspective and future prospects. Am J Altern Agri. 1: 51–7.

Goldstein, A. H. 1995. Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram-negative bacteria. Biol. Agric. Hort. 12: 185-193.

Goldstein, A. H., and S. T. Liu. 1987. Molecular cloning and regulation of a mineral phosphate solubilizing gene from *Erwinia herbicola.* Bio/Technology. 5: 72–4.

Gordon, R. E. 1966. The taxonomy of soil bacteria. In: The ecology of soil bacteria: An International Symposium. T. R. G. Gray, and D. Parkinson (eds.). Liverpool Univ. Press. 293 - 321.

Grass, G., M. D. Wong, B. P. Rosen, R. L. Smith, and C. Rensing. 2002. ZupT is a Zn(II) uptake system in Escherichia coli. J Bacteriol. 184: 864–866.

Greaves, M. P., and D. M. Webley. 1965. A study of the breakdown of organic phosphates by microorganisms from the root region of certain pasture grasses. J Appl Bact. 28: 454–65.

Greenberg, E. L., S. Clerceri, and A. D. Eaton. 1980. Standard methods for the examination of water and waste water. (18th ed.). Am. Publ. Health Assoc. Washington D. C.

Gregsons, S. K., and B. J. Alloway. 1984. Heavy metals in soils contaminated from several sources including sewage sludge. J. Soil Sci. 311(2): 55-61.

Gügi, B., N. Orange, F. Hellio, J. F. Burini, C. Guillou, F. Leriche, and J. F. Guespin-Michel. 1991. Effect of growth temperature on several exported enzyme activities in the psychrotropic bacterium *Pseudomonas fluorescens.* J Bacteriol. 173: 3814–20.

Guibaud, G., N. Tixier, A. Bouju, and M. Baudu. 2003. Relation between extracellular polymers' composition and its ability to complex Cd, Cu and Pb. Chemosphere. 52: 1701–1710.

Gupta, R., R. Singal, A. Shankar, R. C. Kuhad, and R. K. Saxena. 1994. A modified plate assay for screening phosphate solubilizing microorganisms. J Gen Appl Microbiol. 40: 255-260.

Gupta, M., S. Kiran, A. Gulati, B. Singh, and R. Tewari. 2012. Isolation and identification of phosphate solubilizing bacteria able to enhance the growth and aloin-A biosynthesis of *Aloe barbadensis* Miller. Microbiological Research. 167: 358– 363.

Gupta, P. K., 2000. Methods in Environmental Analysis Water, Soil and Air. AGROBIOS (INDIA). PP-171.

Gyaneshwar, P., N. Kumar, L. J. Parekh, and P.S. Poole. 2002. Role of soil microorganisms in improving P nutrition of plants. Plant Soil. 245: 83–93.

Hafeburg, G., and E. Kothe. 2007. Microbes and metals: interactions in the environment. Journal of Basic Microbiology. 47: 453-467.

Hakanson, L. 1992. Sediment variability. In. Burton G. A. jr. (ed) Sediment Toxicity Assessment. Lewis Publishers. Boca Raton. Fl. pp. 19-36.

Halder, A. K., and P. K. Chakrabartty. 1993. Solubilization of inorganic phosphate by *Rhizobium.* Folia Microbiol. 38: 325–30.

Hall, G. E. M., G. Gautheir, J. C. Pelchat, and J. E. Vaive. 1996. Application of a sequential extraction schem to ten geological certified reference materials for the determination of 20 elements. Journal of Analytical Atomic Spectrometry. 11(9): 787-796.

Harris, J. N., P. B. New, and P. M. Martin. 2006. Laboratory tests can predict beneficial effects of phosphate solubilizing bacteria on plants. Soil Biology and Biochemistry. 38: 1521-1526.

Hasnain, S., S. Yasmin, and A. Yasmin. 1993. The effects of lead-resistant pseudomonads on the growth of Triticum aestivum seedlings under lead stress. Environ Pollut. 81: 179–184.

Hassen, A., N. Saidi, M. Cherif, and A. Boudabous. 1998. Resistance of environmental bacteria to heavy metals. *Biosource Technology* 64: 7- 15.

Hayat, S., I. Ahmad, Z. M. Azam, A. Ahmad, A. Inam, and Samiullah. 2002. Effect of long term application of oil refinery wastewater on soil health with special reference to microbiological characteristics. *Biores. Technol.* 84: 159 - 163.

Hayat, S., I. Ahmad, Z. M. Azam, A. Ahmad, A. Inam, and Samiullah. 2002. Effect of long term application of oil refinery wastewater on soil health with spcial reference to microbiological characteristics. Biores. Technol. 84: 159-163.

Hayman, D. S. 1975. Soil Microbiology. Butterworth, London.

Haynes, R. J. and R. S. Swift. 1985. Effects of air-drying on the adsorption and desorption of phosphate and levels of extractable phosphate in a group of New Zealand soils. Geoderma. 35: 145-157.

He, Q. B., and B. R. Singh. 1993. Cadmium distribution and extarctibility in soils and its uptake by plants as affected by organic matter and soil types. J. Soil Sci. 44: 641.

Hemida, S. K., S. A. Omar, and A. Y. Abdel-Mallek. 1997. Microbial populations and enzyme activity in soil treated with heavy metals. Water Air Soil Pollut. 95: 13 - 22.

Henry, J. R. 2000. In An Overview of Phytoremediation of Lead and Mercury. NNEMS Report. Washington. D.C. pp. 3-9.

Herland, B. J., D. Taylor, and K. Wither. 2000. The distribution of mercury and other trace metals in the sediments of the Mersey Estuary over 25 years 1974 - 1998. *Sci. Total Environ*. 253: 45-62.

Hiroki, M. 1992. Effects of heavy metal contamination on soil microbial population. Soil Sci. Plant Nutri. 38: 141-147.

Holleman, A. F. and E. Wiberg. 1985. Lehebuch du Anoranischen chemie. Water de Gruyter, Berlin, pp. 868.

Howard, J. L., and W. J. Vandenbirnk. 1999. Sequential extraction analysis of heavy metals in sediments of variable composition using nitrilotriacetic acid to counteract resorption. Environ. Pollut. Bull. 34: 123-128.

Hu, J., X. Lin, J. Wang, H. Chu, R. Yin, and J. Zhang. 2009. Population size and specific potential of P-mineralizing and P-solubilizing bacteria under long-term P-deficiency fertilization in a sandy loam soil. Pedobiologia. 53: 49–58.

Huang, C., C. Huang, and A. L. Morehart. 1990. The removal of copper from dilute aqueous solutions by *Saccharomyces cerevisiae*. Wat. Res. 24: 433-439.

Huffman, S. A., C. V. Cole, and N. A. Scott. 1996. Soil texture and residue addition effects on soil phosphorus transformations. Soil. Sci. Soc. Am. J. 60(4): 1095-1101.

Hunt, J. 1980. Determination of total sulfur in small amount of plant material. Analyst. 105: 83-85.

Hwangbo, H., R. D. Park, Y. W. Kim, Y. S. Rim, K. H. Park, T. H. Kim, J. S. Such, and K. Y. Kim. (2003) 2-ketogluconic acid production and phosphate solubilization by Enterobacterintermedium. Curr Microbiol 47: 87–92.

Igwe, J. C., I. C. Nnorom, and B. C. G. Gbaruko. 2005. Kinetics of radionuclides and heavy metals behavior in soils: Implications for plant growth. African Journal of Biotechnology 4(B): 1541 - 1547.

Illmer, P., and F. Schinner. 1992. Solubilization of inorganic phosphates by microorganisms isolated from forest soil. Soil Biol Biochem. 24: 389–95.

Islam, A., and B. Ahmed. 1971. Phosphate status of some representative soils of Bangladesh. M. Sc. Thesis. University of Dhaka.

Iyenger, S. S., D. C. Martens, and W. P. Miller. 1981. Distribution and plant availability of soil zinc fractions. Soil Sci Soc. Am. J. 45: 735-739.

Jackson, M. L. 1967. Soil Chemical Analysis. Prentice-Hall of India Pvt. Ltd. New Delhi.

Jackson, M. L. 1973. Soil Chemical Analysis. Prentice Hall of India Pvt. Ltd., New Delhi. p 134.

Jackson, M. L. 1973. Soil Chemical Analysis. Prentice Hall of India Pvt. Ltd. New Delhi. Pp. 326-337.

Jalali, M., Khanlari, Z.V. 2008. Efect of aging process on the fractionation of heavy metals in some calcareous soils of Iran. Geoderma. 143: 26–40.

Jeng, A. S. and Singh, B. R., 1993. Partitioning and distribution of cadmium and zinc in selected cultivated soils in Norway. Soil sci. 156: 240.

Jian-Xianjun, Luo-Yongming, Zhao-Qiguo, Wu-Shengchun, Wu-Longhua, QiIamliang, and Song-Jing. 2000. I. Phytoremediation of heavy metal contaminated soils. II. Quantitative changes of microorganisms in rhizosphere soil of metal accumulator plant Brassica juncea. Soils 32(1): 75.

Jou, A. S. R. and R. L. Fox. 1977. Phosphate sorption characteristics of some benchmark soils of West Africa. Soil Sci. 124: 370-376.

Juwarkar, A. A., A. Nair, K. V. Dubey, S. K. Singh, and S. Devotta. 2007. Biosurfactant technology for remediation of cadmium and lead contaminated soils. Chemosphere. 68: 1996-2002.

Kabala, C., and B. R. Singh. 2001. Fractionation and mobility of copper, lead and zinc in soil profiles in the vicinity of a copper smelter. J. Environ. Qual. 30: 485-492.

Kabata-Pendias, A., and H. Pendias. 1999. Biogeochemistry of trace elements. Warsaw: PWN Scientific Publishing House.

Karczewska, A., L. Szerszen, and C. Kabala. 1998. Forms of selected heavy metals and their transformation in soils polluted by emissions from copper smelters. Avd. Geo. Ecology. 31: 705-712.

Karthanasis, A. D. 2001. Mineral controls in colloid mediated transport of metals in sub surface environments. *In*: Proc. 6th International conference on biogeochemistry of trace elements Guelph.

Kashem, A and Singh, B. R. 2001. Solid- Phase Speciation of Cd, Ni, and Zn in Contaminated and Noncontaminated Tropical Soils. CRC Press LLC. pp: 213-225.

Katznelson, H., E. A. Peterson, and J. W. Rovatt. 1962. Phosphate dissolving microoganisms on seed and in the root zone of plants. Can J Bot. 40: 1181–6.

Kermanshahi, R. K., A. Ghazifard, and A. Tavakoli. 2007. Identification of bacteria resistant to heavy metals in the soils of Isfahan Province. Iranian Journal of Science and Technology, Transaction A. 31(A1): 7 – 16.

Khalil, R. A. and M. M. el-Shinnawi. 1989. Humification of organic matter in soil affecting availability of phosphorus from its mineral compounds. Arid Soil Research and Rehabilitation. 3(1): 77-84.

Khan, A. A., G. Jilani, M. S. Akhtar, S. M. S. Naqvi, and M. Rasheed. 2009. Phosphate Solubilizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production. J. agric. Boil. Sci. 1(1): 48-58.

Khan, J. A., and R. M. Bhatnagar. 1977 Studies on solubilization of insoluble phosphate rocks by *Aspergillus niger* and *Penicillium sp.* Fertil Technol. 14: 329–33.

Khan, M. S., A. Zaidi, M. Ahemad, M. Oves, and P. A. Wani. 2010. Plant growth promotion by phosphate solubilizing fungi – current perspective. Arch Agron Soil Sci. 56: 73–98.

Khan. S., N. N. Khan and N. Iqbal. 1991. Studies on the effects of some organic pollutants on the heavy metal transport in an Indian Soil Environmental Pollution. 70 **(**2): 109-115.

Khattak, R. A., and A. L. Page. 1992. Mechanism of manganese adsorption on soil constituents. In D.C. Adrian (ed.) Biogeochemistry of trace metals. *Lewis Publ., Boca* Raton, FL. 383-400.

Kim, K. Y., D. Jordan, and G. A. McDonald. 1998. Effect of phosphate-solubilizing bacteria and vesicular arbuscular mycorrhizae on tomato growth and soil microbial activity. Biol Fert Soils. 26: 79–87.

Kim, K. Y., G. A. McDonald, D. Jordan. 1997. Solubilization of hydroxyapatite by Enterobacter agglomerans and cloned Escherichia coli in culture medium. Biol Fertil Soils. 24: 347–352.

Kim, K.Y., H. Hwangbo, R. D. Park, K. Y. Seong, Y. W. Kim, B. K. Park, and H. B. Krishnan. 2002. proc. 17th WCSS. Bangkok. pp. 114.

Kirchner, M. J., A. G. Wollum, and L. D. King, 1993. Soil microbial populations and activities in reduced chemical input agroecosystems. Soil Sci Soc Amer J. 57: 1289–95.

Kirk, G. L. D., Yu. Tian-ren, and F. A. Choudhury. 1989. Phosphorus chemistry in relation to water regime. Proceedings of a symposium on Phosphorus Requirement for sustainable Agriculture in Asia and Oceania, 6-10th March. International Rice Research Institute, Manila, Philippines.

Knight, T. J., and P. J. Langston-Unkefer. 1988. Enhancement of symbiotic nitrogen fixation by a toxin releasing plant pathogen. Science 242: 951–954.

Konhauser, K., 2007. Introduction to Geomicrobiology. Oxford: Blackwell.

Koshino, M., 1973. Res. Bulletin: National Institute Agric. Sci. Tokyo, Japan. 13(24): 1-151.

Kotuby-Amacher, J., R. P. Gambrell, and M. C. Amacher. 1992. The distribution and environmental chemistry of lead in soil at an abandoned battery reclamation site. In Engineering Aspects of Metal– Waste Management, pp. 1–24. Edited by I. K. Iskander and H. M. Selim. Boca Raton, FL: Lewis Publishers.

Kremer, R. J., 1994. Determination of soil phosphatase activity using a microplate method. Comun Soil Sci Plant Anal. 25: 319–25.

Krieg, N. R., and J. G. Holt. 1984. Bergey's manual of systematic bacteriology 1. (9th ed.). Williams and Wilkins Company. Baltimore. p. 964.

Krishna Murti, C. R., and P. Vishwanathan. 1991. Toxic metals in the Indian Environment. Tata McGraw Hill Publishing Company Ltd., New Delhi.

Kucey, R. M. N. 1983. Phosphate-solubilizing bacteria and fungi in various cultivated and virgin Alberta soils. Canadian Journal of Soil Science. 63: 671-678.

Kucharski, J., Z. Ciecko, T. Niewolak, T. Niklewska-Larska, 1996. Activity of microrganisms in soils of different agricultural usefulness complexes fertilized with mineral nitrogen. Acta Acad Agric Technicae-Olstenensis. 62: 25–35.

Kuhad, R.C., S. Singh, Lata, and A. Singh. 2011. Chapter 4Phosphate-Solubilizing Microorganisms. A Singh *et al*. (Eds.), Bioaugmentation, Biostimulation and Biocontrol, Soil Biology. PP: 28.

Kummerer, K., 2004. Resistance in the environment. J. Antimicrob. Chemoth. 45: 311-320.

Kundu, B. S., and A. C. Gaur, 1984. Rice response to inoculation with N2-fixing and P-solubilizing microorganisms. Plant Soil. 79: 227–234.

Kundu, B., K. Nehra, R. Yadav, and M. Tomar. 2009. Biodiversity of phosphate solubilizing bacteria in rhizosphere of chickpea, mustard and wheat grown in different regions of Haryana, Indian. J. Microbiol. 49: 120–127.

Kuo, S. 1983. Effect of organic residues and nitrogen transformations on phosphorus sorption and desorption by soil. Agronomy abstracts. ASA, Madison, WI.

Laddaga, R. A., R. Bessen, and S. Silver. 1985. Cadmium resistant mutant of Bacillus subtilis 168 with reduced cadmium transport. J Bacteriol 262: 1106–1110.

Lakzian, A., P. Murphy, A. Turner, J. L. Beynon, and K. E. Giller. 2002. Rhizobium leguminosarum bv. viciae populations in soils with increasing heavy metal contamination: abundance, plasmid profiles, diversity and metal tolerance. Soil Biol. Biochem. 34: 519-529. DOI: 10.1016/S0038-0717(01)00210-3.

Lamo, K., B. N. Korla, and Y. R. Shukla. 2012. Effect on different organic and inorganic sources on seed production of Radish (*Raphanus sativus CV. Chinese pink*). Life Sciences Leaflets. 2: 38-44.

Lebrun, M., A. Andurier, and P. Cossart. 1994. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are present on Tn5422, a novel transposon closely related to Tn917. J. Bacteriol. 176: 3049 - 3061.

Ledin, M. 2000. Accumulation of metals by microorganisms – processes and importance for soil system. Earth Sci. Rev. 51: 1-31. DOI: 10.1016/S0012-8252(00)00008-8.

Lena, Q. M. and N. R. Gade, 1997. Chemical fractionation of cadmium, copper, nickel, and zinc in contaminated soils. J. Environ. Qual. 26: 259.

Leonard, S. S., G. K. Marres, and X. L. Shi. 2004. Metal-induced oxidative stress and signal transduction. Free Rad. Biol. Med. 37: 1921 - 1942.

Li, Z. Y., J. M. Li, M. G. Xu, J. L. Lv, and N. Sun. 2007. Change and availability of soil inorganic phosphorus components influenced by phosphorus fertilizer application. China Soil and Fertilizer. 3: 32-35.

Logan, N. A., and R. C. W. Berkeley. 1984. Identification of Bacillus strains Using the API System. J. Gen. Microbiol. 130: 1871-1882.

Lone, M. I., Z. He, P. J. Stoffella, and X. Yang. 2008. Phytoremediation of heavy metal polluted soils and water: progresses and perspectives. Journal of Zhejiang University Science B (Biomedicine & Biotechnology). 9: 210-220.

Lopez, B. R., Y. Bashan, and M. Bacilio. 2011. Endophytic bacteria of Mammillaria fraileana, an endemic rock-colonizing cactus of the Southern Sonoran Desert. Arch Microbiol. 193: 527–541.

Louw, H. A., and D. M. Webley, 1959. A study of soil bacteria dissolving certain phosphate fertilizers and related compounds. J Appl Bacteriol. 22: 227-233.

Lynch, J. M., 1990. Microbial metabolites. In: Lynch JM, editor. The Rhizosphere. Chichester, England: John Wiley and Sons Ltd, Baffins Lane, Interscience. pp. 177–206.

Ma, L. Q. and G. N. Rao, 1997. Chemical fractionation of cadmium, copper, nickel and zinc in contaminated soils. J. Envir. Qual. 26: 259-264.

Macreadie, I. G., A. K. Sewell, and D. R. Winge. 1994. Metal ion resistance and the role of metallothionein in yeast. *In* Metal Ions in Fungi. pp 279–310. Edited by G. Winkelmann, and D. R. Winge. New York: Marcel Dekker.

Mahantesh, P., and C. S. Patil. 2011. Isolation and Biochemical Characterization of Phosphate Solubilizing Microbes. Intern. Journal of Microbiol., Research. 3 (1): 67-70.

Maier, R. M., I. L. Pepper, and C. P. Gerba. 2000. Environmental Microbiology. San Diego, Academic Press. p. 405.

Maiz, I., I. Arambarri, R. Garcia, and E. Millan. 2000. Evaluation of heavy metal availability in polluted soils by two sequential extraction procedures using factor analysis. Environmental Pollution. 110(1): 3-9.

Makui, H., E. Roig, S. T. Cole, J. D. Helmann, P. Gros, and M. F. Cellier. 2000. Identification of the Escherichia coli K-12 Nramp orthologue (MntH) as a selective divalent metal ion transporter. Mol Microbiol. 35: 1065–1078.

Maliha, R., K. Samina, A. Najma, A. Sadia, and L. Farooq. 2004. Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms under in vitro conditions. Pak J Biol Sci. 7: 187–196.

McGrath, J. W., G. B. Wisdom, G. McMullan, M. J. Lrakin, and J. P. Quinn. 1995. The purification and properties of phosphonoacetate hydrolase, a novel carbon-phosphorus bond-cleaving enzyme from *Pseudomonas fluorescens* 23F. Eur J Biochem. 234: 225–30.

McLaughlin, M. J., K. G. Tiller, R. Naidu, and D. P. Stevens. 1996. Review: the behaviour and environmental impact of contaminants in fertilizers. Australian Journal of Soil Research. 34: 1-54.

Meek, B. D., L. E. Graham, T. J. Donovan and K. S. Mayberry. 1979. Phosphorus availability in a calcareous soil after high loading rates of animal manure. Soil Sci. Soc. Am. J. 43: 741-773.

Meguro, N., Y. Kodama, M. T. Gallegos, and K. Watanabe. 2005. Molecular characterization of resistance-nodulation-division transporters from sol vent- and drug-resistant Bacteria in petroleum-contaminated soil. Appl. Environ. Microbiol. 71(1): 580-586. DOI: 10.1128/ AEM.71.1.580-586.2005.

Mergeay, M., D. Nies, H. G. Schlegel, J. Gerits, P. Charles, and F. van Gijsegem. 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid bound resistance to heavy metals. J. Bacteriol. 3: 691 – 698.

Mergeay, M., D. Nies, H. G. Schlegel, J. Gerits, P. Charles, and F. van Gijsegem. 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid bound resistance to heavy metals. J. Bacteriol. 3: 691 – 698.

Miller, W. P., D. C. Martens, and L. W. Zelazny. 1986. Effect of sequence in extraction of trace metals from soils. Soil science Society of America Journal. 50(3): 598-601.

MoE (Ministry of the Environment), Japan. 1970. Agricultural Land Soil Prevention Law, No. 139. MoE, Japan.

Mohammadi, K. 2012. Phosphorus Solubilizing Bacteria: Occurrence, Mechanisms and Their Role in Crop Production. Resources and Environment. 2: 80-85.

Molla, M. A. Z., A. A. Chowdhury, A. Islam, and S. Hoque, 1983. Micribial mineralization of organic phosphate in soil. Plant soil. 78: 393-399.

Morillo, J. A., M. Aguilera, A. Ramos-Cormenzana, and M. MonteolivaSa´nchez. 2006. Production of a metal-binding exopolysaccharide by Paenibacillus jamilae using two-phase olive-mill waste as fermentation substrate. Curr Microbiol. 53: 189–193.

Murphy, J., and J. P. Riley. 1962. A modified single solution method for the determi nation of phosphate in natural waters. Analytica Chimia Acta. 27: 31–36.

Narwal, R. P., B. R. Singh, and B. Selbu. 1999. Association of cadmium, zinc, copper and nickel with components in naturally heavy metal rich soils studied by parallel and sequential extraction. Commun. Soil Sci. Plant Anal. 30: 1209-1230.

Nautiyal, C. S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol. Lett. 170: 65-270.

Nautiyal, C. S., S. Bhaduria, P. Kumar, H. Lal, R. Mondal, and D. Verma. 2000. Stress induced phosphate solubilization in bacteria isolated from alkaline soil. FEMS. Microbiology Letters. 182(2): 291-296.

Nealson, K. H., R. A. Rosson, and C. R. Mayers. 1992. Microbial reduction of manganese and iron: New approaches to carbon cycling. App Environ Microbiol. 58: 439.

Nies, D. H. 1992a. Resistance to cadmium, cobalt, zinc, and nickel in microbes. Plasmid. 27: 17–28.

Nies, D. H. 1999. Microbial heavy-metal resistance. Appl Microbiol Biotechnol 51: 730–750.

Nies, D. H. and S. Silver. 1995. Ion efflux systems involved in bacterial metal resistances. J Ind Microbiol. 14: 186–199.

Nies, D. H., 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiology Reviews. 27: 313-339.

Norman, U., S. B. Andrew, F. Erick, H. Hans, H. Olivier, L. Mark, P. Cheryl, P. Jules, S. Pedro, S. Nteranya and T. Janice (ed.). 2006. Biological approaches to sustainable soil systems. p - 60.

Nriagu, J. O. 1979. Global inventory of natural and anthropogenic emission of trace metals to the atmosphere. Nature. p. 409-411.

Ochiai, E. 1997. Bioinorganic chemistry; An introduction (Align and Bacon, Boston), 1.

Ohtake, H., H. Wu, K. Imazu, Y. Ambe, J. Kato, and A. Kurod. 1996. Bacterial phosphonate degradation, phosphite oxidation and polyphosphate accumulation. A Res Conserv and Recycling. 18: 125–34.

Oliveira, C.A., V. M. C. Alves, I. E. Marriel, E. A. Gomes, M. R. Scotti, N. P. Carneiro, C. T. Guimaraes, R. E. Schaffert, and N. M. H. Sa. 2009. Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. Soil Biol Biochem. 41: 1782–1787.

Osman, D., and J. S. Cavet. 2008. Copper homeostasis in bacteria. Adv Appl Microbiol. 65: 217–247.

Ostwal, K. P. and V. P. Bhide. 1972. Solubilization of tricalcicum phosphate by soil *Pseudomonas.* Indian J Exp Biol.10: 153–4.

Pal, A., and A. K. Paul. 2008. Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. Indian J Microbiol. 48: 49–64.

Paperi, R., E. Micheletti, and R. De Philippis. 2006. Optimization of copper sorbing-desorbing cycles with confined cultures of the exopolysaccharide-producing cyanobacterium Cyanospira capsulata. J Appl Microbiol. 101: 1351–1356.

Parmar, N., K. R. Dadarwal. 1999. Stimulation of nitrogen fixation and induction of flavonoid like compounds by rhizobacteria. J Appl Microbiol. 86: 36–44.

Patil, R. B., B. J. Rajashekharappa, D. P. Viswanath, and M. V. Shantaram. 1979. Solubilization and immobilization of phosphate by some microorganisms and phosphorus availability to plants. Bull. Ind. Soc. Soil Sci. 12: 550-556.

Paul, A., G. Wauters, and A. K. Paul. 2007. Nickel tolerance and accumulation by bacteria from rhizosphere of nickel hyperaccumulatores in serpentine soil ecosystem of Andaman, India. Plant Soil. 293(1-2): 37-48. DOI: 10.1007/s11104-007-9195-7.

Pazirandeh, M., L. A. Chrisey, J. M. Mauro, J. R. Campbell, and B. P. Gader, 1995. Expression of the *Neurospora crassa* metallothionein gene in *Escherichia coli* and its effect on heavy-metal uptake. Appl. Microbiol.Biotechnol. 43: 1112-1117.

Pečiulyté, D., and V. 2009. Dirginčiuté-Volodkiené. Effect of long-term industrial pollution on soil microorganisms in deciduous forests situated along a pollution gradient next to a fertilizer factory. Ekologija. 55: 67-77.

Pelczar, Jr. M. J., E. C. Chan, and N. R. Kreig. 1993. Microbiology (5th ed.). McGraw-Hill Book Company. New York.

Pelczar, Jr. M. J., E. C. Chan, and N. R. Kreig. 1993. Microbiology (5th ed.). McGraw-Hill Book Company, New York.

Perez, M. P. J. A., R. Garcı´a-Ribera, T. Quesada, M. Aguilera, A. Ramos-Cormenzana, and M. Monteoliva-Sa´nchez. 2008. Biosorption of heavy metals by the exopolysaccharide produced by Paenibacillus jamilae. World J Microbiol Biotechnol. 24: 2699–2704.

Perveen, S., M. S. Khan, A. Zaidi. 2002. Effect of rhizospheric microorganisms on growth and yield of green gram (Phaseolus radiatus). Indian J Agric Sci. 72: 421–423.

Pikovskaya, R. I. 1948. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Microbiology. 17: 362–370.

Piotrowska-Seget, Z., M. Cycoń, and J. Kozdrój. 2005. Metal-tolerant bacteria occurring in heavily polluted soil and mine spoil. Applied Soil Ecology. 28: 237-246.

Ponmurugan, P. C., and C. Gopi. 2006. In vitro production of growth regulators and phosphates activity by phosphate solubilizing bacteria. African Journal of Biotechnology. 5: 348-350.

Poole, R. K., and G. M. Gadd. 1989. "Metals: Microbe Interactions. IRL Press. Oxford. pp. 1-37.

Puente, M. E., C. Y. Li, Y. Bashan. 2009a. Rock-degrading endophytic bacteria in cacti. Environ Exp Bot. 66: 389–401.

Puente, M. E., C.Y. Li, Y. Bashan. 2009b. Endophytic bacteria in cacti seeds can improve the development ctus seedlings. Environ Exp Bot. 66: 402–408.

Puente, M. E., Y. Bashan, C. Y. Li, and V. K. Lebsky. 2004a. Microbial populations and activities in the rhizoplane of rock-weathering desert of caplants. I. Root colonization and weathering of igneous rocks. Plant Biol. 6: 629–642.

Raghu, K., I. C. MacRae. 1966. Occurrence of phosphate-dissolving microorganisms in the rhizosphere of rice plants and in submerged soils. J Appl Bacteriol. 29: 582–6.

Rajapaksha, R. M. C. P., M. A. Tabor-Kapłon, and E. Bååth. 2004. Metal toxicity affects fungal and bacterial activities in soil differently. Applied and Environmental Microbiology. 70: 2966-2973.

Rajbanshi, A. 2008. Study on heavy metal resistant bacteria in Guheswori sewage treatment plant. Our Nature. 6: 52 – 57.

Rajendran, P., J. Muthukrishnan, and P. Gunasekaran. 2003. Microbes in heavy metal remediation. Indian J. Experiment. Bio. 41(9): 935-944.

Ramkumar, V. S., and E. Kannapiran. 2011. Isolation of total heterotrophic bacteria and phosphate solubilizing bacteria and in vitro study of phosphatase activity and production of phytohormones by PSB. Archives of Applied Science Research. 3: 581-586.

Ramos, L., L. M. Hernandez, and M. J. Gonzalez. 1994. Sequential fraction of copper, lead, cadmium and zinc in soils from or near Donana National Park. J. Environ. Qual. 20: 50.

Ratuzny, T., Z. Gong, and B. M. Wilke. 2009. Total concentrations and speciation of heavy metals in soils of the Shenyang Zhangshi Irrigation Area, China. Environmental Monitoring and Assessment. 156(1-4): 171-180.

Reddy, K. R., M. R. Overcash, R. Khaleal and P. W. Westerman. 1980. Phosphorus sorption and desorption characteristics of two soils utilized for disposal of animal manure. J. Env. Qual. 9: 86-92.

Renella, G., D. Egamberiyeva, L. Landi, M. Mench, and P. Naniepieri. 2006. Microbial activity and hydrolase activities during decomposition of root exudates released by an artificial root surface in Cd-contaminated soils. Soil Biol. Biochem. 38: 702-708. DOI:10.1016/j.soilbio.2005.06.021.

Rengel, Z., and P. Marschner. 2005. Nutrient availability and management in the rhizosphere: exploiting genotypic differences. New Phytology. 168: 305–312.

Rensing, C. H., D. T. Newby, and I. L. Pepper. 2002. The Role Of Selective And Selfish Dna In Horizontal Gene Transfer And Soil Microbial Community Adaptation. Soil. Biol. Biochem. 34: 285-296.

Reyes, I., R. Baziramakenga, L. Bernier, and H. Antoun. 2001. Solubilization of phosphate rocks and minerals by a wild type strain and two UV induced mutants of Penicillium regulosum. Soil Biol Biochem. 33: 1741–1747.

Richardson, A. E., and P. A. Hadobas. 1997. Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. Can J Microbiol. 43: 509–16.

Richardson, E. A., and R. J. Simpson. 2011. Soil Microorganisms Mediating Phosphorus Availability. Plant Physiology. 156: 989–996.

Roane, T. M. 1999. Lead resistance in two bacterial isolates from heavy metal contaminated soils. Microb Ecol. 37: 218–224.

Roane, T. M., and I. L. Pepper. 2000. Microbial responses to environmentally toxic cadmium. Microbial Ecology. 38: 358 – 364.

Roane, T. M., and S. T. Kellogg. 1996. Characterization of bacterial communities in heavy metal contaminated soils. Can. J. Microbiol. 42: 593 - 603.

Rodriguez, H., and R. Fraga. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances. 17(4-5): 319-359.

Rodríguez, H., I. Goire, and M. Rodríguez, 1996. Caracterización de cepas de *Pseudomonas* solubilizadoras de fósforo. Rev ICIDCA. 30: 47–54.

Rodriguez, H., R. Fraga, T. Gonzalez, Y. Bashan. 2006 .Genetics of phosphate solubilization and its potential application for improving plant growth-promoting bacteria. Plant Soil. 287: 15–21.

Rosen, B. P. 2002. Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. Comp Biochem Physiol. 133: 689–693.

Rossolini, G. M., S. Schippa, M. L. Riccio, F. Berlutti, L. E. Macaskie, and M. C. Thaller. 1998.Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. Cell Mol Life Sci. 54: 833–850.

Rouch, D. A., B. T. D. Lee, and A. P. Morby. 1995. Understanding cellular responses to toxic Agents: A model for mechanism choice in bacterial metal resistance. J. Ind. Microbiol. 14: 132 - 141.

SAB (Society of American Bacteriologist). 1957. Manual of microbiological methods. McGraw Hill Book Company Inc., New York. p. 315.

Saber, K., L. D. Nahla, and A. Chedly. 2005. Effect of P on nodule formation and N fixation in bean. Agron Sustain Dev. 25: 389–393.

Sag, Y., D. Ozer and T Kutsal. 1995. A comparative study of the biosorption of lead(II) ions to Z. ramigera and R. arrhizus. Process Biochem. 30: 169–174.

Sahu, S. N., and B. B. Jana, 2000. Enhancement of the fertilizer value of rock phosphate engineered through phosphate-solubilizing bacteria. Ecological Engineering. 15: 27-39.

Salbu, B., T. Krekling, and D. H. Oughton. 1998. Characterisation of radioactive particles in the environment. Analyst. 123: 843.

Salehizadeh, H. and S. A. Shojaosadati. 2003. Removal of metal ions from aqueous solution by polysaccharide produced from Bacillus firmus. Water Res 37: 4231–4235.

Samuel, J. T., G. Sposito, D. Hesterberg and U. Kafkafi. 1986. Effects of pH and organic acids on organophosphate solubility in an acid montmorillonitic Soil. Soil Sci. Soc. Am. J. 50: 45-52.

Sandaa, R. A., V. Torsvik, and O. Enger. 2001. Influence of long-term heavy metal contamination on microbial communities in soil. Soil Biol. Biochem. 33: 287-295.

Saxena, A. K., and K. V. B. R. Tilak. 1994. Interaction among beneficial microorganisms. Indian J Microbiol. 34: 91–106.

Saxena, A. K., and K. V. B. R. Tilak. 1997. Interaction of soil microorganisms with vesicular *arbuscular mycorrhiza.* In: J. P. Tiwari, G. Saxena, I. Tiwari, N. Mittal, B. P. Chamola. (eds). New approches in microbial ecology. Aditya Books, New Delhi, India. pp 231–250.

Scervino, J. M., V. L. Papinutti, M. S. Godoy, J. M. Rodriguez, I. D. Monica, M. Recchi, M. J. Pettinari, and A. M. Godeas. 2011. Medium pH carbon and nitrogen concentrations modulate the phosphate solubilization efficiency of *Penicillium purpurogenum* through organic acid production. Journal of Applied Microbiology. 110: 1215-1223.

Scheffer, F., and P. Schachtschabel. 1992. Lehrbuch der Bodenkunde. Ferdinand enke Verlag, Stuttgart, Germany.

Schwab, A. P., Y. H. He and M. K. Banks. 2005. The influence of organic ligands on the retention of lead in soil. Chemosphere. 61: 856– 866.

Sharma, R., R. K. Baghel, and A. K. Pandey. 2010. Dynamics of acid phosphatase production of the ectomycorrhizal mushroom *Cantharellus tropicalis*.African Journal of Microbiology Research. 4: 2072-2078.

Sharma, S., V. Kumar, and R. B. Tripathi. 2011. Isolation of Phosphate Solubilizing Microorganism (PSMs) From Soil. The J. Microbiol. Biotech. Res. 1: 90-95.

Sheng, X. F., J. J. Xia, C. Y. Jiang, L. Y. He, and M. Qian 2008.Characterization of heavy metal-resistant endophytic bacteria from rape (Brassica napus) roots and their potential in promoting the growth and lead accumulation of rape. Environ Pollut. 156: 1164– 1170.

Shin, M. N., J. Shim, Y. You, H. Myung, K. S. Bang, M. Cho, S. Kamala-Kannan, and B. T. Oh. 2012. Characterization of lead resistant endophytic Bacillus sp. MN3-4 and its potential for promoting lead accumulation in metal hyperaccumulator Alnus firma. J Hazard Mater 199–200, 314–320.

Siegel, F. R. 2002. Environmental Geochemistry of Potentially Toxic Metals. Berlin: Springer-Verlag. p. 218.

Silver, S., and L. T. Phung. 1996. Bacterial heavy metal resistance: new surprises. Annu Rev Microbiol. 50: 753–789.

Silver, S., and L. T. Phung. 2009. Heavy metals, bacterial resistance. *In* Encyclopedia of Microbiology, Edited by M. Schaechter. Oxford: Elsevier. pp. 220–227.

Sindhu, S. S., S. K. Gupta, S. Suneja, and K. R. Dadarwal. 2002b. Enhancement of greengram nodulation and growth by Bacillus sp. Biol Plantarum. 45: 117–120.

Sindhu, S. S., S. Suneja, A. K. Goel, N. Parmar, and K. R. Dadarwal. 2002a. Plant growth promoting effects of Pseudomonas sp. on coinoculation with Mesorhizobium sp. cicer strain under sterile and wilt sick soil conditions. Appl Soil Ecol. 19: 117–120.

Singal, R., R. Gupta, and R. K. Saxena. 1994. Rock phosphate solubilization under alkaline conditions by *Aspergillus japonicus* and A. foetidus. Folia Microbiol. 39: 33–36.

Singh, A. K., P. L. Gupta, and H. P. Agrawal. 2001. Chromium contamination of water resources and soils and of carpet industrial area of eastern Uttar Pradesh. J. Ind. Soc. Soil Sci., 49(4)**:** 776-778.

Singh, S. M., L. Yadav, S. K. Singh, P. Singh, P. N. Singh, and R. Ravindra. 2011. Phosphate solubilizing ability of two Arctic *Aspergillus niger* strains. Polar Research. 30: 7283. doi: 10.3402/polar.v30i0.7283.

Singh, S. P., F. M. G. Tack, and M. G. Verioo. 1996. Extractability and bioavailability of heavy metals in surface soils derived from dredged sediments. Chem Spec Bioavail. 8: 105–110.

Singh, S. P., F. M. Tack, and M. G. Verloo. 1998. Heavy metal fractionation and extractability in dredged sediment derived surface soils. Water Air Soil Pollut.102: 313.

Skrary, F. A., and D. C. Cameron. 1998. Purification and characterization of a *Bacillus licheniformis* phosphatase specific for D-alpha-glycerphosphate. Arch Biochem Biophys. 349: 27–35.

Smith, P. B., K. M. Tomfohrde, D. L. Rhoden, and A. Balows, 1972. API system: a multitube micromethod for identification of Enterobacteriaceae. Appl. Micro- biol. 24: 449-452.

Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt. 1986. Bergey's manual of systematic bacteriology (9th ed.). Williams and Wilkins. Baltimore. London. 2: 1599.

Sobolev, D., and M. F. T. Begonia. 2008. Effects of heavy metal contamination upon soil microbes: lead-induced changes in general and denitrifying microbial communities as evidenced by molecular markers. International Journal of Environmental Research and Public Health. 5: 450-456.

Son, T. T. N., C. N. Diep, and T. T. M. Giang. 2006. Effect of bradyrhizobia and phosphate solubilizing bacteria application on soybean in rotational system in the mekong delta. Omonrice. 14: 48–57.

Son, T. T. N., V. U. Vu van Thu, and H. Kobayashi. 2003. Effect of organic and bio fertilizer application on rice-soybean-rice cropping systems. In: Proceedings of the final workshop of JIRCAS Mekong Delta Project – "Development of new technologies and their practice for sustainable farming systems in the Mekong Delta", November 25–26, 2003. pp 65–81.

Sood, R. D. and R. S. Minhas. 1989. Studies on the mineralization of organic phosphorus in some soils Himachal Pradesh. J. Indian Soc. Soil Sci. 37(1): 29-34.

Sparks, D. L. (2005). Toxic metals in the environment: the role of surfaces elements. 1: 193–197.

Sperber, J. I. 1958. The incidence of apatite solubilizing organisms in the rhizosphere and soil. Aus. J. Agr. Res. 9: 778-787.

Sperber, J. I. 1958a. The incidence of apatite-solubilizing organisms in the rhizosphere and soil. Aust J Agr Res. 9: 778–781.

Sperber, J. I. 1958b. Solubilization of apatite by soil microorganisms producing organic acids. Aust J Agr Res. 9: 782–787.

Sperberg, J. I. 1985. The incidence of apatite-solubilizing organisms in the rhizosphere and soil. Aust J Agric Res. 9: 778.

Spiro, T. G. and W. M. Stigliani. 2002. Chemistry of the Environment. p. 356. Upper Saddle River, NJ: Prentice-Hall.

Sposito, G., J. Lund, and A. C. Chang. 1982. Trace metal chemistry in arid-zone field soils amended with sewage sludge: I Fractionation of Ni, Cu, Zn, Cd and Pb in solid phases. Soil Sci. Soc. Am. J. 46: 164-260.

Sprocati, A. R., C. Alisi, L. Segre, F. Tasso, M. Galletti, and C. Cremisini. 2006. Investigating heavy metal resistance, bioaccumulation and metabolic profile of a metallophile microbial consortium native to abandoned mine. Science of the Total Environment. 366: 649-658.

Sridevi, M. A. Ranjan, and M. R. Mahalakshmi. 2013. Isolation and Characterization of Phosphate-solublizing bacterial species from different crop fields of Slem, Tamil Nadu, India. International Journal of Nutrition, Pharmacology, Neurological disease, 3: 1.

Srinivasan, R., A. R. Alagawadi, S. Mahesh, K. K. Meena, and A. K. Saxena. 2012. Characterization of phosphate solubilizing microorganisms from salt-affected soils of India and their effect on growth of sorghum plants [*Sorghum biocolor* (L). Moench]. Ann Microbiol. 62: 93-105.

Stewart, J. W. B. 1981. The importance of P cycling and organic P in soils, in better crops with plant food. Am. Potash-Phosphate Institute, Atlanta, Georgia. Pp. 16-19.

Stibal, M., A. M. Anesio, C. J. D. Blues, and M. Tranter. 2009. Phosphatase activity and organic phosphorus turnover on a High Arcticglacier. Biogeosciences. 6: 913-922.

Stietiya, M. H., and J. J. Wang. 2014. Zinc and cadmium adsorption to aluminium oxide nanoperticles affected by naturally occurring ligands. Journal of environmental quality. *Inc.* Soil Science Society of America. 43(2): 498-506.

Stohs, S. J., and D. Bagchi. 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Rad. Biol. Med.* 18: 321 - 336.

Subba, R. N. S. 1993. Biofertilizers in agriculture and forestry. Oxford, New Delhi: Oxford Press and IBH Publishing Co.

Sumio, I. 2002. Application of mechanistic model for P uptake by barley under lower temperature condition. Soil Sci. Plant Nutr. 48(3): 441-445.

Sun, F. and Z. Shao. 2007. Biosorption and bioaccumulation of lead by Penicillium sp. Psf-2 isolated from the deep sea sediment of the Pacific Ocean. Extremophiles. 11: 853–858.

Surange, S. M. 1985. Comparative phosphate solubilizing capacity of some soil fungi. Curr Sci. 54: 1134-1135.

Syers, J. K., A. E. Johnston, and D. Curtin. 2008. Efficiency of soil and fertilizer phosphorus use: reconciling changing concepts of soil phosphorus behavior with agronomic information. Rome: Food and Agriculture Organization of the United Nations.

Taghavi, S., M. Mergeay, and D. vanderLelie. 1997. Genetics and physical map of the *Alcaligenes eutrophus* CH34 mega-plasmid pMOL28 and it derivative pMOL50 obtained after temperature induced mutagenesis and mortality. Plasmid. 37: 22 – 34.

Taha, S. M., S. A. Z. Mahmood, A. H. El-Damaty, and A. M. A. El-Hafez. 1969. Activity of phosphate dissolving bacteria in Egyptian soils. Plant and soil. 31: 149-160.

Tanaskovic, M. 1987. Monthly report on river water quality within the Belgrade Territory. City Institute for Health Protection. Belgrade. 29.

Tandon, H. L. S. 1987. Phosphorus Research and Agricultural Production in India pp. 160 (New Delhi: Fertilizer development and consultation organization).

Tarafdar, J. C., and N. Claassen. 1988. Organic phosphorus compounds as a phosphorus source for higher plants through the activity of phosphatases produced by plant roots and microorganisms. Biol Fertil Soils. 5: 308–12.

Tessier, A., P. G. C. Campbell, and M. Bisson. 1979. Sequential extraction procedure for the speciation of particulate trace metals, Anal. Chem. 51: 844-851.

Thaller, M. C., F. Berlutti, S. Schippa, P. Iori, C. Passariello, and G. M. Rossolini. 1995b. Heterogeneous patterns of acid phosphatases containing low-molecular-mass Polipeptides in members of the family *Enterobacteriaceae.* Int J Syst Bacteriol. 4: 255–61.

Theodorou, M. E. and W. C. Plaxton. 1993. Metabolic adaptations of plant respiration to nutritional phosphate deprivation; Pl. Physiol. 101: 339-344.

Tilak, K.V.B.R. 1993. Bacterial fertilizers. New Delhi, India: Indian Council of Agricultural Research.

Tisdale, S. L., W. l. Nelson, and J. D. Beaton. 1985. Soil Fertility and Fertilizers. 4^{th}ed. MacMillan Publishing Company, New York.

Tomsett, A. B., A. K. Sewell, S. J. Jones, J. R. Miranda, and D. A. Thurman. 1992. Metal binding proteins and metal regulated gene expression in higher plants. In Inducible plant proteins, Soc. Exp. Biol. Sem. Series, edited by Wary j. L. (Cambridge University Press, New York). 1.

Tornabene, T. G. and H. W. Edwards. 1972. Microbial uptake of lead. Science 176: 1334–1335.

Tornabene, T. G. and S. L. Peterson. 1975. Interaction of lead and bacterial lipids. Appl Microbiol. 29: 680–684.

Trajanovska, S., M. L. Britz, and M. Bhave. 1997. Detection of heavy metal ion resistance genes in gram-positive and gram-negative bacteria isolated from a lead-contaminated site. Biodegradation. 8: 113–124.

Trevors, J. T., G. W. Stratton, and G. M. Gadd. 1986. Cadmium transport, resistance, and toxicity in bacteria, algae, and fungi. Can. J. Microbiol. 32: 447 - 464.

Turner, B. L., and P.M. Haygarth. 2005. Phosphatase activity in temperate pasture soils: potential regulation of labile organic phosphorus turnover by phosphor di-esterase activity. Sci. Total Environ. 344: 27–36.

Tyler, G. 1974. Heavy metal pollution and soil enzymatic activity. Plant and Soil. 41: 303-311.

Tynecka, Z., Z. Gos, and J. Zajac. 1981. Reduced cadmium transport determined by a resistance plasmid in Staphylococcus aureus. J Bacteriol. 147: 305–312.

Ure, A. M., P. H. Quevauviller, H. Muntau, and B. Griepink. 1993. Speciation of heavy metals in soils and sediments. An account of the improvement and harmonization of extraction techniques undertaken under the auspices of the BCR of the commission of the European communities. International Journal of Environmental Analytical Chemistry. 51: 135-151.

Vallee, B. L. and D. D. Ulmer. 1972. Biochemical effects of mercury, cadmium, and lead. Annu Rev Biochem. 41: 91–128.

Van Ho, A., D. M. Ward, and J. Kaplan. 2002. Transition metal transport in yeast. Annu Rev Microbiol. 56: 237–261.

Vazques, P., G. Holguin, M. E. Puente, A. Lopez-Cortes, and Y. Bashan. 2000. Biol. Fertil. Soils. 30: 460-468.

Vázquez, P. 1996. Bacterias solubilizadoras de fosfatos inorgánicos asociadas a la rhizosfera de los mangles: *Avicennia germinans* (L.), *Laguncularia racemosa* (L.) Gerth. Tesis para el título de Biologo Marino. Univ. Autónoma de Baja California Sur. La Paz, B.C.S. México.

Vazquez, P., G. Holguin, M. Puente, A. Lopez-cortes, and Y. Bashan. 2000. Phosphate solubilizing microorganisms associated with the rhizosphere of mangroves in a semi-arid coastal lagoon. Biol Fertil Soils. 30: 460–468.

Veglio, F., F. Beolchini, and A. Gasbarro. 1997. Biosorption of toxic heavy metals: an equilibrium study using free cells of *Arthrobacter* sp. Process Biochem. 32: 99-105.

Vikram, A. 2007. Efficacy of Phosphate Solubilizing Bacteria Isolated from Vertisols on Growth and Yield Parameters of Sorghum. Research Journal of Microbiology. 2: 550-559.

Violante, A., P. M. Huang, and G. M. Gadd. (ed.) 2008. Biophysicochemical Processes of Heavy Metals and Metalloids in Soil Environments. Chichester: Wiley.

Vyas, P., and A. Gulati. 2009. Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent Pseudomonas. BMC Microbiol. 9: 174.

Wada, K., Li. Xue-Yuan, and P. W. Moody. 1989. Chemistry of adverse upland soils. Proceedings of a Symposium on Phosphorus Requirement for Sustainable Agriculture in Asia and Oceania, 6-10th March, 1989. International Rice Research Institute, Manila, Philippines, pp. 307-323.

Wainwright, M., W. Nevell and S. J. Grayston. 1986. Potential use of heterotrophic sulphur oxidizing microorganisms as soil inoculants. Sulphur Agric. 10: 248-253.

Wang, Y. T., and H. Shen. 1995. Bacterial reduction of hexavalent chromium. *J. Ind. Microbiol*. 14: 159 - 163.

Wani, P. A., M. S. Khan, and A. Zaidi. 2007. Synergistic effects of the inoculation with nitrogen fixing and phosphate-solubilizing *rhizobacteria*

on the performance of field grown chickpea. J Plant Nutr Soil Sci. 170: 283–287.

Weast, R. C. 1984. CRC handbook of chemistry and physics. 64 (edn).CRC. Boca Raton. Fla.

Weinberg, E. D. 1990. Roles of trace metals in transcriptional control of microbial secondary metabolism. Biology of Metals. 2: 191-196.

Welte, B. N., and Montiel, A. 1983. Study of the different methods of speciation of heavy metals in the sediments: II. Applications. Environ. Technol. Lett. 4: 223-238.

Whitelaw, M. A., T. J. Harden, and K. R. Helyar, 1999. Phosphate solubilization in solution culture by the soil fungus *Penicillium radicum*. Soil Biol Biochem. 32: 655–665.

Winge, D. R., R. N. Reese, K. Mehra, E. B. Tarbet, A. K. Hughes, and C. T. Daeron. 1989. Structural aspects of metal-r-glutamyl peptides. *In*: Ion Homeostasis: Molecular Biology and Chemistry. D. H. Hamer, and D. R Winge (eds.). Alan R. Liss. New York. p. 301 - 311.

Witter, E. 1989. Agricultural use of sewage sludge, Controlling metal contamination of soils. National Environment Protection Board. Stockholm, Sweden. Rep. 3620.

Wuertz, S., and M. Mergeay. 1997. The impact of heavy metals on soil microbial communities and their activities. *In:* Modern Soil Microbiology. J. D. Van Elsas, J. T. Trevors, and E. M. H. Wellington (eds.). p. 607 – 639. Marcel Dekker, New York.

Wyszkowska, J., and M. Wyszkowski. 2002. Effect of cadmium and magnesium on microbiological activity in soil. Polish journal. of Environmental Studies 11(5): 585 – 591.

Xu, J. G., and R. L. Johnson. 1995. Root growth, microbial activity and phosphatase activity in oil-contaminated, remediated and uncontaminated soils planted to barley and field pea. Plant Soil. 173: 3–10.

Yasmin, H., and A. Bano. 2011. Isolation and characterization of Phosphate Solublizing Bacteria From Rhizosphere Soil Of Khewra Salt Range And Attock. Pak. J. Bot. 43: 1663-1668.

Yokel, J. and D. A. Delistraty. 2003. Arsenic, lead, and other trace elements in soils contaminated with pesticide residues at the Hanford site (USA). Environ Toxicol. 18: 104–114.

Zaidi, A., M. S. Khan, and M. Amil. 2003. Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea (*Cicer arietinum* L.). Eur J Agron. 19: 15–21.

Zanardini, E., V. Andreoni, S. Borina, F. Cappitellia, D. Daffonchio, P. Talottaa, C. Sorlinia, G. Ranallib, S. Brunic, and F. Cariatic. 1997. Lead-resistant microorganisms from red stains of marble of the Certosa of Pavia, Italy and use of nucleic acid-based techniques for their detection. *Int* Biodeter Biodegr. 40: 171– 182.

Zeien, H., and G. W. Brummer. 1989. Chemische extraction zur bestimmung von schwermetallbindungsformen in boden. Metteilungen der Deutschen Bodenkundlichen Gesellschaft. 39: 505-510.

Zhang, J., W. W. Huang, and Q. Wang. 1990. Concentration and partitioning of particulate trace metals in the Changing (Yangze river). Water Air Soil Pollut. 52: 57.

Zhang, X., B. Kosier, U. B. Priefer. 2001. Symbiotic plasmid rearrangement in Rhizobium leguminosarum bv. viciae VF39SM. J. Bacteriol. 183: 2141-2144. DOI: 10.1128/ JB.183.6.2141-2144.2001.

Zhao, X. R., and Q. M. Lin. 2001. A review of phosphate – dissolving microorganisms. Soil Fertilizer. 3: 7-11.

Zimdahl, R. L. and R. K. Skogerboe. 1977. Behavior of Lead in soil. Environ. Sci. Tehnol. 1: 1202-1207.

Web: http://blast.ncbi.nlm.nih.gov

Dhaka University Institutional Repository

APPENDICES

APPENDIX A

Composition of the growth media used in this study

(In each case, pH was adjusted before sterilization).

A 1 Acid and gas production From Glucose (SAB, 1957)

Typical Formula	gm/litre
Beef extract	3.0 g/L
Peptone	5.0 g/L
Carbohydrate (glucose)	5.0 g/L
Phenol red	0.018 g/L
Distilled Water	1000 ml
pH	6.8

A 2 Methyl Red/Voges-proskauer broth medium (Bryan, 1950)

Typical Formula	gm/litre
Peptone	7.0 g/L
Glucose	5.0 g/L
Di-potassium hydrogen phosphate	5.0 g/L
Distilled water	1000 ml

pH 7.0 ± 0.2 @ 25°C

A 3 Deep glucose agar medium (SAB, 1957)

Typical Formula	**gm/litre**
Beef extract	3.0 g/L
Peptone	5.0 g/L
Glucose	10.0 g/L
Agar	15.0 g
Distilled water	1000 ml

A 4 Motility medium (Eklund and Lankford, 1967)

Typical Formula	**gm/litre**
Nutrient broth	100 ml
Agar	0.5 g
Tetrazolium chloride	0.001 g

A 5 Nutrient agar medium (Bryan, 1950)

Typical Formula	**gm/litre**
Beef extract	3.0 g/L
Peptone	5.0 g/L
NaCl	5.0 g/L
Agar	15.0 g/L
Distilled water	1000 ml/L
pH	7.2

A 6 Tryptic Soy Agar

Typical Formula	gm/litre
Enzymatic Digest of Casein	15 g
Enzymatic Digest of Soybean Meal	5g
Sodium Chloride	5g
Agar	15 g
Final pH 7.3 ± 0.2 at 25°C	

A 7 Nutrient broth medium (Bryan, 1950)

Typical Formula	gm/litre
Beef extract	3.0 g/L
Peptone	5.0 g/L
NaCl	5.0 g/L
Distilled water	1000ml
pH 7.0 ± 0.2 @ 25°C	

A 8 Nutrient gelatin agar medium (Collins and Lyne, 1984)

Typical Formula	gm/litre
Beef extract	3.0 g/L
Peptone	5.0 g/L
Gelatin	8.0 g/L
Agar	15.0 g/L
Distilled water	1000ml

A 9 Phenylalanine agar medium (Sneath *et al*,. 1986)

Typical Formula	**gm/litre**
Yest extract	3.0g/L
Di-phenylalanine	2.0 g/L
Na_2HPO_4	1.0 g/L
NaCl	5.0 g/L
Agar	12.0 g/L
Distilled water	1000 ml
pH	7.3

A 10 Phenol red broth (Sneath *et al.,* 1986)

Typical Formula	**gm/litre**
Pancreatic digest of casein	10.0 g/L
NaCl	5.0 g/L
Phenol red	0.018 g/L
Carbohydrate solution	20 mL
Distilled Water	1000 ml
pH	7.4-7.5

A 11 Physiological Saline

Typical Formula	**gm/litre**
NaCl	0.85 g
Distilled water	100 ml

A 12 Simmon's citrate agar (Claus, 1995)

Typical Formula	**gm/litre**
Magnesium sulphate	0.2g/L
Ammonium Di-hydrogen phosphate	0.2g/L
Sodium ammonium phosphate	0.8g/L
Sodium citrate, tribasic	2.0g/L
Sodium chloride	5.0g/L
Bromothymol Blue	0.08 g/L
Agar	15.0 g/L
pH 7.2 ± 0.2 @ 25°C	

A 13 Starch agar medium (Claus, 1995)

Typical Formula	**gm/litre**
Beef extract	3.0 g/L
Peptone	5.0 g/L
Soluble starch	10.0 g/L
Agar	15.0 g/L
Distilled water	1000 ml
pH 7.0 ± 0.2 @ 25°C	

A 14 Tryptone broth medium (SAB, 1957)

Typical Formula	**gm/litre**
Tryptone	10 g/L
Distilled water	1000 ml
pH	7.2

Dhaka University Institutional Repository

A 15 NaCl Glycine Kim and Goepfert Agar (NGKG)

Typical Formula	gm/litre
Peptone	1.0
Yeast extract	0.4
Sodium chloride	4.0
Glycine	3.0
Polymyxin 3 sulfurate	50,000 U
Phenol red	0.025
Agar	18.0
pH 6.8 ± 0.2 @ 25°C	

A 16 Potato Dextrose agar (PDA)

Typical Formula	gm/litre
Potato, infusion form	200g
Dextrose	20g
Agar	15g
pH (at 25^0C)	5.6±0.2

A 17 Sorbitol MacConkey Agar

Typical Formula	gm/litre
Peptone	20.0
Sorbitol	10.0
Bile salts No.3	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
pH 7.1 ± 0.2 @ 25°C	

A 18 Manitol Salt Agar

Typical Formula	gm/litre
Protease peptone	10.0
Beef extract	1.0
D-manitol	10.0
Sodium chloride	75.0
Agar	15.0
pH 7.4 ± 0.2 @ 25°C	

A 19 Cetrimide Agar Base

Typical Formula	gm/litre
Pancreatic digest of gelatin	20.0
Magnesium chloride	1.4
Potassium sulphate	10.0
Cetrimide	0.3
Agar	15.0
pH 7.2 ± 0.2 @ 25°C	

A 20 Eosine Methylene Blue Agar

Typical Formula	gm/litre
Peptone	10.0g/L
Lactose	10.0g/L
Di-potassium hydrogen phosphate	2.0g/L
Eosin Y	0.4g/L
Methylene blue	0.065g/L
Agar	15g/L
pH: 7.1 ±0.2 at 25°C	

A 21 Pikovskayas Agar (PVK)

Typical Formula	gm/litre
Glucose	10
Yeast extract	0.5
Calcium phosphate ($Ca_3(PO_4)_2$)	5
Ammonium sulphate $(NH_4)_2SO_4$	0.5
Potassium chloride (KCl)	0.2
Sodium chloride (NaCl)	0.2
Magnesium sulphate ($MgSO_4.7H_2O$)	0.1
Manganese sulphate ($MnSO_4.2H_2O$)	0.0001
Ferrous sulphate ($FeSO_4.7H_2O$)	0.0001
Agar	15
pH	7

A 22 National Botanical Research Institute's Phosphate Growth Medium (NBRIP)

Typical Formula	gm/litre
Glucose	10g
Calcium phosphate ($Ca_3(PO_4)_2$)	5g
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	5g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.25g
Potassium chloride (KCl)	0.2g
Ammonium sulphate $(NH_4)_2SO_4$	0.1g
Agar	15g
pH	7

APPENDIX B

Composition of the reagents used in this study

B1. Ammonium oxalate crystal violet solution (Claus, 1995)

Solution A

Crystal violet (85% dye content)	2.0 g
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 m

Solution A and B were mixed

B2. Alkaline Methylene blue solution (SAB, 1957)

Solution A

Methyl blue	0.3 g (90% dye content)
Ethyl alcohol (95%)	40 ml

Solution B

KOH (0.01% by wt)	100 ml

Solution A and B were mixed.

B3. Iodine Solution (Claus, 1995)

Iodine	1.0 g
Potassium Iodide	2.0 g
Distilled water	300 ml

B4. Kovac's reagent (SAB, 1957)

Paradimethyl amino-benzaldehyde	5.0g
Butyle alcohol	5 ml
HCl (Conc.)	25 ml

B5. Methyl red solution (Bryan, 1950)

Methyl red	3.0 g
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

B6. α-Napthol solution (Bryan, 1950)

α-Napthol	5.0g
Ethyl alcohol (95%)	100 ml

B7. Oxidase test reagent (Collins and Lyne, 1984)

Tetramethyl – p- phenylene- Diamine dihydro-chloride	1.0 g
Distilled water	100 ml

B8. Safranin solution (SAB, 1957)

Safranin	0.5 g
Distilled water	100 ml

B9. Baritt's Reagent/ (VP1 & VP2)

VP1: 5% α-naphthol was dissolved in absolute ethanol (w/v).

VP2: 40% potassium hydroxide was dissolved in de-ionized water (w/v).

Both reagents were covered with aluminium foil and stored at 4ºC.

B10. Catalase Reagent

3% hydrogen peroxide solution in distilled water.

B11. NIT1 and NIT2 Reagents (Reagents for Nitrate Reduction Test)

NIT1: 0.4g sulfanilic acid and 30g acetic acid was dissolved in 70ml distilled water.

NIT2: 0.6g of N,N-dimethyl-1-naphthylamine and 30g acetic acid was dissolved in 70ml distilled water. Both reagents were stored away from light at 4ºC.

B12. Zinc Reagent

Zinc dust (powdered form of zinc) was used.

B13. Tryptophan Deaminase Reagent (TDA Reagent)

10 g of Iron (III) chloride was dissolved in 100ml distilled water.

B14. Zym A and Zym B Reagents

Zym A: 25g Tris, 11ml of 37% hydrochloric acid and 10g of sodium lauryl sulfate was dissolved in 100ml of distilled water.

Zym B: 0.12 g of Fast blue BB (active ingredient) was dissolved in 40ml methanol and 60ml dimethyl sulfoxide.

B15. Ninhydrin Solution

7g ninhydrin was dissolved in 2-methoxyethanol.

APPENDIX C

C 1 Release of soluble phosphate and changes of pH values in NBRIP medium.

Isolates ID	Grown on NBRIP medium, initial pH 7							
	pH Days of incubation				P released (µg/ml)			
	3	7	11	15	3	7	11	15
Control	7.0	7.0	7.0	7.1	0.3	0.3	0.8	0.8
C2	6.78	6.70	6.72	6.76	8.3	17.5	35.9	28.5
C3	6.55	6.47	6.45	6.46	28.5	45.1	40.2	32.5
C4	6.27	6.24	6.21	6.22	32.3	84.5	113.2	108.7
A1	6.45	6.42	6.40	6.41	9.3	20.2	57.3	35.5
A3	6.47	6.45	6.44	6.44	15.3	27.4	42.7	33.7
A4	6.80	6.75	6.74	6.75	7.2	18.5	31.2	27.5
A5	6.40	6.35	6.34	6.34	12.5	41.9	93.7	80.5
A6	6.75	6.65	6.65	6.66	9.6	15.73	39.2	30.4
A7	6.76	6.73	6.73	6.73	7.9	16.5	33.4	26.2

C 2 Release of soluble phosphate and changes of pH values in PVK medium (Pikovskaya, 1948).

Isolates ID	Grown on PVK medium, initial pH 7							
	pH Days of incubation				P released (µg/ml)			
	3	7	11	15	3	7	11	15
Control	7.0	7.0	7.0	7.02	0.2	0.2	0.5	0.5
C2	6.12	5.94	5.88	5.90	7.5	18.6	23.5	20.4
C3	5.84	5.76	5.71	5.69	18.5	27.6	41.2	30.4
C4	5.65	5.59	5.55	5.54	8.3	20.5	54.4	39.2
A1	5.74	5.66	5.60	5.61	11.2	29.4	43.4	35.5
A3	5.83	5.78	5.74	5.75	13.5	29.5	38.9	27.5
A4	6.35	6.20	6.18	6.19	5.5	11.5	18.2	15.5
A5	4.95	4.84	4.80	4.81	23.3	45.9	62.4	51.2
A6	5.93	5.82	5.79	5.80	9.4	20.5	26.4	23.2
A7	6.25	6.15	6.15	6.16	6.1	15.5	21.2	14.5

C 3 Release of soluble phosphate and changes in pH values by phosphate dissolving microorganisms in NBRIP*[1] modifying medium.

Isolates ID	Grown on NBRIP*[1] medium, initial pH 7							
	pH Days of incubation				Phosphate release (µg/ml)			
	3	7	11	15	3	7	11	15
Control	7.0	7.0	7.0	7.0	0.3	0.3	0.8	0.8
C2	5.15	5.12	5.10	5.10	12.5	25.5	33.5	26.5
C3	5.03	4.88	4.82	4.83	47.15	79.95	87.2	83.8
C4	4.32	4.28	4.26	4.26	177.2	335.6	486.3	457.5
A1	4.60	4.55	4.51	4.53	94.7	133.5	173.5	162.5
A3	5.02	4.92	4.87	4.89	23.2	39.4	45.5	34.25
A4	5.12	5.10	5.8	5.9	13.2	27.5	34.5	29.2
A5	4.65	4.46	4.40	4.40	138.5	275.5	377.6	332.5
A6	5.17	5.14	5.11	5.12	10.5	24.4	32.2	28.5
A7	5.05	4.99	4.96	4.98	18.5	32.7	49.3	31.5

NBRIP*[1] modified medium, source of phosphorus was compost (OM_1)

C 4 Mineralization of organic phosphate and changes in pH values by phosphate dissolving microorganisms in NBRIP*[2] modifying medium.

Isolates ID	Grown on NBRIP*[2] medium, initial pH 7							
	pH Days of incubation				P released (µg/ml)			
	3	7	11	15	3	7	11	15
Control	7.0	7.0	7.0	7.0	0.3	0.3	0.7	0.7
C2	4.61	4.42	4.30	4.30	10.5	23.5	40.2	28.5
C3	4.15	4.09	4.08	4.09	15.2	47.5	84.6	62.4
C4	4.21	3.95	3.89	3.92	6.9	93.6	133.1	121.5
A1	4.20	4.08	4.02	4.04	29.75	52.2	87.5	68.2
A3	5.95	4.15	4.09	4.11	12.3	35.5	72.7	58.4
A4	4.33	4.29	4.26	4.27	12.5	25.6	32.3	30.5
A5	4.12	4.03	3.99	4.01	42.1	73.6	102.7	90.6
A6	4.81	4.62	4.50	4.52	8.5	19.6	29.4	25.55
A7	4.30	4.24	4.20	4.18	13.2	27.2	45.5	37.7

NBRIP*[2] modified medium, source of phosphorus was oil cake (OM_2)

C 5 Release of soluble phosphate and changes of pH values by phosphate dissolving microorganisms in PVK*[1] modifying medium.

Isolates ID	Grown on PVK*[1] medium, initial pH 7							
	pH Days of incubation				P released (µg/ml)			
	3	7	11	15	3	7	11	15
Control	7.0	7.0	7.0	7.02	0.3	0.3	0.5	0.5
C2	5.44	5.34	5.30	5.31	24.5	42.5	64.2	52.7
C3	4.92	4.86	4.82	4.83	49.9	72.3	111.3	96.25
C4	4.74	4.52	4.46	4.46	103.2	157.8	227.6	211.3
A1	4.80	4.74	4.70	4.71	54.6	84.2	130.5	117.5
A3	4.73	4.61	4.56	4.58	153.4	187.5	226.7	208.8
A4	5.21	5.06	4.99	5.01	30.1	54.4	74.8	65.7
A5	4.50	4.46	4.42	4.44	182.5	207.2	292.4	250
A6	5.32	5.25	5.24	5.24	27.5	48.5	67.2	49.5
A7	5.15	4.93	4.89	4.90	33.4	57.6	85.9	77.5

PVK*[1] modified medium, source of phosphorus was compost (OM_1)

C 6 Mineralization of organic phosphate and changes of pH values by phosphate dissolving microorganisms in modified PVK*[2] medium (Pikovskaya, 1948).

Isolates ID	Grown on PVK*[2] medium, initial pH 7							
	pH Days of incubation				P released (µg/ml)			
	3	7	11	15	3	7	11	15
Control	7.0	7.0	7.0	7.02	0.2	0.2	0.5	0.5
C2	4.55	4.49	4.44	4.45	4.25	8.5	15.7	13.6
C3	4.44	4.38	4.33	4.35	11.3	26.3	31.5	22.5
C4	4.25	4.11	4.10	4.09	21.4	44.5	70.2	62.6
A1	4.18	4.13	4.11	4.10	18.5	27.6	41.2	39.4
A3	4.38	4.32	4.30	4.31	11.2	20.6	35.5	29.7
A4	4.50	4.42	4.41	4.42	7.5	15.7	23.2	17.5
A5	4.12	4.06	4.01	4.02	62.4	90.3	117.5	115.2
A6	4.52	4.44	4.42	4.43	5.5	12.5	19.5	12.2
A7	4.48	4.40	4.37	4.36	9.4	16.5	26.5	22.4

PVK*[2] modified medium, source of phosphorus was oil cake (OM_2)

C 7 Minimum inhibitory concentrations (MICs) against Lead and Cadmium in three broth media.

Isolates	MICs against Pb^{2+} in µg/ml			MICs against Cd^{2+} in µg/ml		
	NB	NBRIP	PVK	NB	NBRIP	PVK
C2	100	200	150	150	250	200
C3	75	200	150	100	150	150
C4	200	300	250	100	150	120
A1	200	350	300	200	250	250
A3	100	250	150	100	250	250
A4	150	350	300	75	120	150
A5	200	350	300	50	150	120
A6	120	250	200	100	200	150
A7	75	150	120	120	150	120

C 8 Maximum tolerable concentrations (MTCs) concentrations against Lead and Cadmium in three media.

Isolates	MTCs against Pb^{2+} in µg/ml			MTCs against Cd^{2+} in µg/ml		
	NB	NBRIP	PVK	NB	NBRIP	PVK
C2	100	250	200	120	200	150
C3	75	150	120	75	120	120
C4	100	300	300	75	200	150
A1	150	300	250	150	150	200
A3	75	200	150	75	200	200
A4	120	300	250	50	200	200
A5	150	300	250	30	120	120
A6	100	200	150	75	100	100
A7	50	150	120	100	100	100

C 9 Concentration of Pb in individual fraction of silt loam soils after 24 hours of incubation.

Sample ID	Concentration of Pb in individual fraction (µg/g)							
	F1	F2	F4	F5	F6	Sum	Total	Recovery (%)
T1B	1052.5	3633.7	5416.2	608.19	28.420	10739	15000	71.593
T1C	632.23	2445.2	6223.2	538.24	33.845	9872.7	15000	65.818
T1O	751.81	2033.8	7380.8	579.48	8.4508	10754	15000	71.696
T1R	991.61	1440.1	4867.8	575.54	41.114	7916.1	15000	52.774
T2B	2010.1	1531.5	8615.1	603.83	29.197	12790	20000	63.949
T2C	501.66	3222.1	7061.0	631.81	10.269	11427	20000	57.134
T2O	1461.2	2353.7	6238.3	529.43	72.959	10656	20000	53.278
T2R	1187.5	1897.1	6491.2	719.17	4.5855	10300	20000	51.498
T3B	2786.9	4456.3	6467.3	884.56	49.663	14645	30000	48.816
T3C	3929.1	3907.6	6055.6	1295.5	83.585	15271	30000	50.905
T3O	5025.8	2531.1	7141.6	683.94	63.943	15446	30000	51.488
T3R	5391.8	2262.7	10809	695.23	72.202	19231	30000	64.102

C 10 Concentration of Pb in individual fraction of silt loam soils after 15 days of incubation

Sample ID	Concentration of Pb in individual fraction (µg/g)							
	F1	**F2**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	269.53	1972.4	2951.2	7773.8	187.77	13155	15000	87.698
T1C	669.33	3863.2	3740.8	1884.8	28.689	10187	15000	67.912
T1O	564.35	3432.2	102.18	3512.4	214.44	7825.6	15000	52.171
T1R	442.80	1182.5	54.715	5044.9	245.28	6970.2	15000	46.468
T2B	2321.3	2786.3	102.59	5427.9	259.27	10897	20000	54.487
T2C	2728.0	7501.8	6.5285	6480.8	24.803	16742	20000	83.710
T2O	2656.1	5900.2	150.05	2937.9	196.82	11841	20000	59.205
T2R	1148.4	4773.2	724.97	2938.3	84.145	9669.0	20000	48.345
T3B	5454.0	8883.9	365.91	2196.3	4.0933	16904	30000	56.347
T3C	12263	2465.8	365.60	7510.2	194.23	22799	30000	75.995
T3O	6353.6	11231	173.99	2171.9	103.81	20035	30000	66.783
T3R	6510.6	5870.7	3549.6	2028.7	2.0207	17962	30000	59.872

C 11 Concentration of Pb in individual fraction of silt loam soils after 30 days of incubation.

Sample ID	Concentration of Pb in individual fraction (µg/g)							
	F1	**F2**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	723.73	1350.1	1047.0	1286.6	93.472	4500.9	15000	30.006
T1C	357.82	1349.7	1429.7	1382.1	137.24	4656.6	15000	31.044
T1O	494.82	1421.5	3631.9	1262.3	55.627	6866.1	15000	45.774
T1R	312.44	1733.1	2783.7	1071.2	49.881	5950.3	15000	39.669
T2B	2688.8	3743.8	376.79	1214.8	62.124	8086.4	20000	40.432
T2C	1728.8	3743.5	1740.9	1262.4	56.150	8531.8	20000	42.659
T2O	1637.3	2402.9	3847.4	1262.3	40.083	9189.9	20000	45.950
T2R	1043.6	3767.8	472.54	2770.8	85.440	8140.2	20000	40.701
T3B	6481.9	2570.9	4661.7	1334.5	54.093	15103	30000	50.343
T3C	5704.7	2379.1	352.54	2124.1	54.596	10615	30000	35.383
T3O	7121.2	2881.7	400.31	1238.3	32.311	11674	30000	38.913
T3R	6756.1	2810.3	711.92	1286.6	69.896	11635	30000	38.783

C 12 Concentration of Pb in individual fraction of silty clay loam soils after 24 hours of incubation.

Sample ID	Concentration of Pb in individual fraction (µg/g)							
	F1	**F2**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	2998.6	1329.1	6198.2	155.53	29.695	10711	15000	71.384
T1C	1744.7	1271.3	2616.1	425.99	56.076	6114.1	15000	40.747
T1O	2580.5	1614.4	2392.2	536.35	66.528	7189.9	15000	47.933
T1R	3401.5	1454.9	2616.4	637.36	103.81	8214.1	15000	54.742
T2B	3222.4	6469.3	3914.8	417.77	70.051	14094	20000	70.454
T2C	4968.2	827.82	3690.7	389.75	59.122	9935.6	20000	49.665
T2O	6221.7	1051.6	2526.5	541.02	31.452	10372	20000	51.848
T2R	6177.4	2529.4	8302.4	584.06	49.492	17643	20000	88.192
T3B	7520.5	3648.7	11481	381.52	68.782	23101	30000	76.990
T3C	11908	2394.8	9511.0	464.37	71.812	24350	30000	81.152
T3O	11594	2708.1	12108	517.56	60.640	26988	30000	89.946
T3R	11684	872.89	12690	517.97	53.553	25819	30000	86.048

C 13 Concentration of Pb in individual fraction of silty clay loam soils after 15 days of incubation.

Sample ID	Concentration of Pb in individual fraction (µg/g)							
	F1	F2	F4	F5	F6	Sum	Total	Recovery (%)
T1B	6179.5	4421.6	48.629	937.77	62.640	11650	15000	77.642
T1C	6882.7	5171.8	95.228	1242.3	126.33	13518	15000	90.093
T1O	6929.5	1607.0	118.58	1336.0	122.01	10113	15000	67.399
T1R	5874.6	1443.2	72.081	5487.4	115.69	12993	15000	86.591
T2B	9368.9	4492.0	165.89	867.41	92.843	14987	20000	74.917
T2C	11432	3061.1	48.325	1476.9	31.406	16050	20000	80.230
T2O	9626.5	3295.5	798.68	1031.2	97.137	14849	20000	74.227
T2R	10588	2076.4	72.081	2133.8	62.893	14934	20000	74.650
T3B	12535	3186.2	118.98	1078.5	75.838	16994	30000	56.639
T3C	12042	3377.6	71.777	1453.4	133.44	17078	30000	56.918
T3O	14200	4297.6	71.675	1382.9	69.980	20022	30000	66.728
T3R	12089	3646.2	118.98	1969.6	184.21	18008	30000	60.018

C 14 Concentration of Pb in individual fraction of silty clay loam soils after 30 days of incubation.

Sample ID	Concentration of Pb in individual fraction (µg/g)							
	F1	F2	F4	F5	F6	Sum	Total	Recovery (%)
T1B	5697.3	6872.9	268.12	735.84	59.645	13634	15000	90.861
T1C	5298.3	6335.3	282.74	688.63	68.766	12674	15000	84.463
T1O	5368.5	2977.4	216.55	547.82	56.832	9167.1	15000	61.094
T1R	6095.9	2574.8	210.56	876.55	68.782	9826.6	15000	65.489
T2B	11021	2574.8	265.99	642.03	56.599	14560	20000	72.783
T2C	11067	3559.5	410.66	1017.0	62.421	16117	20000	80.565
T2O	9214.6	2171.5	399.90	876.14	11.909	12674	20000	63.354
T2R	9895.1	3022.5	364.06	1345.6	177.66	14805	20000	74.006
T3B	18080	3649.3	297.97	1204.9	23.858	23256	30000	77.507
T3C	16227	4857.9	336.04	758.98	49.731	22229	30000	74.086
T3O	14257	5350.3	321.02	1110.7	27.898	21067	30000	70.210
T3R	16532	3828.4	257.46	1251.8	49.239	21919	30000	73.051

C 15 Concentration of Pb in individual fraction of sandy loam soils after 24 hours of incubation.

Sample ID	Concentration of Pb in individual fraction (µg/g)								
	F1	F2	F3	F4	F5	F6	Sum	Total	Recovery (%)
T1B	9.8492	297.49	4047.6	7601.9	143.22	17.704	12118	15000	80.785
T1C	20.101	79.799	4180.3	3657.0	212.56	30.251	8180.0	15000	54.533
T1O	15.779	58.593	6352.0	2548.8	351.76	14.920	9341.9	15000	62.279
T1R	11.960	63.216	4180.6	2372.0	195.98	17.704	6841.4	15000	45.609
T2B	3.5176	778.69	5022.7	4721.0	293.07	37.553	10857	20000	54.283
T2C	22.211	284.52	5864.5	4100.2	256.88	17.688	10546	20000	52.730
T2O	68.543	793.07	4978.0	5607.0	324.32	42.307	11813	20000	59.066
T2R	5.6281	69.548	4712.5	5075.6	415.48	27	10306	20000	51.528
T3B	7.7387	177.19	6574.0	8133.8	470.35	43.834	15407	30000	51.356
T3C	32.764	582.11	9454.6	8044.8	467.94	64.171	18646	30000	62.155
T3O	13.668	582.01	7238.4	8000.4	533.27	73.211	16441	30000	54.803
T3R	9.8492	111.76	4934.1	8621.3	713.07	44.337	14434	30000	48.115

C 16 Concentration of Pb in individual fraction of sandy loam soils after 15 days of incubation.

Sample ID	Concentration of Pb in individual fraction (µg/g)								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	10.653	56.482	4654.3	7773.0	609.45	63.266	13167	15000	87.781
T1C	2.6131	24.523	4875.6	7063.5	476.18	76.065	12518	15000	83.457
T1O	4.7236	70.854	4742.5	6620.2	609.05	73.548	12121	15000	80.806
T1R	2.9146	54.372	4787.2	6576.3	520.80	54.221	11996	15000	79.972
T2B	1.7035	96.583	5407.7	10255	786.73	53.467	16601	20000	83.006
T2C	1.5075	123.72	4166.4	10388	875.08	97.171	15652	20000	78.258
T2O	4.7236	45.528	2482.1	13623	963.62	86.111	17205	20000	86.026
T2R	6.2312	79.698	5407.7	10122	786.73	921.31	17324	20000	86.619
T3B	4.0201	90.251	7402.2	15884	1185.6	135.63	24702	30000	82.339
T3C	8.1407	51.960	6914.4	16371	1274.0	109.73	24729	30000	82.431
T3O	8.0402	132.06	7313.2	16282	1229.5	96.412	25062	30000	83.538
T3R	4.0201	113.47	5141.8	17081	1806.1	89.899	24236	30000	80.786

C 17 Concentration of Pb in individual fraction of sandy loam soils after 30 days of incubation.

Sample ID	Concentration of Pb in individual fraction (µg/g)								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	16.080	171.76	2582.4	6757.0	456.08	44.472	10028	15000	66.852
T1C	15.779	89.146	2803.7	8263.6	449.45	162.80	11785	15000	78.563
T1O	11.457	424.62	2670.7	9548.8	445.13	39.176	13140	15000	87.599
T1R	24.523	53.568	1740.3	6889.9	430.75	16.332	9155.4	15000	61.036
T2B	5.5276	106.33	2139.2	14646	656.58	81.658	17636	20000	88.178
T2C	15.779	82.814	2360.5	14823	656.28	57.020	17996	20000	89.978
T2O	11.457	456.28	3158.2	14167	649.85	22.090	18465	20000	92.323
T2R	20.302	47.236	6615.7	10360	631.26	22.111	17696	20000	88.481
T3B	3.4171	245.63	4045.0	13627	1937.7	41.960	19901	30000	66.335
T3C	1.0050	131.36	5152.8	14513	1133.3	43.955	20975	30000	69.918
T3O	34.673	384.52	8432.5	13671	1122.6	218.82	23864	30000	79.546
T3R	1.3065	131.66	2892.7	17369	1342.5	43.970	21781	30000	72.603

C 18 Concentration of Cd in individual fraction of silt loam soils after 24 hours of incubation.

Sample ID	Concentration of Cd in individual fraction (µg/g)							
	F1	**F2**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	7407.2	2711.9	885.58	65.084	9.8192	11080	15000	73.864
T1C	7711.7	2955.6	853.48	54.308	7.4824	11583	15000	77.217
T1O	6928.3	2807.6	880.89	76.354	6.4715	10700	15000	71.331
T1R	9426.2	2859.5	994.79	54.301	4.8736	13340	15000	88.931
T2B	10280	3251.6	881.01	55.478	7.4876	14475	20000	72.376
T2C	9296.0	2990.4	926.60	76.008	9.2440	13298	20000	66.491
T2O	9752.9	3225.4	912.88	94.141	9.4249	13995	20000	69.974
T2R	8681.9	2824.6	917.10	51.810	7.2311	12483	20000	62.413
T3B	9948.9	3713.0	954.12	68.641	9.2492	14694	30000	48.980
T3C	9887.9	3791.2	912.89	63.557	3.0523	14659	30000	48.862
T3O	9739.9	3512.7	967.72	74.931	8.6995	14304	30000	47.680
T3R	9913.6	3460.1	944.52	65.329	8.0342	14392	30000	47.972

C 19 Concentration of Cd in individual fraction of silt loam soils after 15days of incubation.

Sample ID	Concentration of Cd in individual fraction (µg/g)							
	F1	**F2**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	7949.3	2783.6	1424.1	413.55	17.965	12588	15000	83.923
T1C	6426.9	3793.8	619.83	237.28	10.654	11089	15000	73.924
T1O	6261.1	3410.4	503.76	232.09	23.659	10431	15000	69.540
T1R	5985.1	3379.0	1398.7	325.01	22.398	11110	15000	74.068
T2B	6933.8	2773.2	770.20	201.12	16.851	10695	20000	53.476
T2C	8188.6	2767.9	1055.1	278.74	19.722	12310	20000	61.550
T2O	6151.3	2850.8	977.34	258.00	16.017	10253	20000	51.267
T2R	5968.5	2803.8	780.09	299.10	16.776	9868.3	20000	49.342
T3B	13136	3198.1	998.18	252.93	5.7373	17591	30000	58.639
T3C	11246	3021.8	775.27	180.29	15.007	15238	30000	50.793
T3O	10873	3280.9	738.99	408.26	14.799	15315	30000	51.051
T3R	12509	3306.4	749.01	195.47	14.160	16775	30000	55.915

C 20 Concentration of Cd in individual fraction of silt loam soils after 30days of incubation.

Sample ID	Concentration of Cd in individual fraction (µg/g)							
	F1	**F2**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	6933.0	2788.1	1585.8	267.39	19.753	11594	15000	77.294
T1C	7160.9	2964.2	1248.9	283.87	43.893	11702	15000	78.012
T1O	7461.4	3161.1	1658.2	184.37	20.913	12486	15000	83.240
T1R	6704.6	3741.0	1367.7	221.33	24.393	12059	15000	80.394
T2B	10715	2259.6	1326.7	263.24	21.540	14587	20000	72.933
T2C	10436	3212.9	1528.7	217.54	62.027	15457	20000	77.283
T2O	10612	3161.1	1782.5	229.97	26.897	15812	20000	79.061
T2R	11295	3264.4	1357.3	208.89	21.802	16148	20000	80.738
T3B	17907	4073.1	1404.4	246.66	16.126	23647	30000	78.825
T3C	19140	3927.9	2046.8	263.14	22.779	25401	30000	84.670
T3O	19555	4104.1	1539.0	229.97	18.504	25446	30000	84.821
T3R	20319	3979.4	1403.9	225.47	21.206	25949	30000	86.496

C 21 Concentration of Cd in individual fraction of silty clay loam soils after 24hours of incubation.

Sample ID	Concentration of Cd in individual fraction (µg/g)							
	F1	**F2**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	8356.0	1620.7	376.81	30.860	4.9178	10389	15000	69.262
T1C	7716.3	1697.4	399.10	29.016	3.8213	9845.6	15000	65.638
T1O	8031.8	1637.7	403.56	35.279	5.1914	10114	15000	67.424
T1R	8074.1	1500.9	524.10	34.934	5.6310	10140	15000	67.598
T2B	10079	1578.1	515.60	32.254	5.7553	12210	20000	61.052
T2C	10373	1688.9	479.69	30.061	4.6589	12576	20000	62.880
T2O	6070.4	1757.1	506.54	28.309	4.7091	8367.0	20000	41.835
T2R	6939.9	1662.9	662.89	37.722	5.6563	9309.1	20000	46.546
T3B	9865.4	1910.7	452.92	36.436	6.4914	12272	30000	40.907
T3C	10130	2047.0	569.23	33.198	5.2680	12784	30000	42.615
T3O	9660.7	1936.2	506.54	31.445	5.0898	12140	30000	40.466
T3R	9805.3	2353.7	555.44	39.116	5.1995	12759	30000	42.529

C 22 Concentration of Cd in individual fraction of silty clay loam soils after 15days of incubation.

Sample ID	Concentration of Cd in individual fraction (µg/g)							
	F1	F2	F4	F5	F6	Sum	Total	Recovery (%)
T1B	7205.7	2362.9	426.45	324.15	7.8289	10327	15000	68.847
T1C	8322.4	1692.7	558.32	440.80	12.088	11026	15000	73.508
T1O	9936.6	2048.0	517.71	136.22	9.8284	12648	15000	84.322
T1R	6992.1	1844.6	1466.6	146.03	12.273	10462	15000	69.744
T2B	11368	1855.2	670.10	116.03	7.6766	14017	20000	70.086
T2C	9114.2	1743.5	629.39	557.55	7.7477	12052	20000	60.262
T2O	10089	1916.0	695.37	120.99	9.8792	12831	20000	64.156
T2R	10302	1915.7	491.98	125.73	10.293	12845	20000	64.227
T3B	13358	3845.1	695.48	253.09	7.3213	18159	30000	60.530
T3C	15419	1926.2	695.38	131.16	7.5193	18179	30000	60.597
T3O	15571	1966.8	685.22	115.92	7.5442	18347	30000	61.155
T3R	15916	2047.7	527.51	120.65	8.6437	18620	30000	62.068

C 23 Concentration of Cd in individual fraction of silty clay loam soils after 30days of incubation.

Sample ID	Concentration of Cd in individual fraction (µg/g)							
	F1	F2	F4	F5	F6	Sum	Total	Recovery (%)
T1B	8638.4	1764.6	659.79	108.50	10.139	11181	15000	74.543
T1C	6560.2	1886.3	1020.1	75.909	9.4990	9552	15000	63.680
T1O	7612.6	1886.3	669.83	71.838	10.133	10251	15000	68.338
T1R	9111.8	1926.6	816.69	87.736	14.887	11958	15000	79.718
T2B	14554	2221.4	598.87	76.010	10.519	17461	20000	87.305
T2C	12808	2160.4	1390.6	100.27	12.545	16472	20000	82.358
T2O	13078	1226.4	751.05	84.020	12.823	15153	20000	75.764
T2R	11548	2068.7	852.22	91.797	13.720	14575	20000	72.874
T3B	21864	2597.1	664.86	80.071	10.976	25217	30000	84.057
T3C	17816	3652.8	1055.6	92.152	14.601	22632	30000	75.439
T3O	18114	2617.3	2223.1	92.142	15.793	23063	30000	76.875
T3R	19671	2525.6	501.97	95.858	13.517	22808	30000	76.025

C 24 Concentration of Cd in individual fraction of sandy loam soils after 24hours of incubation.

Sample ID	Concentration of Cd in individual fraction (µg/g)								
	F1	F2	F3	F4	F5	F6	Sum	Total	Recovery (%)
T1B	9532.1	1801.4	344.31	60.030	5.6107	0.57789	11744	15000	78.294
T1C	8558.0	1653.5	280.89	55.497	6.2002	0.42211	10554	15000	70.364
T1O	10257	1368.6	323.10	68.784	3.0849	15.824	12036	15000	80.241
T1R	8258.3	1790.4	343.86	108.33	12.749	0.35427	10514	15000	70.093
T2B	11903	1643.1	323.21	117.65	27.692	0.90452	14016	20000	70.079
T2C	11931	1885.7	396.97	64.362	18.966	0.52261	14298	20000	71.489
T2O	11382	2107.3	375.86	64.352	9.9854	0.47186	13940	20000	69.702
T2R	11115	1864.2	312.20	37.417	10.679	0.35427	13340	20000	66.698
T3B	17771	2023.0	312.65	60.030	5.6107	11.834	20184	30000	67.280
T3C	16589	2445.0	344.21	24.472	24.142	0.57286	19427	30000	64.757
T3O	17025	2350.0	407.52	42.191	4.4650	5.0447	19834	30000	66.114
T3R	16391	2170.3	417.73	37.417	19.304	4.2990	19040	30000	63.468

C 25 Concentration of Cd in individual fraction of sandy loam soils after 15days of incubation.

Sample ID	Concentration of Cd in individual fraction (µg/g)								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	7698.2	2371.4	623.54	111.11	11.317	2.5744	10818	15000	72.121
T1C	7446.8	2690.4	697.31	119.87	12.271	2.0920	10969	15000	73.126
T1O	7778.5	2658.8	686.74	115.43	23.447	1.9910	11265	15000	75.099
T1R	6521.9	2690.1	485.90	106.22	32.181	2.0744	9838.3	15000	65.589
T2B	11929	2743.3	518.01	221.91	18.070	2.3231	15433	20000	77.165
T2C	11316	2922.6	591.78	159.76	21.980	1.9161	15014	20000	75.071
T2O	12231	2880.4	633.98	195.21	10.362	2.0663	15953	20000	79.764
T2R	11346	2795.6	591.43	128.38	23.106	2.3005	14887	20000	74.434
T3B	18298	3355.4	549.67	146.56	10.472	1.9965	22362	30000	74.541
T3C	18187	3302.5	612.88	124.30	9.5276	1.9663	22238	30000	74.128
T3O	18979	3239.2	549.56	137.59	12.261	1.8905	22920	30000	76.400
T3R	15467	3418.2	528.11	128.38	11.497	1.9739	19555	30000	65.183

C 26 Concentration of Cd in individual fraction of sandy loam soils after 30days of incubation.

Sample ID	Concentration of Cd in individual fraction (µg/g)								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**Sum**	**Total**	**Recovery (%)**
T1B	9624.1	2521.5	441.40	112.37	11.839	2.9523	12714	15000	84.761
T1C	9258.2	2553.0	789.54	112.27	13.216	3.0477	12729	15000	84.862
T1O	10201	2468.6	673.45	129.99	12.362	3.8010	13489	15000	89.927
T1R	10271	2531.6	757.53	120.78	23.206	3.4070	13707	15000	91.383
T2B	13803	2553.1	631.35	112.37	22.814	3.2286	17126	20000	85.631
T2C	14324	2310.3	1021.7	116.70	13.427	3.3744	17789	20000	88.946
T2O	14563	2394.7	504.60	138.85	15.528	3.2482	17620	20000	88.100
T2R	14028	2362.7	799.74	114.62	34.814	2.0352	17342	20000	86.709
T3B	21162	2869.7	652.45	116.80	13.106	2.6759	24817	30000	82.724
T3C	21430	2626.9	694.56	125.57	15.749	1.7915	24894	30000	82.981
T3O	21711	2605.8	684.00	121.13	12.573	2.1935	25137	30000	83.789
T3R	20895	2594.9	588.68	103.06	15.608	2.5025	24199	30000	80.664

Sum- (F1+F2+F3+F4+F5+F6); Total- Aqua regia-extractable;
Recovery %: (F1+F2+F3+F4+F5+F6)/ Aqua regia-extractable*100.