ENDOPHYTIC ENHANCED BIOREMEDIATION OF HEPTACHLOR FROM PESTICIDE CONTAMINATED SOIL

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Soils - Analysis

Soil remediation.

Heptachlor

Endophytic Enhanced Bioremediation of Heptachlor from Pesticide Contaminated Soil

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FINAL APPROVAL

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DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

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LIST OF ABBREVIATIONS

Acronym	Abbreviation
DDT	Dichlorodiphenyltrichloroethane
HCH	Hexachlorocyclohexane
OCPs	Organochlorine pesticides
PAHs	Polycyclic aromatic hydrocarbons
TCE	Trichloroethylene
MSM	Mineral salt medium
DCPIP	dichlorophenol indophenol
ACC	aminocyclopropane-1-carboxylate
SDW	Sterile distilled water
CRD	completely randomized design
SM	Soil moisture
EC	Electrical conductivity
OM	Organic matter
SOM	Soil organic matter
MBC	Microbial biomass Carbon
TOC	Total organic carbon
DO	Dissolved oxygen

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ABSTRACT

Heptachlor is an organochlorine pesticide applied to control a wide range of pests of agricultural crops. The present study reports the isolation of endophytic bacterial strain RP7 through enrichment technique. RP7 endophytic bacterial strain isolated from white poplar *Populus alba* grown on pesticide non-contaminated soil. RP7 was inoculated into soil treatments containing different concentrations of Heptachlor (5%, 15% and 25%). Results revealed that endophytic bacterial isolate RP7 utilized heptachlor as carbon and energy source even at high concentrations. Biodegradation rate of heptachlor among different treatments was observed (35 % ,73%, and 80%) during 5 10 and 15 days of incubation. The concentration of heptachlor was determined by GC-MS analysis. The results of present study showed that endophytic bacterial strain RP7 may have potential for the biodegradation of heptachlor.

1 INTRODUCTION

About some decades ago, crop yields depended on rainfall patterns, biological control mechanisms and recycling of organic matter all these processes played significant role in agricultural system to meet food demands while in modern era expansion of agricultural activities is required to increase supply of food to feed growing population. Therefore, expanding utilization of agrochemicals has led the pollution of soil and water with various kind of pesticides cause devastating consequences on environment. Pesticides are substances consist of various products used to kill destroy and manage pests at acceptable levels. Organochlorine pesticides used worldwide for the control of weeds in forestry, non-crop soils and agriculture.

Pakistan is an agricultural country and its economy largely depends on the production of major cash crops including sugarcane, rice, cotton, wheat and tobacco. Therefore, increase in the demands has pushed towards the extensive use of synthetic agrochemicals to obtain more yield of crops. In this situation organochlorine pesticides, have been used for the management of pests cause harmful effects on crop production for many years. Some organochlorine pesticides DDT, HCH, Lindane, Aldrin, Endosulfan, Heptachlor have been banned due to their devastating effects on environment However, some organochlorine pesticides Endosulfan, Heptachlor and HCH are still being used in Pakistan for pest management of cotton crops. Studies reported that extensive use of organochlorine pesticides cause accumulation of pesticides compounds in soil of those areas which are under the cultivation of cotton crops (Hussain *et al.*, 2001). Scientific research evidences showed that organochlorine pesticides can be found in food items e.g. milk, cotton seeds, vegetables and tobacco, that can interrupt human food chain (Masud and Hasan, 1992).

Excessive use of pesticides, insecticides, herbicides and fungicides has resulted the accumulation of variety of chemicals in environmental matrix. Thus, production of these organic chemicals has forced to implement innovative technologies to eliminate them from natural environment. Earlier techniques like recycling of waste, landfills, incineration,

pyrolysis and some other technologies are expensive and cause adverse effects on environment by the formation of toxic metabolites. All these methods proved to be expensive and hard to implement in case of organochlorine pesticides (Debarati *et al.*,2005).

Bioremediation is a low-cost technology that is being used for the remediation of contaminated sites. In effective bioremediation process, natural biodegradation rate of contaminants can be enhanced by accomplishing these microbes with electron donors, carbon sources, and nutrients. This process can also be done by using microbes of specific characteristics and enriched culture of microorganisms. In the bioremediation process, complete mineralization of toxic contaminants occurs without the formation of harmful intermediates (Kumar and Philip, 2006).

Successful bioremediation for the degradation and removal of toxic compounds requires efficient bacterial strains, availability of metabolite or pesticide to the microorganism, survival of pesticide degrading bacteria, and physiological status of the microorganisms. Soil moisture, temperature, pH of soil, organic matter, sufficient supply of nutrients to microbes and the quantity of pesticides in soil are key factors for pesticides degrading microorganisms. Bioremediation process targets harmful compounds by transformation, mineralization and alternation. Microbial biodegradation is considered a reasonable and environmentally approachable strategy for the elimination of toxic and harmful contaminants in the industrialized world due to the discovery of several types of microorganisms that are efficient in degradation of pollutants toxic to environment (Silva *et. al.*, 2004).

Plants and their bacterial endophytes interaction is another novel approach which has shown much potential in enhancing bioremediation. Endophytes microbes have natural capacity to degrade pesticides in addition to providing several benefits to the plants species such as stress tolerance, phosphate solubilizing and nitrogen fixation. Endophytic microorganisms live within plants they do not cause harm and disease to their host. These

microbes produce alkaloids, antibiotics, enzymes that protect plant from stress conditions (Ryan *et al.*, 2008).

These microbes interact with their host most closely as compared to rhizosphere and phylosphere. Endophytic microbes enhance plant growth and development thus plant provides residency and nutrients to these microbes. Various plants have diverse types and composition of endophytic bacteria, most of the strains are related to common soil bacterial of genera *Pseudomonas, Arthrobacter, Burkholderia and Entrobacter (*Luo *et al.*,2011).

Heptachlor is an organochlorine pesticide belongs to persistent organic pollutants POPs that are banned in Pakistan. It may enter food chain become bio magnify and pose serious threats on ecosystem and human health. Many organochlorine pesticides function by impeding normal nervous system functions damage the nervous system and cause convulsions in brain. Chronic exposure to heptachlor and its metabolite can cause destruction of nerves as well as muscle tissue damage. Heptachlor converts into chlordane, heptachlor epoxide and other metabolites products after biodegradation in environment. Therefore, its removal from natural environment should be priority for many contaminated sites of environment. Heptachlor and its metabolic products have been found in food crops which grown in soils contaminated with heptachlor. Therefore, there is a need to degrade heptachlor and products by the action of endophytic microbes (Vaccari *et al*, 2006).

1.2 Significance of study

This study will provide strategy to enhanced bioremediation of Heptachlor using endophytic bacterial isolates isolated from white poplar and highlight the importance and remediation potential of these microbes. The present study will also provide informations for further research and analysis of organochlorine pesticides in soil.

1.2.1 Objectives

- Isolation of Endophytic bacteria from white poplar *Populus alba* tree grown on non-contaminated soil for the remediation of heptachlor contaminated soil.
- To assess degradation potential of Heptachlor from contaminated soil by endophytic bacteria.

LITERATURE REVIEW

2 LITERATURE REVIEW

The rapidly growing industrialization and wide spread use of manmade xenobiotic cause excessive accumulation of wide variety of synthetic chemicals in environment. 80-90% pesticides are used on cotton crop in Pakistan and remaining and remaining 10 20% consumed on paddy, sugarcane, fruits and vegetables.Due to this reason, remarkable efforts are needed to implement modern technologies such as bioremediation to derive benefit from the ability of microorganisms to remove pollutants from contaminated environmental sites. Bioremediation is an alternative, effective, nonhazardous, economical feasible and environmental friendly treatment method (Finley *et al.*, 2010).

2.1 Impact of Modern Agriculture on Environment

After World War II, advancement in science and technology has resulted in the extensive use of synthetic chemicals. Although, these chemicals are beneficial in certain industrial and agricultural processes, but their harmful effects on living organisms and natural environment have been reported in many studies. Pakistan is an agriculture country and its economy is depending on the production of agricultural crops such as wheat, sugarcane, cotton and rice etc. 25 % land of Pakistan is under cultivation (Jain *et al.*, 2005) therefore, today farmers are more integrated into international economics rather than environment, the important biological life disappears due to the use of synthetic fertilizers and harmful pesticides. pesticide contamination can occur in surface soil. surface water, ground water vadose and saturated zones. The presence of OCPs in the natural environment has been of extraordinary concern due to their persistency, potential for transport, toxicity to biodiversity and people. These organic pollutants bioaccumulate in organic tissues of organisms and disturb food chain (Nakata *et al.*, 2002).

2.2 Use of Pesticides in Agriculture

Over the years, the need of agrochemicals has increased to produce more agricultural yield. Pesticides have been used to control extensive variety of agriculture, horticulture and public-sector pests but the present use of pesticides is more concerned to cotton which is important cash crop and export commodity in Pakistan (Tieyu *et al.*, 2005).

Pesticides are chemical substances which are used to destroy, repel, and control pests at acceptable levels to diminish their harmful effects. Pesticides used in various situations like horticulture, cropping, forestry, and home gardening etc. pesticides can be derived from inorganic, organic (natural or synthesized as complex organic compounds in laboratory. Persistent organic pollutants are halogenated compounds for low water solubility, prompting their biomagnifications and bioaccumulation (Poolpak *et al.*, 2008).

2.2.1 Use of Heptachlor in Crop Production

Heptachlor belongs to class of organochlorine pesticides used widely from the 1940s through the 1960s on agricultural crops and mosquito control. People can be exposed to heptachlor through inhalation in an area where it was recently applied. It can also be ingested from dairy products, fishes, and other fatty that are contaminated because of its hydrophobic nature it can be accumulate in milk, meat, and fats, thereby transferred to human through food chain (Lemaire *et al.*, 2004).

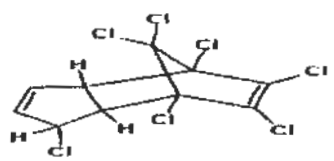
Heptachlor was used as broad -spectrum insecticide on may crops in united states, with major use on corn. It had also been used for nonagricultural purposes for termite control and seed treatment in homes and gardens. The heptachlor applied against several serious pests e.g. aphid, bollworms and white fly these pests have direct and indirect effect on reduction of crop yield (Kim *et al.*, 2007).

2.2.2 Properties of Heptachlor

Heptachlor is chlorinated cyclodiene used as a pesticide and insecticide. Its chemical name is 1.4.5.6.7.8.8- heptachloro-3a,4,7,7a-tetrahydro-4,7-methanol-H-indene. Heptachlor is insoluble in water and in pure form it exits as a white crystalline solid. it is soluble in organics like acetone, carbon tetra chloride and xylene .it exists stable in day light and become oxidized biologically in heptachlor epoxide. It can also be photo degraded and volatilized in environment.it is less persistent and slowly vaporize from soil (Gao *et al.*, 2008).

Heptachlor tied to silty loam soil and metabolize by soil bacteria and fungi to many different metabolites by many metabolic pathways. chlordane, heptachlor epoxide, chlordane epoxide ,1-hydroxychlordene are the products of the microbial degradation of heptachlor (Nwachukwu and Osuji, 2007).

Table 2.1: Physical and chemical properties of Heptachlor

Chemical name	Heptachlor, Heptachlorane, Heptamul,3-chlorodene, Rhodiachlor
Molecular formula	$C_{10}H_5Cl_7$
Structure	
Molecular weight	373.3 g/mol
Density	1.6 g/cm^3
Boiling point	293°F
Melting point	95-96 deg C
Vapour pressure	Pa at 25°C: 0.053
Solubility	Acetone, benzene, alcohol, xylene Carbon tetrachloride, Cyclohexanone,

2.2.3 Environmental and Health Hazards of Heptachlor

Heptachlor and its degradation products adsorb to sediments and become bioconcentrated in terrestrial and aquatic organisms. heptachlor readily changed into to heptachlor epoxide and move up through trophic level and become biomagnified because it is more persistent in nature and lipophilic (Murano *et al.*, 2009).

Heptachlor can enter the body through contaminated air, and absorption from contaminated soils. Heptachlor exposure showed harmful health impacts on humans and animals. It causes liver damage and decreased fertility. Although degradation of Heptachlor in soil is very slow it can be absorbed by soil components and enter the human food chain. However, bioremediation is viable technology for the decontamination of polluted sites and microorganisms play very effective role in the removal of pesticides from soil .so that there is a need to study the Heptachlor residues in soil in relation to physicochemical properties of soil (Zhang *et al.*, 2006).

2.3 Persistency of Pesticides in Soil

Pesticides persists in soil the persistence of pesticide in soil usually expressed in terms of half-life of pesticide. Half -life of pesticide is the time required for the breakdown of original quantity. Pesticides can be divided into three groups according to their half-lives: non-persistent, moderately persistence and persistence pesticides (Saier, 2005).

However, the degradation products of most of the pesticide are mineral, water and carbon dioxide. but, sometime intermediate metabolites products of some pesticides are formed. Therefore, half -life values of intermediate products should be determined. Generally, pesticides residues present on ground water and canopy cover foliage are less persistent than soil residues. Despite their mechanism of persistence, they are considered highly stable organic contaminants (Weir *et al.*, 2006).

2.4 Fate of Pesticides in Environment

Pesticides in soil can be inactivated, destroyed and removed by various mechanisms. These mechanisms include volatilization, leaching of chemicals through the soil surface, adsorption of the pesticide by soil particles, photochemical destruction process, plant removal from pesticide contaminated soil, chemical reactions, and biological detoxification process. The specific mechanism of removal of pesticide from contaminated environmental media depends on the type of soil, and environmental condition to which pesticides exposed to. Some pesticides may disappear entirely from volatilization, leaching and other destroyed by microbial activities (Tariq *et al.*, 2007).

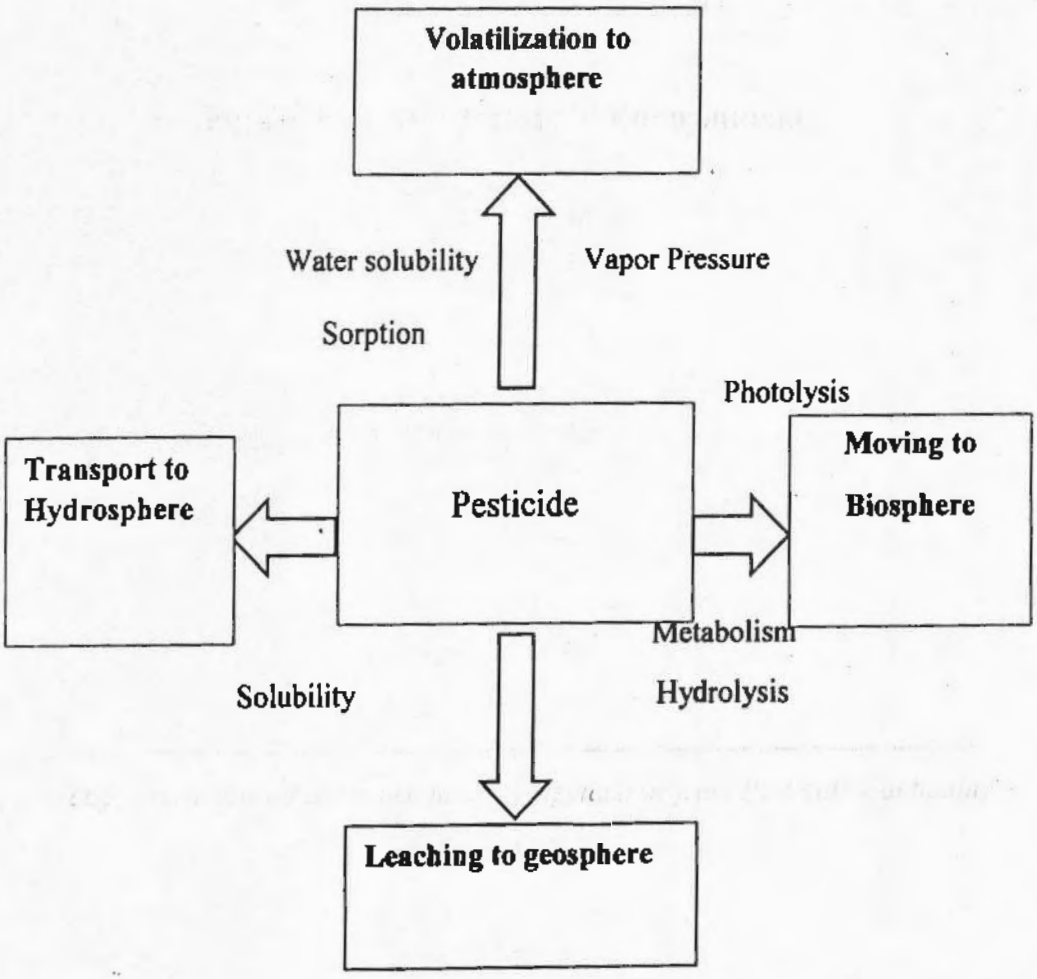


Fig 2.1 Fate of Pesticide in Environment

2.5 Microbial Degradation of Pesticides

Earlier physical and chemical soil clean up processes are very expensive, thus the potential of microbes in the bioremediation processes should be exploited to remove pesticides from contaminated soils. Metabolism of contaminant by microbial activity is considered as the base of bioremediation process. Bioremediation process aims to reduce pollution level in soil to nontoxic or acceptable levels (Alexander 1999). Degrading microorganism obtain energy, nitrogen and carbon from pesticides molecules. Bioremediation technology eliminates the need of soil excavation on site and it preserve soil structure and require less energy. Therefore, it has become apparent that the principles of bioremediation and economically efficient methods are required to treat the pesticides contaminated soils (Andreoni and Gianfreda ,2007).

Nutritional and favorable conditions provide to microbes, make them capable to incorporate organic substance into their cell and oxidize them into different products. However, degradation of recalcitrant or refractory substances is slower. Therefore, these complex organic compounds may inhibit the microorganism's growth and metabolic activities. thus, such compounds need special physical and biological techniques for active remediation (Zhang *et al.*, 2005).

Pesticide accumulation in food products and water supplies triggered the need to progress safe and efficient methods for pesticides remediation. Biodegradation of pesticides from many bacteria such as *Bacillus sp, Arthrobacter sp, Pseudomonas sp* have been reported (Zeyaullah *et al.*, 2009).

2.6 Bioremediation

The process of bioremediation is described as a technique which use microorganisms to destroy waste material and remove contaminants from environment. This is also known as the process of detoxification which targets the harmful pollutants by the process of transformation, mineralization, and alternation. Some years ago, civilizations used natural bioremediation process to treat waste water. But most recent development is the use of bioremediation technique for hazardous wastes (Shanahan, 2004).

Physicochemical characteristics of environment such as pH, oxygen availability, soil moisture, temperature and substrate influence biodegradation of pesticides. There are two important factors co-metabolism and consortia condition to degrade pesticide. Organochlorine pesticides degrade co-metabolically therefore these pesticides required another substrate (Siciliano *et al.*, 2001).

The present study is conducted to develop an efficient, economical feasible and eco-friendly bio treatment process by using endophytes for the remediation of heptachlor contaminated soils. Endophytic microbes can be isolated from roots, stems, leaves, fruit plants, inflorescences of weeds, and vegetables (Bulgari *et al.*, 2012).

2.7 Potential of Endophytic Bacteria for Bioremediation

Endophytes since they harbour inside and are in a proximity with the plants have the potential to become preferred substitutions for some of the routinely used conventional synthetic products. Hence, these bio degraders can significantly contribute to the sustainable production of ecofriendly and less toxic residue products. Extensive use of some organochlorine pesticides for agricultural and nonagricultural purposes leads the contamination of soil, water and sediments. Endophytic microbes not only increase crop yield but also eliminate contaminants (Sharma *et al.*, 2014).

Endophytic bacteria live within the internal tissues of plants as compared to rhizospheric microbial community living on and around the plant roots. Endophytic bacteria isolated from plants utilized to improve the remediation efficiency of plants. Endophytic bacteria are microbes that reside in inner tissues of living plants without causing any negative symptoms and infections in plants. Endophytic bacteria bring opportunity to degrade contaminants by intercellular deoxygenases (Weyens *et al.*, 2010).

This situation emphasized that proper attention should be paid to promote the scientific research which provides essential information for the removal of heptachlor. Endophytic microorganisms live within plants. Endophytes are defined as asymptomatic because they don't cause any harm to their hosts (Elliot *et al.*, 2000). These microbes produce alkaloids, antibiotics, enzymes to assist plant in results plant provide nutrients and protection (Azevedo *et al.*, 2000). These organisms are also linked to vegetables and show applications in medicine.

2.8 Bioremediation of Pesticides

Most of the research studies showed that endophytic microbes have natural capacity to degrade xenobiotics (Ryan *et al.*, 2008) providing benefits to the plants, such as phosphate solubilization, stress tolerance and nitrogen fixation (Compant *et al.*, 2010). non-native or genetically modified endophytic microbes to enhance the biodegradation of contaminated soil has been explored in many studies (Barac *et al.*,2004).

Endophytic microbes enhance plant growth by stimulating defense mechanism of plant which increase plant resistant to organic contaminants. The efficiency of two endophytic bacterial strains P1 *Stenotrophomonas sp* and P3 *Pseudomonas sp* has been explored in a study conducted on degradation of polycyclic hydrocarbons. Naphthalene, phenanthrene, fluorene, pyrene, and benzo pyrene were used as the sources of carbon and energy and reduced concentration of PAHs within 7 days after inoculation it is also suggested that biodegradation of PAHs could be enhanced by adding more carbon and nitrogen nutrients (Zhu *et al.*, 2016).

Another bacterial strain PDN3, which isolated from poplar tree growing on TCE contaminated soil degraded TCE to chloride without the addition of any phenolic compounds (Won *et al.*, 2012). Degradation of pesticides usually involved more than one microorganisms because each organism contributes in the degradation process it is essential for effective remediation. Some important biodegrades belong to different classes have been reported in studies are *Aerobacter, Acinobacter, Moraxella, Pseudomonas, Plesiomonas, Burkholderia, Neisseria*, Sphingomonas (Hayatsu *et al.*,2000).

Although bacterial strains have been proved as a good bio remediators specie in many studies but some fungi plants and algae also have a capability to degrade pesticide too. Bioremediation strategy can be improved by knowing the mechanism of metabolism of bio degrader species or strains by the process of bio stimulation, bioaugmentation (Mattozzi *et al.*, 2006). Fast growth and easy handling of bacterial species make them more suitable for the bioremediation process. Unfortunately, disposal of bacterial biomass, bioactivation among other organisms and pathogenicity are disadvantages concerned with bacteria. Bacteria found everywhere in environment like in soil, water or in air. Therefore, only a small fraction of bacteria can be cultured in laboratory conditions (Cycon M *et al.*, 2009).

Bacterial biodegradation of contaminants could take place in both anaerobic or aerobic conditions with different enzymatic reactions. The process of mineralization is expected to occur in both, aerobic and anaerobic degradation (Langerhoff A *et al.*,2001). Studies showed that anaerobic metabolism is more appropriate for dichlorination of organochlorine pesticides (Baczynski *et al.*, 2010).

Absence of adequate enzymes and oxidative damage make organochlorine to become more persistent in anaerobic conditions while, aerobic metabolism produce cleavage of aromatic and aliphatic metabolites. The first step in biodegradation is the removal of hetero atoms or heteroatom-containing groups, monooxygenases, peroxidases and, dioxygenases catalyzed all steps and generate enormous quantities of free radicals in

aerobic metabolism. Therefore, anaerobic conditions are more suitable for biodegradation of organochlorine pesticides, while aerobic conditions are better for degradation of hydrocarbon metabolites (Qureshi *et al.*, 2009).

HCH is a persistent organic pollutant, it could be biodegraded in situ (Langerhoff *et al.*, 2001). HCH has been shown to be degraded by bacterial species belongs to *Burkholderia* ,*Flavobacterium*, *Pseudomonas*, (Murthy and Manonmani,2007).it is found that HCH mineralization need anaerobic conditions, sometimes oxygen could be bio available while on other hand soil provide niches for metabolism. Bacteria grown on coffee beans have been found more effective those in medium alone (Barragán *et al.*, 2007). HCH mineralization need anaerobic conditions, while comparing biodegradation times for DDT, HCH and endosulfan showed increasing order of degradation that varies according to the strains or consortium used (Kumari *et al.*,2002).

It has been revealed from literature that some bacterial strains related to *Entrobacter* are capable to degrade and mineralize chlorpyrifos, parathion, diazinon, coumaphos and isazofos (Singh *et al.*, 2004) likewise, it has also been reported that a bacterial strain related to *Serria could* degrade diazinon (Abo, 2011). Rate of biodegradation of endosulfan pesticide by bacterial cultures was more efficient as compared to fungal cultures (Siddique *et al.*, 2003).

Biodegradation of endosulfan pesticide by bacterial species Staphyloococcus, *Bacillus circulans* in mixed and pure culture have been studied. Endosulfan has been shown to be degraded in mixed culture, 71.82%.76.04% and 93.3% degradation tare was observed after four weeks of incubation period while, in pure culture 89.95% 76.73% and 82.9% degradation potential was observed after 14 days of incubation (Kumar and Philip, 2006).

A Pseudomonas bacterial isolate isolated from diazinon contaminated soil is most effective specie of bacteria grown in mineral salt medium (MSM) supplemented with diazinon as an energy and carbon source responsible for the degradation of insecticide

within 14 days of incubation. Degradation rate was accelerated by adding more glucose in mineral salt medium with decrease pH values, after glucose utilization (Amer,2011).

It is reported that in spiked soil isolates and their consortium showed efficient degradation of insecticide (100 mg kg1 soil). While in non-spiked soil not supplemented with diazinon, showed less degradation, in which 2 % degradation of dose was observed (Cycon *et al.*, 2009). *Bacillus cereus, Staphylococcusi* and *Pseudomonas* sp have been found more effective to degrade petroleum, diesel oil and gasoline. These endophytic strains were isolated from plants that were present in asphalt mud impacted areas. Verification of petroleum and its derivate was performed in ELSA plates. After Discoloration of DCPIP which showed positive reading total nine bacterial strains were tested but three of them showed best degradative results. (Natalia *et al.*,2012). Endophytic microbes reduce toxicity of organic and inorganic pollutants to minimum level iron chelators, organic acids, 1-aminocyclopropane-1-carboxylate (ACC) deaminase and many other degrading enzymes (Choi *et al.*,2009). Bacterial endophytes, able to degrade herbicides, by the process of phytoremediation of the organochlorine herbicide 2, 4-dichlorophenooxyacetic acid (Germaine *et al.* 2006).

It is found in study that the endophytic bacterial strains have ability to efficiently colonized with roots of plant and showed no symptoms of toxicity in plants. In another study, it has been found that the endophytic *Enterobacter* strains, having plant growth-promoting ACC deaminase activity, was considered more efficient for the plant growth and hydrocarbon degradation as compared to those bacterial isolates have only alkane degradation activity (Yousaf *et al.*,2011). engineered endophyte, *Burkholderia* enhanced plant biomass and reduced phytotoxicity of trichloroethylene contaminated soil (Weyens *et al.*,2010).

In present study, the widely-used organochlorine pesticide (Heptachlor) has been taken for bioremediation under controlled environmental conditions. Endophytic bacterial strains were isolated by an enrichment technique from roots, shoots and leaves of white poplar *Populus alba* tree grown on pesticide non-contaminated soil. After two weeks of

spiking with Heptachlor the RP7 endophytic bacterial strain was inoculated in to soil containing 5%,15% and 25% Heptachlor respectively. Degradation of Heptachlor was assessed after two weeks of incubation period. Biodegradation rate of heptachlor in different treatments was 35%, 73% and 80% observed during 5 10 and 15 days of incubation period. Results of present study showed that endophytic bacterial strain RP7 isolated from white poplar tree has been found effective for bioremediation of organochlorine pesticide (Heptachlor) persistent in agricultural soil environment.

MATERIALS AND METHODS

3 MATERIALS AND METHODS

3.1 Methodology

A series of experiments were conducted to isolate Endophytic bacteria from white poplar *Populus alba* to enhance the bioremediation of Heptachlor from contaminated soil.

3.2 Collection of Plant material

Healthy leaves, stems, and roots of white poplar *Populus alba were* collected from Rangeland Research Institute Department of National Agriculture Research Centre Islamabad (NARC) for the isolation of endophytic bacterial strains. Samples were placed in clean plastic bags, brought to the laboratory and used for further experimental purpose.

3.2.1 Pre-treatment of Plant material

The endophytic bacterial strains were isolated from roots, stem, and leaves of white poplar *Populus alba*. The isolation of bacterial endophytes was done from the plant after collection. The leaves, root and stem of a plant were washed under running tap water for 15 to 20 minutes to remove adhering soil particles, air dried and plant parts were separated out. The separated plant parts roots, leaves and stem were weighted up to one gram weighing balance. The weighed samples were soaked in distilled water for five minutes and drained.

3.2.2 Surface Sterilization

Freshly collected plant parts were washed under tap water after that again washed in Taween 20 (1 drop in 200 mL sterile distilled water [SDW]) for 1 minute and then were cleaned three times with SDW in the laminar air flow cabinet. sterilizing agent's sodium hypochlorite: 1-5% for 2-10 minutes, ethanol: 70-95% for 30seconds -4minutes, hydrogen peroxide and mercuric chloride 0.05-0.2% for 2-5 minutes were used, 2% sodium hypochlorite, 70% ethanol and 0.1%, mercuric chloride at different treatment duration and combination was used for the surface sterilization agent for the current study for the process of surface sterilization (McInroy and Kloepper, 1994).

3.2.3 Isolation of Endophytic Bacteria through Enrichment technique

After proper drying of surface sterilized plant material, using aseptic procedure the surface of the stems was removed using a sterile scalpel in the laminar air flow cabinet and leaves were cut into pieces and each piece was placed on nutrient agar medium supplemented with antifungal agents. Culture of bacterial strains were prepared through enrichment technique, for this purpose suspension was prepared in 250 ml conical flasks containing mineral salt medium (MSM) and 100 µmol/l at 100 mg l^{-} amount of heptachlor as a source of carbon then suspension in a 250 ml of conical flasks was inoculated. suspension was prepared in different conical flasks for each sample. Covered flasks incubated at 35 °C in a shaking incubator at 180 rpm for seven days. Degradation rate of contaminant was measured by centrifugation at 10,000 rpm for 15 min to remove cells. Dilution plate technique was used to spread 1 ml culture suspension on agar plate after seven days of incubation period. Total ten dilutions (10^{-1}-10^{-10}) were prepared from which only last three dilutions were spread on agar medium. Bacterial colonies which showed prolific growth in the medium were selected. Pure culture of bacterial isolates was made by streaking by spreading selected colonies on fresh agar medium. The process was repeated twice to obtain accurate results. The initial streaks were spread out across the plate in successive cycles of sterilization and cross streaking. All selected isolates were sub cultured in nutrient agar slants and finally, all the purified endophytes were maintained at 4°C till further used.

3.3 Collection of soil samples

Uncontaminated soil was collected from National Agricultural Research Centre. The soil samples were ground, sieved with 2 mm pore size sieve and air dried.

3.3.1 Soil Texture

For the analysis of particle size distribution of soil samples reliable method of Bouyoucos hydrometer was used.

Table 3.1: Physical Characteristics of Soil

Soil type	Sandy clay loam
Silt (%)	16
Clay (%)	19.8
Sand (%)	64.2
Moisture (%)	33.82

3.4 Soil Incubation Experiments

Soil incubation experiments were carried out to assess the degradation of heptachlor. Endophytic bacteria isolated from poplar were selected leaves, roots and stems of poplar were collected from National Agriculture Research Centre Islamabad. Experiment was carried out in the Department of Environmental Sciences, International Islamic university, Islamabad. For incubation experiment mixed culture of endophytic strains were added into petri dishes containing contaminated soil after 15 days of spiking with Heptachlor. Incubation experiment was performed with three replications using completely randomized design (CRD). To conduct incubation experiment petri dishes were

incubated at temperature 30°C to 37°C for 15 days. To assess the degradation potential of heptachlor soil samples were collected after 5, 10 and 15 days of incubation. Field capacity of soil per Petri dish was also maintained at 50 percent to provide enough moisture content for bacterial growth and activity.

3.5 Physico-chemical analysis of soil

Soil samples were analyzed for physico-chemical parameters before spiking the soil with heptachlor, after 15 days of aging, before filling the Petri plates and after incubation experiment. The analysis was performed by following the standard procedures as described below:

3.5.1 Soil moisture (SM)

Pre-weighed (10 g) soils were put in a Petri plate, weighed again. Soils samples dried at 105 °C for 24 hours in digital oven to remove moisture contents from soil. Analysis of soil samples to calculate moisture content was performed by using the formula below:

$$\text{Soil Moisture \%} = \frac{Loss\ of\ weight\ in\ soil\ samples}{Weight\ of\ oven\ dried\ soil} \times 100$$

3.5.2 pH

The pH meter was calibrated by buffer solution of pH 4,7 and 10 to measure the pH of soil samples (APHA, 2005). The model of the pH meter was used (BMS pH-200L).

3.5.3 Electrical Conductivity

EC meter (DIST HI 98303 model) was used to calculate electrical conductivity of soil in micro semen (µS/cm) (Muhammad *et al.*, 2008).

3.5.4 Organic Matter

Analysis of soil organic matter was carried out by (Walkley, 1997) titration method. One gram air dry soil was added into a 500ml flask. 10 ml of normal solution of potassium dichromate and 20ml of concentrated sulphuric acid were added using a dispenser and swirl the flask to mix the suspension well and then solution allowed to stand for 30 minutes. 200 ml distilled water and 10 ml concentrated ortho-phosphoric acid was added and allowed the mixture to cool. Titrated the contents of flask with 0.5 M solution of ferrous ammonium sulfate until unless the color of the solution was changed from violet blue to green.

Calculations for percentage organic matter in soil:

$$M = \frac{10}{V\,\text{blank}} \times 100$$

$$\%\ \textbf{Oxidize able organic carbon w/w} = \frac{(\text{Vblank} - \text{Vsample}) \times 0.3 \times M}{\text{Wt}}$$

% Total Organic carbon (w/w) = 1.334× % Oxidize able organic carbon

%Organic matter (w/w) = 1.724 × % Total organic carbon

3.5.5 Microbial Biomass Carbon (MBC)

Analysis of Microbial biomass carbon was performed by modified method of rapid microwave irradiation and extraction (Islam and Weil, 1988).

MBC (mg C kg soil$^{-1)}$ = (MWCext - C_{ext}) ×2.64

MWCext = Microwaved extracted carbon

C_{ext} = Un microwaved extracted carbon

3.6 Heptachlor Extraction from Soil

Soil samples were analyzed for heptachlor at the beginning and at the end of the experiment.

3.6.1 Preparation of Soil Samples for Heptachlor

For the extraction of heptachlor from soil 1 g soil samples were shifted into 100 ml Teflon tubes then 5 ml of dichloromethane was added to each sample. samples were extracted for 2 hours in water bath at temperature of 38°C. After water bath extraction samples were centrifuged at 4000 r min^{-1} for 5 min to separate the supernatant from soil. land 2 ml mix of n-hexane: dichloromethane (v/v 50:50) was added to extract after that the supernatant extract for pesticides and then dried by sparging with N_2; 1 ml of acetonitrile was added to re dissolved solid residues (Zhang *et al.*,2006).

3.7 Gas Chromatography- Mass Spectrometry (GC-MS)

The Gas chromatography- Mass spectrometry (GC-MS) technique was used to perform the analysis of heptachlor. The standards of pesticides mixture were run on GC-MS and retention times were optimized with individual pesticides then samples were run on GC- MS (Rhind *et al.*, 2013).

3.8 Statistical Analysis

The data was formulated in excel and statistical analysis of data was performed on EXCEL.

RESULTS AND DISSCUSION

4 RESULTS AND DISSCUSION

Contamination of surface of soil is an environmental problem posed by repeated application of pesticides in agricultural field. The present study was carried out to establish effective remediation method for persistent organochlorine pesticide (Heptachlor) using endophytic bacteria isolated from poplar tree grown on non-contaminated soil.

4.1 Soil Analysis

Properties of soil such as electrical conductivity, organic matter, pH, soil moisture and temperature effect the pesticide degradation process in soil. These physicochemical properties also effect the persistence of pesticides under field conditions. Moisture content in soil act as an important factor for the movement of pesticide in soil and proper functioning of microbes. Most of the pesticides show slow degradation rate in low moisture content and in dry soil. pH of soil effect adsorption process and degradation rate of pesticides. Organic matter content in soil also effect the microbial degradation process by stimulating pesticide or enhance activity of microbes by the process of co-metabolism. For the active growth of microbial population sufficient nutrients should be available to microbes for degradation of pesticides.

In the present study, physicochemical properties, soil pH, electrical conductivity (EC), total organic carbon, organic matter, Microbial biomass carbon, soil moisture content were determined.

4.1.1 Effect of heptachlor on Soil pH

In present work, variations in soil pH was observed at the start and end of experiment. Figure 4.1 represents change in soil pH among different treatments (T1, T2, T3, T4) after spiking and then after the incubation period of soil samples. pH of the soil in different treatments was 6.79,8.53,7.78,7.45. After two weeks of incubation period, a significant decrease was found in all treatments inoculated with endophytic bacteria which is 6.51, 7.87, 6.71 and 6.48. Several studies have shown that soil pH greatly influence the degradation rate of pesticide. Most studies suggest that best degradation rate is around pH 7 (neutral pH) and below this range breakdown become slow (Andrea, 1994).

The pH of soil may affect adsorption, both biotic and abiotic pesticide degradation processes. It influences mobility and bioavailability of pesticides in soil. Degradation process of pesticide also influenced by pH which depends on whether a pesticide goes under acidic hydrolysis or alkaline hydrolysis. pH also influenced sorptive behavior of pesticide molecule on clay and organic surfaces in the soil (Zhang *et al.*, 2006). In present study decreasing trend of soil pH was observed in all treatments. The reason behind the reduction of soil pH is the formation of organic acids produced from intense fermentation of carbohydrates (Dibble and Bartha, 1979). It is reported that pH between 6 and 9, bacterial strain is more able to degrade pesticide. However, when pH become less than 6 or more than 9, the activities of hydrolases may inhibit (Tripathi *et al.*,2010).

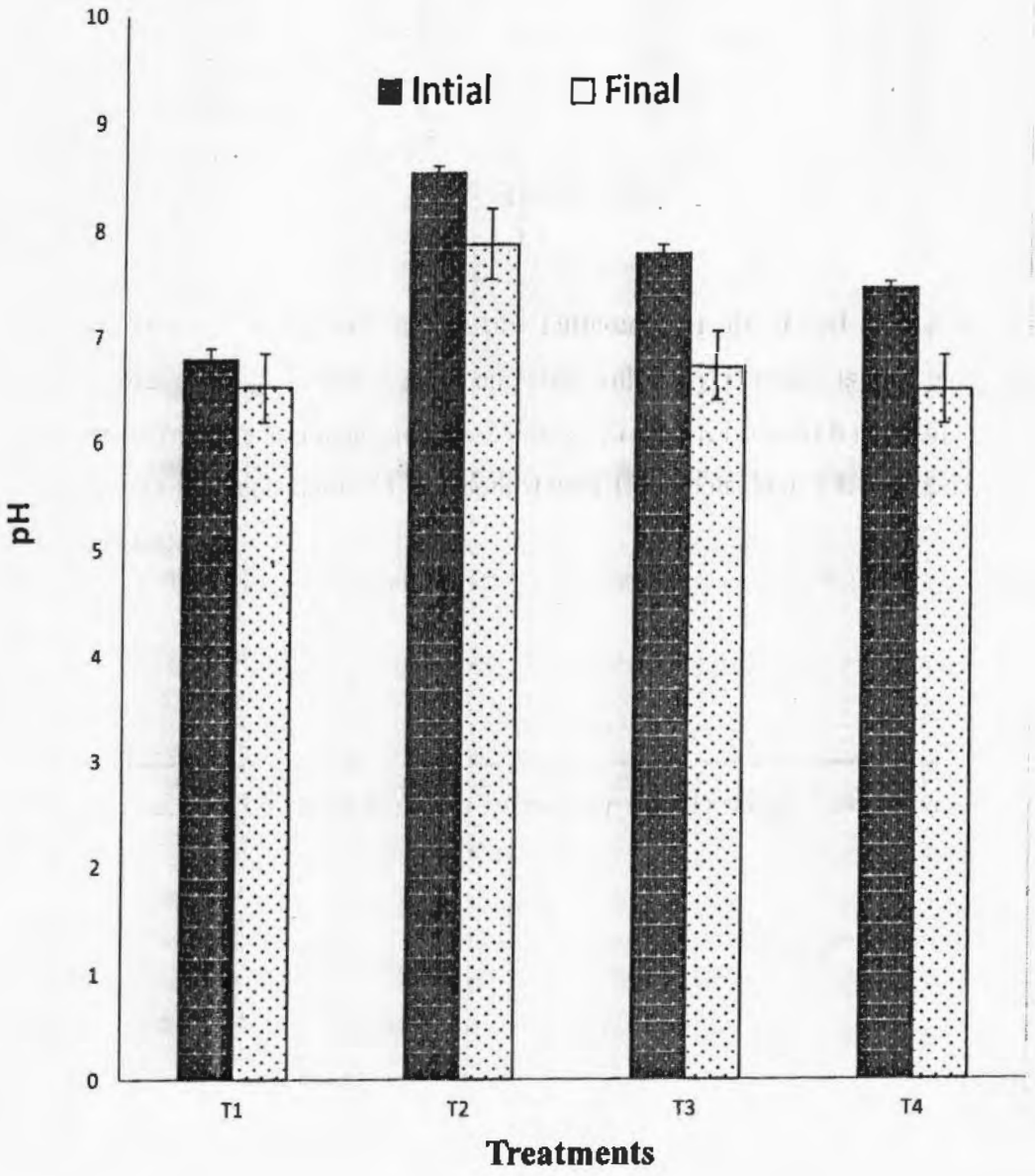


Figure 4.1. Effect of heptachlor on soil pH. Difference in pH of soil among different treatments after spiking for two weeks compared with the soil which is inoculated with endophytic RP7 isolate and incubated for 2 weeks, T1: control soil with no pesticide, T2: soil spiked with 5% heptachlor, T3: soil spiked with 15% heptachlor, T4: soil spiked with 25% heptachlor.

4.1.2 Effect of heptachlor on Soil Electrical conductivity (EC)

Figure 4.2 shows the variations in electrical conductivity of soil among different treatments (T1, T2, T3, T4) after spiking and then after two weeks of incubation of soil .EC of the soil with different treatment was 80.98(µs/cm), 96.71 (µs/cm),117.12 (µs/cm),177.02 (µs/cm) respectively. At the end of experiment the soil which is inoculated with endophytic bacterial strain showed a significant increase in EC. The lowest values of EC were 80.98 (µs/cm) and 96.71 (µs/cm) shown by T1 and T2, while T3 and T4 showed comparatively higher values 117.12 (µs/cm) and 177.02 (µs/cm). The EC values increased and finally after 2 weeks of incubation period the higher values were 115.39 us/cm,184.63 µs/cm, shown by treatments T2 and T3, while maximum value was observed in T4 (250.12 µs/cm). As value of T1 is not much higher because it does not contain heptachlor. It was found in present work, during the process of bioremediation EC values were increasing from 80.98(µs/cm) to 250.12(µs/cm). The electrical conductivity of the soil inoculated with endophytic bacterial strain increased with increasing incubation time because of the ions released during the organic matter mineralization process (García *et al.*,1994). Aeration and moistening during bioremediation cause release of dissolved solutes and increase in electrical conductivity (Raman *et al.*, 2015).

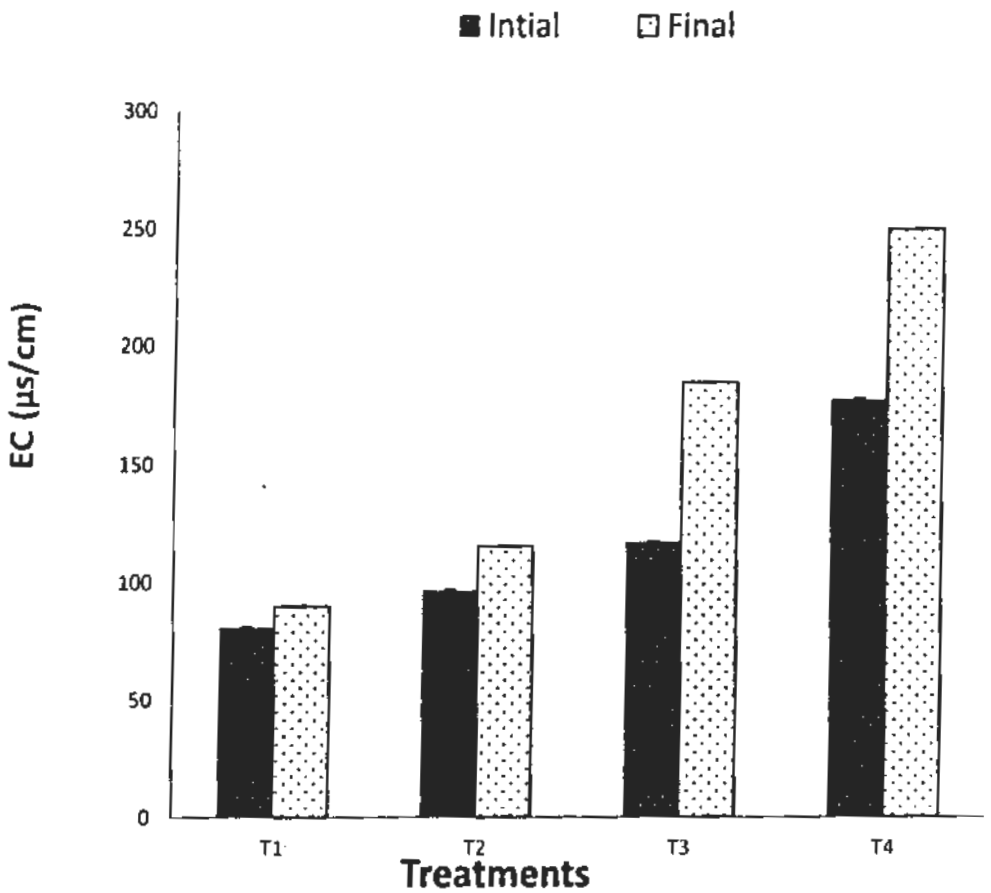


Figure 4.2 Effect of heptachlor on soil Electrical Conductivity. Difference in Electrical conductivity of soil among different treatments after spiking for two weeks compared with the soil which is inoculated with endophytic bacterial isolate RP7 and incubated for 2 weeks, T1: control soil with no pesticide, T2: soil spiked with 5% heptachlor, T3: soil spiked with 15% heptachlor, T4: soil spiked with 25% heptachlor.

4.1.3 Effect of heptachlor on Soil Organic matter (OM)

Addition of endophytic bacterial isolate RP7 to soil had an influence on the organic matter content in soil shown in figure 4.3. The amount of organic matter of the soil samples at the start of the experiment was observed to be T1: 1.80% and it varied in different treatments T2: 1.75%, T3 :1.69%, and T4 :1.59 %. The spiked soil was then inoculated with endophytic isolate and incubated for two weeks. After two weeks of incubation period soil spiked with heptachlor and endophyte bacterial strain showed decrease amount of organic matter in figure 4.3. The variations in organic matter of the soil treatments in which endophytic isolate was inoculated are recorded as (1.50%.1.40%,1.32%. and 1.29 %) in figure 4. 3. (Anderson and Flanagan ,1989) reported that organic matter in soil used by small micro-organisms in soil, as food as these microbes break organic matter, excess number of nutrients (N, P and S) are released into the soil. This release process of nutrients in to soil is called mineralization (Phillips *et al.*, 2005). Organic matter consists sugars, starches, and proteins which are rapidly decomposed by microbes. Fresh organic residues become decomposed under favorable conditions, a warm, moist and well aerated soil with pH 6 and 7 provides ideal conditions for decomposition of organic matter thus rapid reduction in the volume of organic matter takes place (Ahuja *et al.*, 2001).

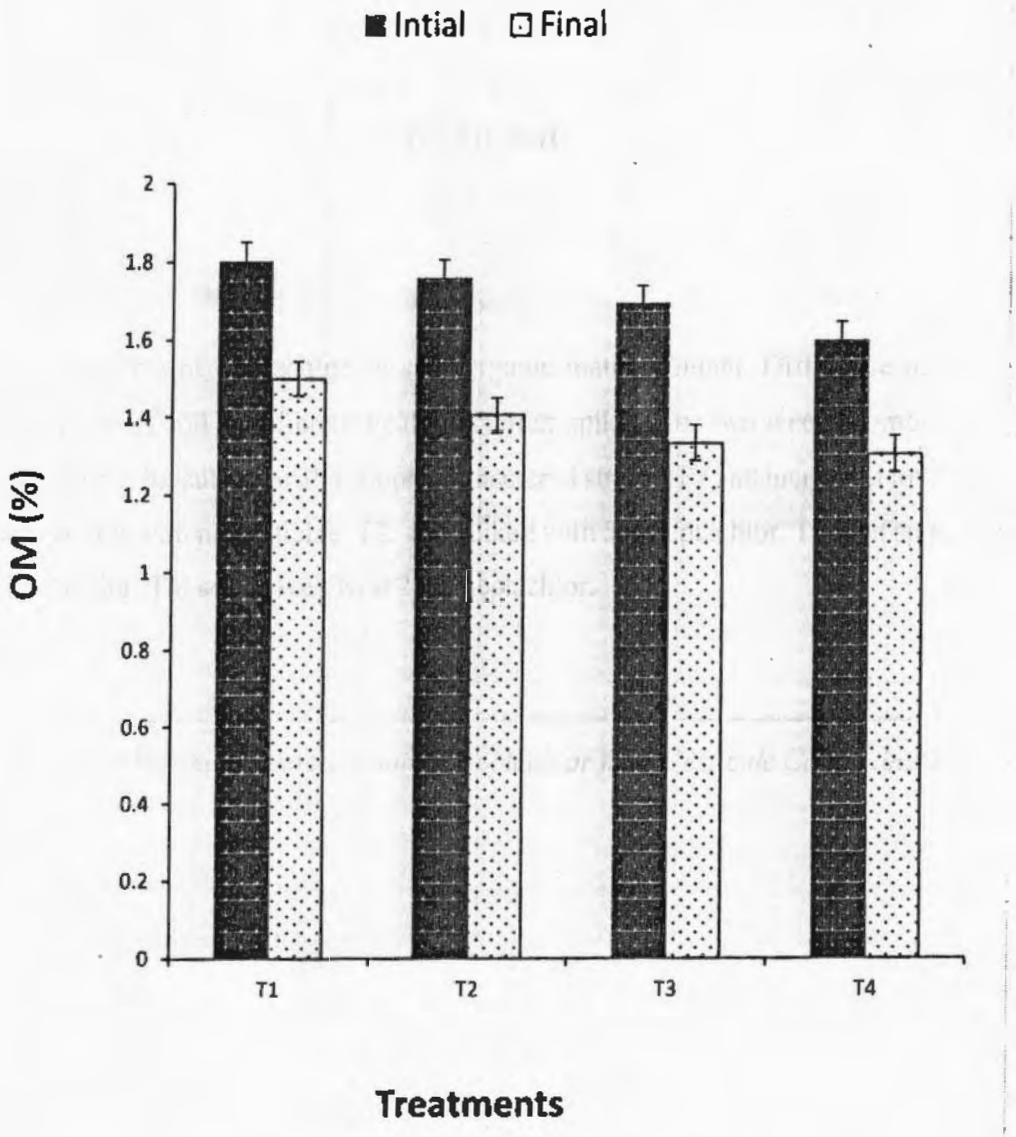


Figure 4.3: Effect of Heptachlor on soil Organic matter content. Difference in Organic matter content of soil in different treatments after spiking for two weeks compared with the soil which is inoculated with endophytic bacterial strain RP7 and incubated for 2 weeks, T1: control soil with no pesticide, T2: soil spiked with 5% heptachlor, T3: soil spiked with 15% heptachlor, T4: soil spiked with 25% heptachlor.

4.1.4 Effect of heptachlor on soil Total organic carbon (TOC)

Figure 4.4 represents the total organic carbon concentrations among different treatments after spiking in comparison with treatments after incubation experiment. Initially the concentration of total organic carbon was 1.98%.1.96 %,1.89%,1.87% in treatments. After two weeks of incubation period a significant decrease in the TOC content was observed in all treatments. Figure 4.4 is showing the variations in the soil treatments which are inoculated with endophytic bacterial strain RP7. After two weeks, a significant decrease in the TOC values are recorded as (1.58%,1.54% ,1.59% and 1.56%) as compared to the initial concentration. Decrease in total organic carbon values were recorded during bioremediation process, due to bacterial metabolic activities (Chefetz *et al.*, 1998). Soil treated with diverse types of pesticide like carbamate, organochlorine, organophosphorus pyrethroid groups of insecticide at recommended levels showed higher rate of organic carbon mineralization as compared to soil with no pesticide. Enhanced biodegradation process of insecticide stimulates the growth and metabolic activates of microbes which increase the mineralization of organic matter content of soil. Along with the process of mineralization microbes also transform plant nutrients to derive energy and carbon for their cellular activities, thus amount of organic carbon become lower in soil (Sanchez *et al.*, 2004).

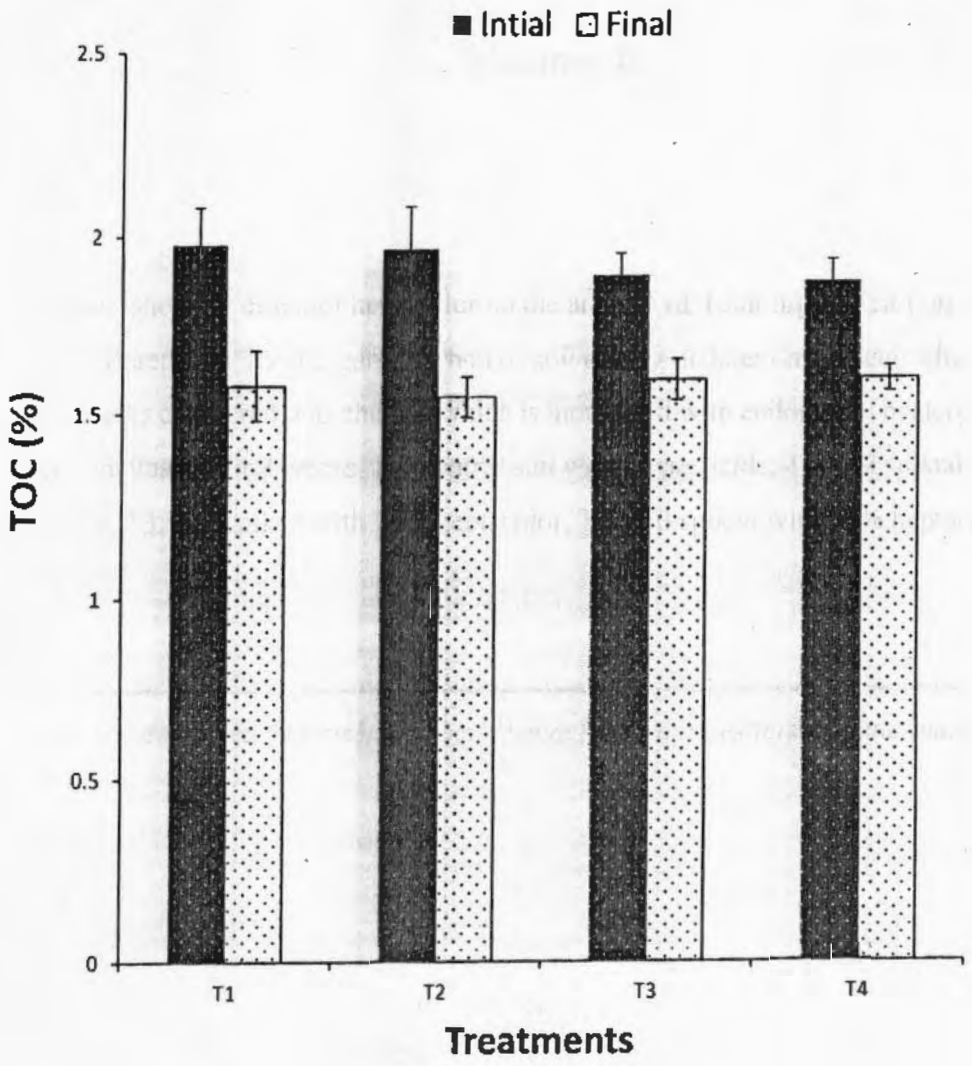


Figure 4.4: showing effect of heptachlor on the amount of Total organic carbon (TOC %) in soil. Difference in Total organic carbon of soil among different treatments after spiking for two weeks compared with the soil which is inoculated with endophytic bacterial isolate RP7 and incubated for 2 weeks, T1: control soil with no pesticide, T2: soil spiked with 5% heptachlor, T3: soil spiked with 15% heptachlor, T4: soil spiked with 25% heptachlor.

4.1.5 Effect of heptachlor on soil Microbial biomass carbon (MBC)

Results showed that concentration of microbial biomass carbon was significantly influenced in the presence of endophytic bacterial strain RP7. After two weeks of spiking treatments T1, T2, T3and T4 showed lower MBC contents (1.51% ,1.12%.1.08%.1.00%), While other treatments with endophytic bacterial consortium showed higher amount of MBC. After 2 weeks of incubation experiment, the treatments inoculated with bacterial isolate RP7 showed higher MBC content (1.70% ,1.62%, 1.55%, 1.49%) compared to other treatments of soil spiked with heptachlor figure 4.5. Pesticide degradation capacity is strongly indicated by the amount of microbial biomass in soil because both pesticide degradation capacity and amount of microbial biomass show positive relation (Voos and Cn-oflinan, 1997). It is reported that most pesticides show no harmful and toxic effects to beneficial microorganisms in soils when applied in soil at recommended levels and interval of time (Patnaik *et al.*, 1995). Microbial biomass carbon content increased in control samples with no pesticide both under flooded and non-flooded condition in the presence of 2,4-D herbicide and its analog 2,4,5-T, after 30 days of incubation period. similarly, HCH is also stimulatory to microbial biomass when it is applied on soil a significant increase in the amount of microbial biomass carbon content was observed. Some pesticides showed no significant harmful effect on microbial activity and their composition and increase microbial respiration. Microorganisms degrade pesticide in soil environment and assimilate degradation products of these pesticides resulting in increased activities of microorganisms and their size (Tyess *et al.*, 2006). Sometimes, at the initial concentrations of pesticides microbial population is affected by pesticide application but with time after a certain period of acclimation, the population returns to normal amount or even increases in amount (Fliessbach and Mader,2004).

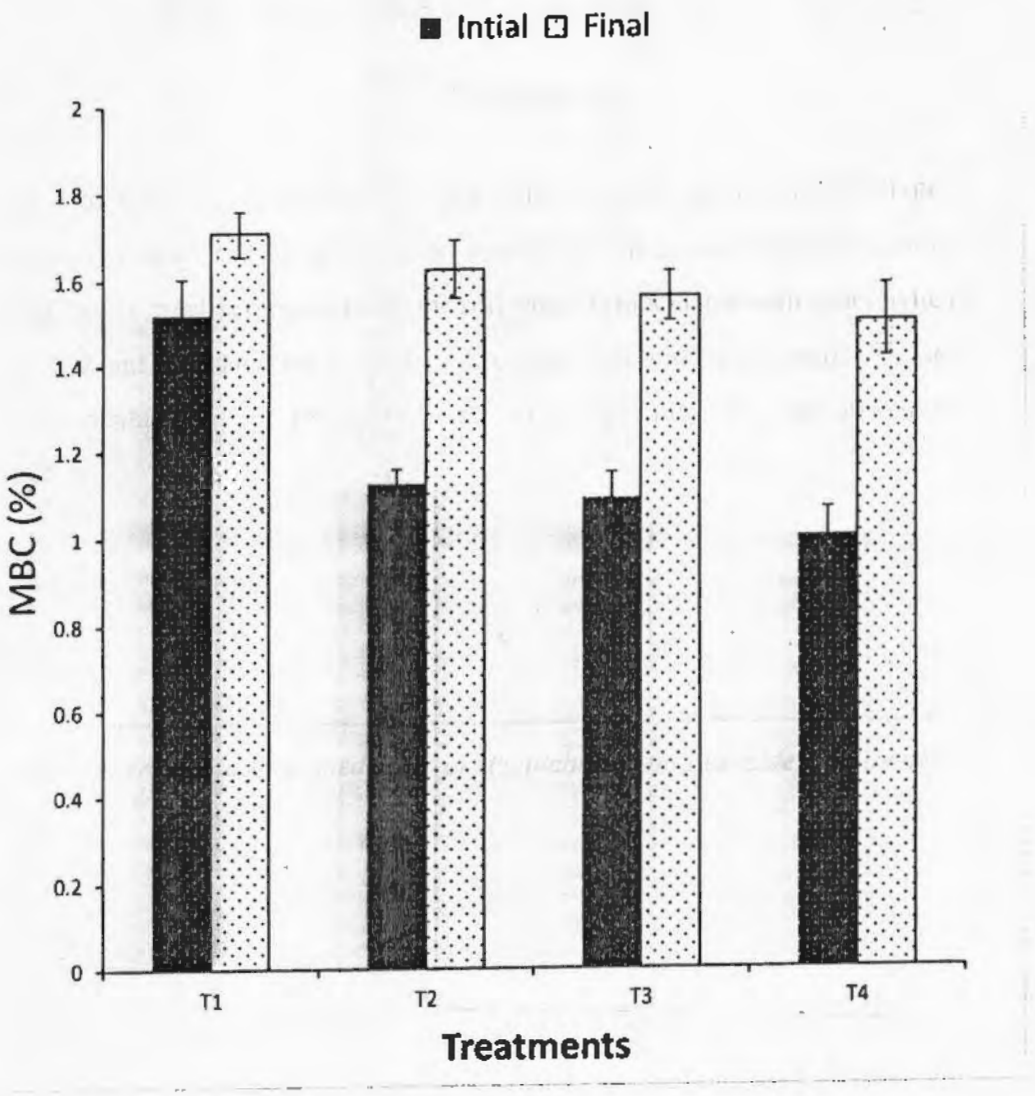


Figure 4.5: Effect of heptachlor on Microbial biomass carbon (MBC%) in the soil. Difference in Microbial biomass carbon content of soil among different treatments after spiking for two weeks compared with the soil which is inoculated with endophytic bacterial strain RP7 and incubated for 2 weeks, T1: control soil with no pesticide, T2: soil spiked with 5% heptachlor, T3: soil spiked with 15% heptachlor, T4: soil spiked with 25% heptachlor.

4.2 Isolation of Endophytic Bacteria

Heptachlor degrading endophytic bacterial strains were isolated from roots, stem, and leaves of white poplar (*populous alba)*. The most efficient endophytes were screened based on their effectiveness to consume heptachlor as the sole source of carbon. The most efficient bacterial endophytes were further evaluated for their potential to promote the degradation of heptachlor in controlled conditions. The results of these experiments are discussed below.

4.2.1 Isolation and screening of effective Endophytic Bacterial Isolates capable to degrade Heptachlor

Number of endophytic bacterial strains were isolated from root, stem and leaves of white popular by using enrichment technique in which heptachlor was used as the source of carbon and energy in the Dworkin and Foster (DF) salt minimal medium (Dworkin and Foster, 1958). DF medium with pH 7.2 contained 50 mg l-1 amount of heptachlor. All chemicals were purchased from Sigma-Aldrich and ACC was purchased from ACROS Organics.

Further, Isolation of endophytic bacterial strains was done using dilution plate technique in which suspension of 200 µl from each dilution were plated onto agar medium containing heptachlor after that medium was incubated at 30 °C for 48h. Initially, total 89 endophytic bacterial strains were isolated these isolated bacterial strains were again tested on solid (agar) medium containing (50 mg l-1) amount of heptachlor. Out of 89, 28 endophytic bacterial isolates showed prolific growth on the medium containing heptachlor and these isolates were selected for evaluating their degradation potential for heptachlor degradation under controlled conditions.

For screening of effective bacterial isolates, mineral salt medium (MSM) spiked with heptachlor. Heptachlor was added to 2 mL in pre-sterilized small vials. For each vial inoculation was done separately using 40 (micro L) bacterial culture of uniform cell density (OD = 0.7 +- 0.02). Reduction in amount of heptachlor was observed by taking 1.5 mL of aliquot and centrifuging it at 8,000 * g approx. for 10 min to remove bacterial cells.

spectrophotometer was used to determine the concentration of heptachlor (Modified MA 02052, USA) at 540 nm. Based on the bacterial efficiency to reduce heptachlor was selected for further screening.

Table 4.1: Screening of endophytic bacteria capable of degrading heptachlor in liquid medium under static conditions

Bacterial isolates	Percent reduction Mean ±SE[a]
RP2	23.5±1.02
RP19	45.6±1.30
RP7	55.7±3.20
SP3	6.76±4.30
SP19	24.9±2.44
SP20	33.4±3.67
SP22	19.7±1.23
LP5	28.6±2.13
LP8	35.4±3.88
LP17	7.58±0.81

[a]Standard Error

4.4 Biodegradation of Heptachlor by endophytic bacterial isolate RP7 in soil

Pesticides could increase agricultural productivity but also pose devastating consequences on environment. Pesticides degraded by physical, biological and chemical means in soil. The amount of pesticide available in the soil stimulate the microorganism's population and their enzymatic systems to degrade pesticide. The efficiency of pesticide degradation process can be determined by soil type and soil physicochemical properties (Sanchez *et al.*, 2004). In the present Research study, the Incubation experiment conducted to determine the tolerance of endophytic bacterial isolates isolated from white poplar populous alba grown on pesticide non-contaminated soil. Number of endophytic bacterial strains were isolated from roots, stem and leaves of white poplar on Mineral salt medium (MSM) in which heptachlor was used as a carbon and energy source but some of them showed growth in medium. RP7 bacterial isolate was used in the present study, which showed 56 % reduction of heptachlor in medium so that RP7 isolate was found best showed the highest tolerance rate towards different concentration of Heptachlor 4.3µg/g, 9µg/g and 12µg/g with maximum biodegradation rates.

Results showed that the isolate RP7 removed up to 35%,73% and 80% amount of heptachlor after 5,10 and 15 days of incubation period among different treatments shown in Fig.4.4 as same results was observed in other study in which soil inoculated with bacterial strain incubated at 35°C showed 62.9%, 75.6% 71.6% and 82% biodegradation rates (Maryam *et al.*, 2014). Comparable results were observed in study in which Endosulfan has shown degradation rate of 71.82% and 76.04 %, 75.96 % after two weeks of incubation (Kumar and Philip, 2006).

Heptachlor in the soil was gradually depleted by the isolate from 4.3 µg/g on day 5 to 2.8µg/g ,1.2µg/g on day 8 and finally to 0.9 µg/g on day 15 in treatment T2. For the treatment T3 heptachlor in soil decreased by bacteria from 9µg/g on 5 days to 7.3µg/g, 5.6µg/g on day 8 and 1.8µg/g on day 15 shown in table 4.4. In treatment T4 endophytic bacterial isolate depleted heptachlor amount from 12µg/g to 10.2µg/g,7.3µg/g and 3.8µg/g on day 5 10 and 15 respectively. Results indicated that Heptachlor concentration among

different treatments with no bacterial isolates decreased progressively throughout the 15 days of incubation period. Figure 4.4 is showing the dissipation behavior of heptachlor in different treatments of soil. For the first concentration in treatment T2 during the 5, days of incubation, Heptachlor was biodegraded at slower rate with 35%, while after the 10 and 15 days of incubation 73% and 79% biodegradation rate was observed as compared to treatment T3 and T4.

In untreated natural soil (without endophytic bacterial isolate), heptachlor was more persistent then soil samples inoculated with endophytic bacterial isolate RP7. After 5d, 10d and 15 d of incubation,35 %, 73% and 79% degradation rate was observed in treatment T2, while in treatment T3: 19% ,38%, 80% degradation rate was observed on 5,10 and 15days of incubation period. Increase in biodegradation rate with increase in incubation time might be due to the ability of bacterial isolates to survive in the maximum concentration, it stimulates expression of bacterial enzymes that hydrolyze heptachlor (Yonar *et al.*, 2014). It is obvious from previous studies that higher concentrations of heptachlor might induce rate of biodegradation (Lalucat *et al.*, 2006).

Finally, Treatment T4 showed 15% degradation on day 5, 39.16% on day 8 and 68.33% was observed on day 15, respectively. Results showed that decrease rate in treatment T4 might be due to the high concentration of pesticide had adverse effect on the bacterial growth. High concentration may inhibit the enzymatic activity involved in biodegradation process, similar pattern of bacterial response to high concentration also observed in other studies (Singh *et al.*, 2013). Data revealed that degradation rate has decreased with increasing pesticide concentration after certain level of concentrations this may bc due to the stressful conditions the bacterial culture exposed to (Jilani *et al.*, 2004). less availability of dissolved oxygen could be another reason because increase organic load may reduce the amount of dissolved oxygen OD (Battaglin and Fairchild, 2002). After 15 days of incubation, the biodegradation of Heptachlor became stable. Therefore, 15 days were optimal time for Heptachlor biodegradation.

The reduction in concentration of Heptachlor during bioremediation in soil were mentioned in Table 4.4. During this period, maximum 73% ,79%, and 80% degradation of

heptachlor was achieved shown in Figure 4.4. This might be slightly high rate of degradation performed by endophytic bacterial isolate RP7. it could be due to the bacterial enzymes secreted from bacterial cell being exposed to pollutant (Atit *et al.*, 2013). The incubation period to examine degrading activity of endophytic bacterial isolate RP7 also varied from 5d to 15d.

The results for 15days of incubation period Table 4.4 is showing that the bacterial isolate RP7 showed different degradation profile as compared of results of 5 and 8 days of incubation. For best degradation activity isolates need 1 to 2 weeks in invitro degradation of pesticides (Wongsa *et al.*,2004). The obtained results conclude that endophytic bacterial strain RP7 isolated from white poplar grown on non-contaminated soil is capable to degrade heptachlor at maximum rate so that this endophytic isolate RP7 is found more effective for the treatment of heptachlor contaminated soil as compared to other isolates which showed less reduction percentage in medium.

Table 4.2: Biodegradation of Heptachlor contaminated soil

Days	5% heptachlor	15% heptachlor	25% heptachlor
0 day	4.3 ± 0.2	9± 0.4	12 ±0.5
5 day	2.8 ±0.3	7.3 ± 0.6	10.2 ±0.3
10 day	1.2 ± 0.05	5.6± 0.4	7.3 ±0.2
15 day	0.9± 0.02	1.8± 0.08	3.8± 0.1

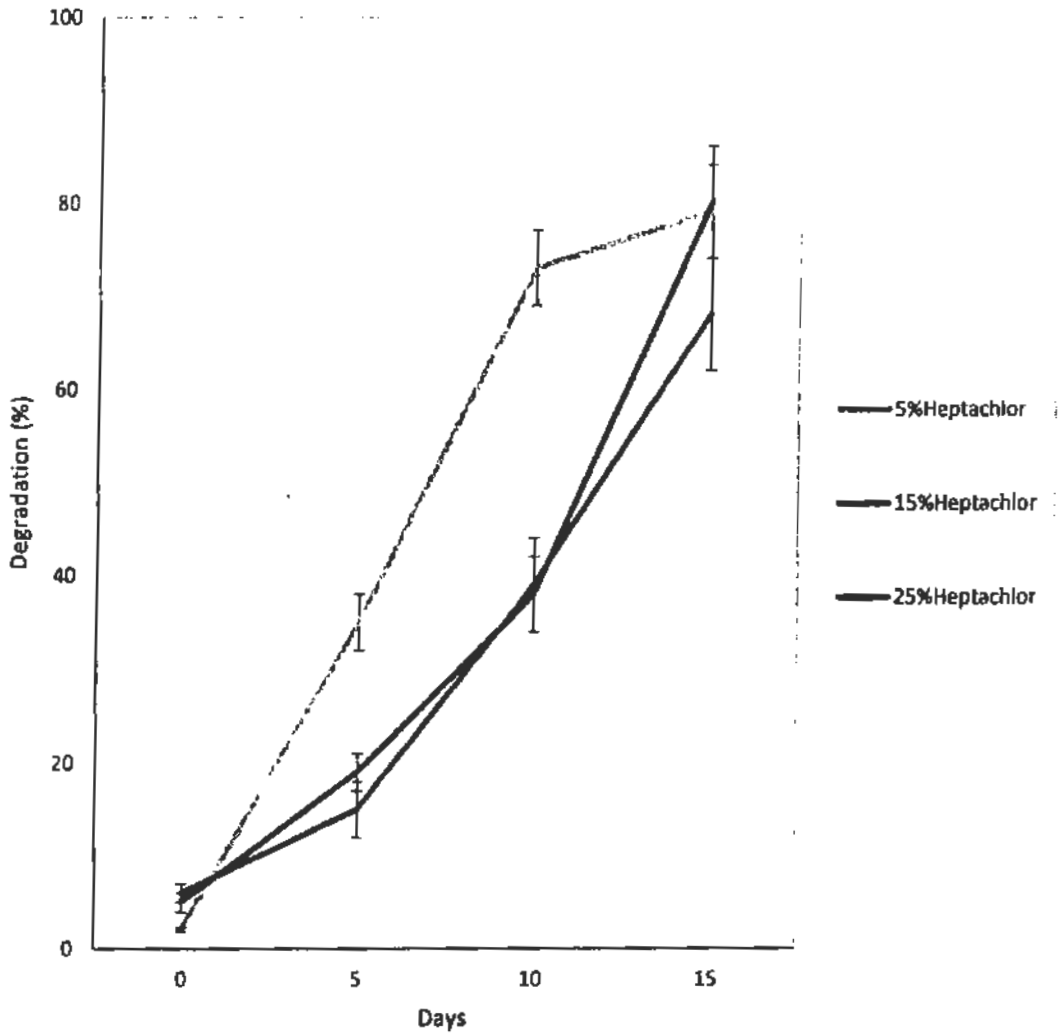


Figure 4.6: Biodegradation of Heptachlor in different treatments of soil with endophytic bacterial isolate RP7.

4.5 Conclusion

The contamination of environmental media with pesticide is a widespread problem. Although, pesticides increase agricultural productivity but because of pesticides application devastating consequences on environment occur. To reduce pesticide contamination from soil and water, bioremediation could be an effective and viable strategy. Endophytic bacterial strains that can degrade various pesticides could be isolated from plants and from woody tree species showing tolerance and resistance to different organic pollutants and cultured in the laboratory under controlled environmental conditions. The present study provides initial explanation of use of endophytic diversity isolated from poplar grown on pesticide non-contaminated soil, showing that even in unfavorable condition endophytic microbes show growth and enhance bioremediation process of organochlorine pesticide. For these organochlorine pesticide (Heptachlor), the incubation period of 5 to 15days was seen to be sufficient for the bioremediation.

4.5.1 Future work

Use of endophytic microbes for bioremediation of organochlorine pesticide (Heptachlor) requires an understanding of all important biochemical steps involved in pesticide degradation. Future studies should be focused to explain specific mechanisms involved in the metabolism of heptachlor like transport of heptachlor into bacterial cells, degradation pathways of heptachlor, and degrading enzymes of endophytic microbes should be studied. The future research on endophytic microbes should be focused on the practical use of endophytic bacterial strains to remediate contaminated sites. Biomolecular

engineering technique should be used to enhance the endophytic bacterial strains and their enzymes in bioremediation process.

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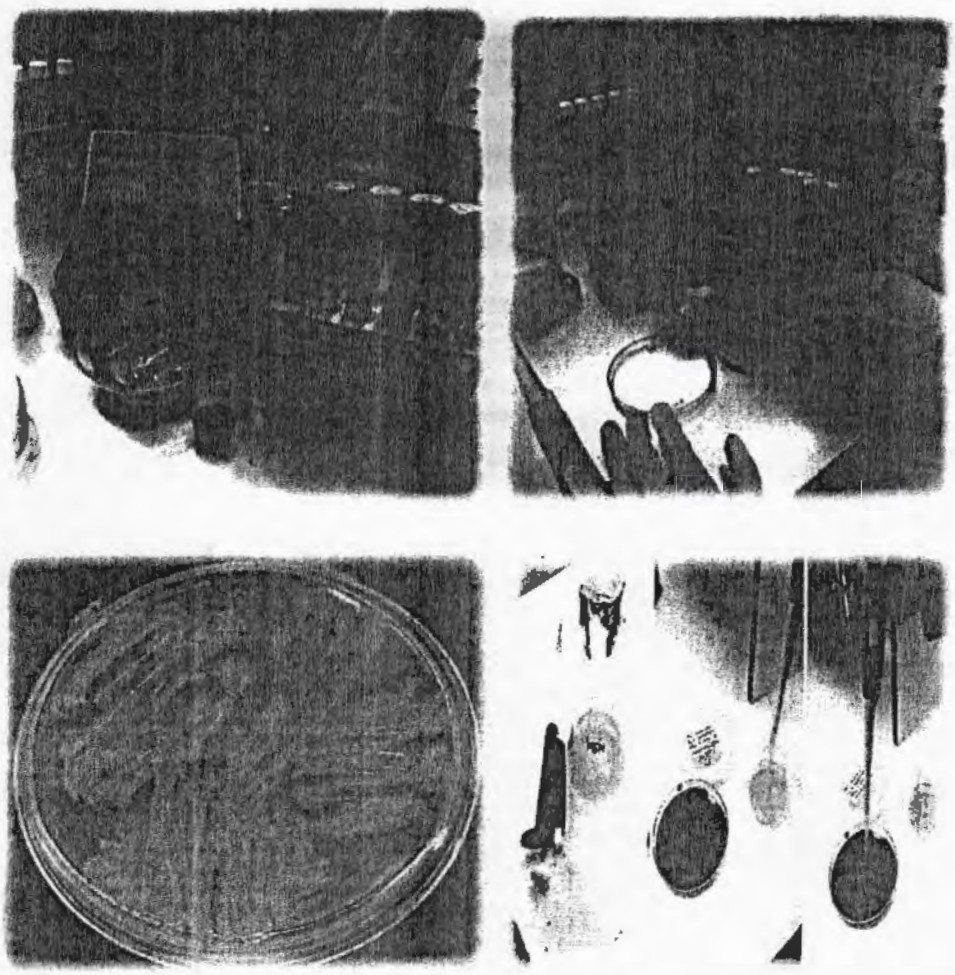
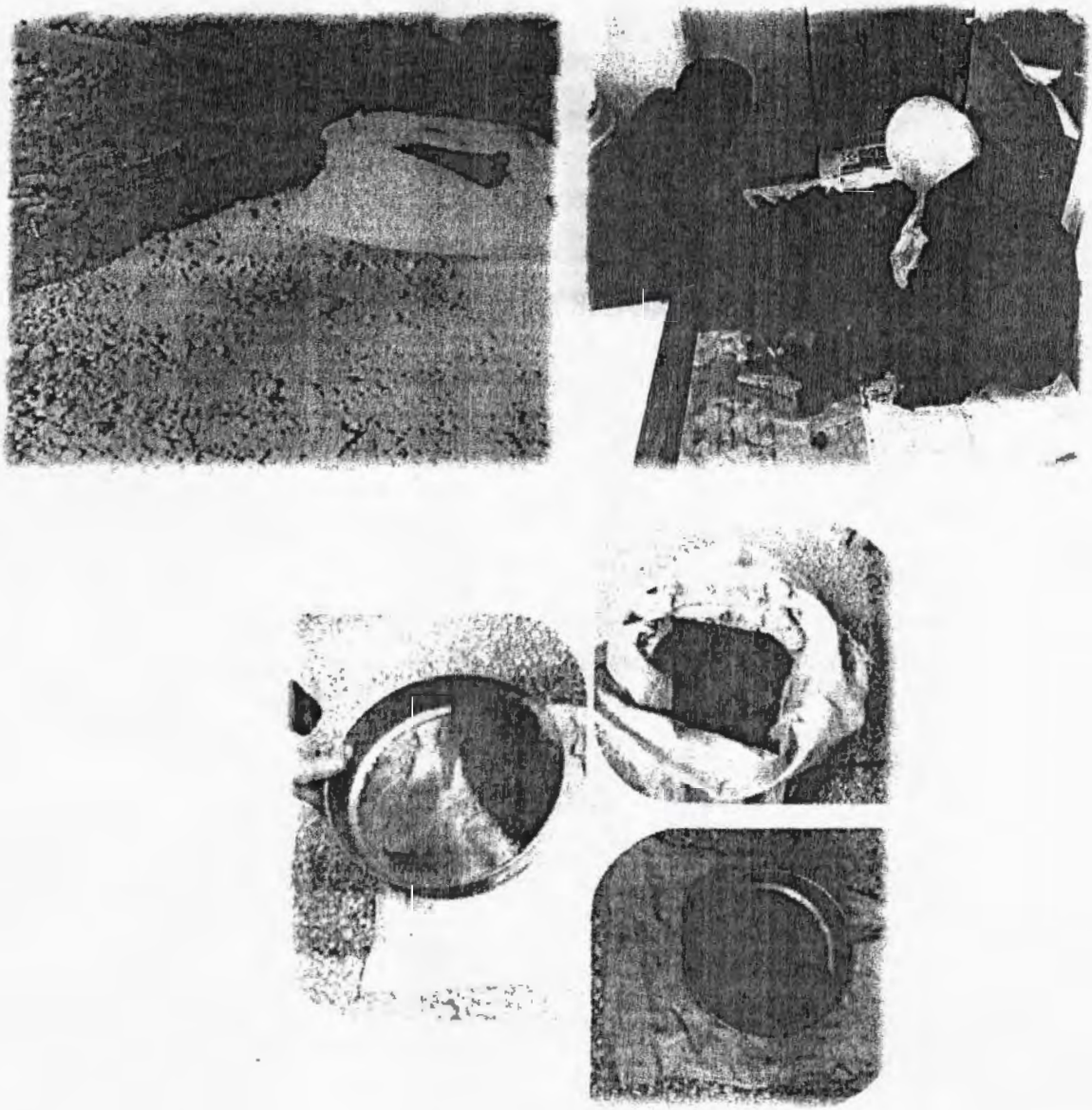
Annexure: A



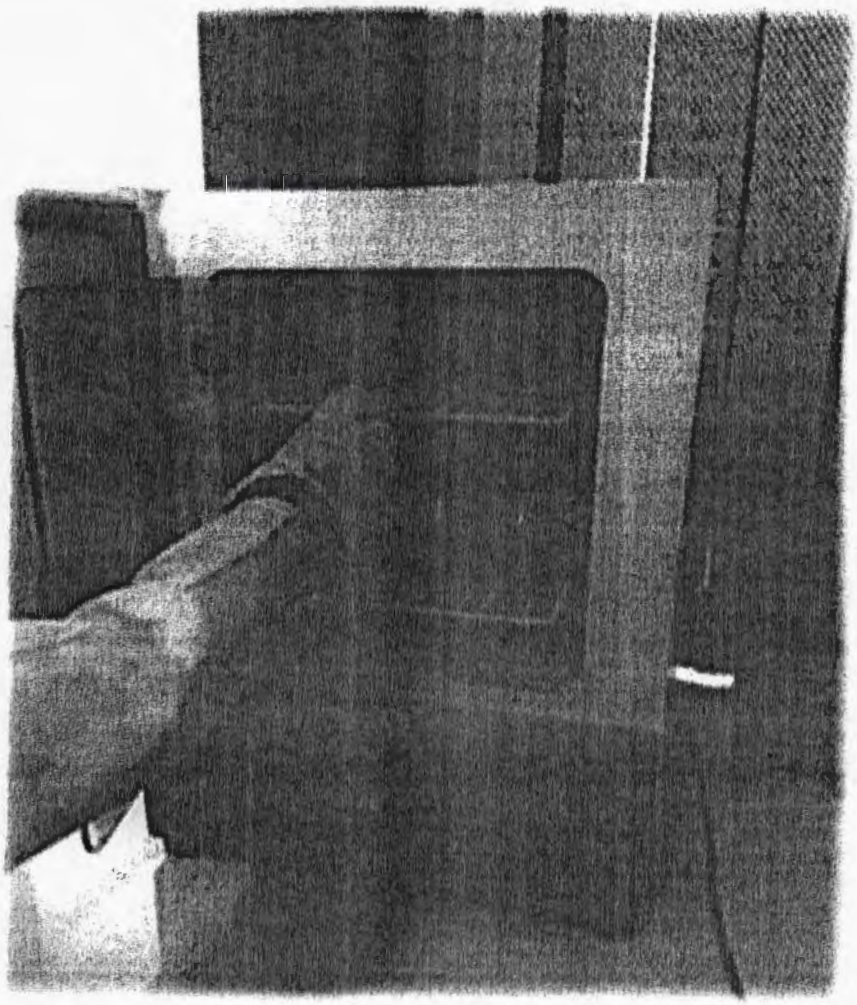
Isolation of Bacterial strains through Enrichment technique

Annexure: B



Preparation of Soil Samples

Annexure: C

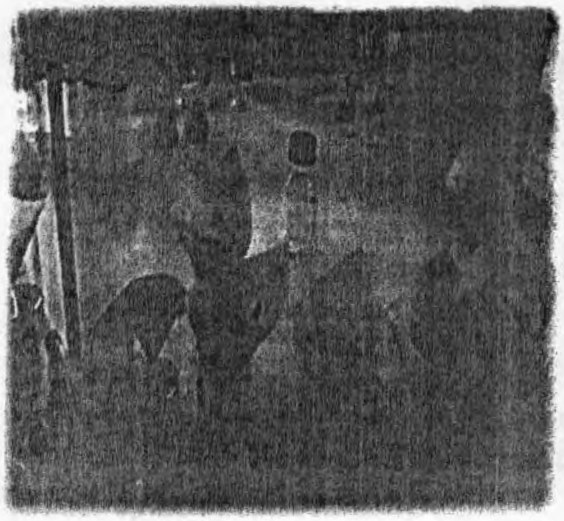


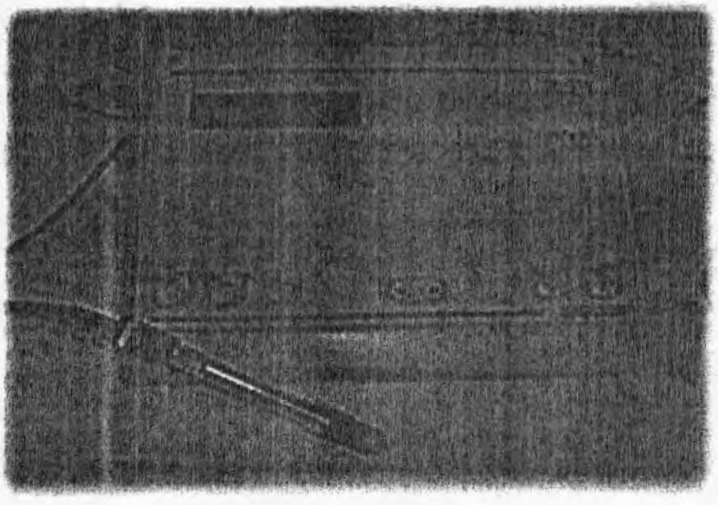
Incubation of soil samples

Annexure: D

Soil samples Spiked for 2 weeks

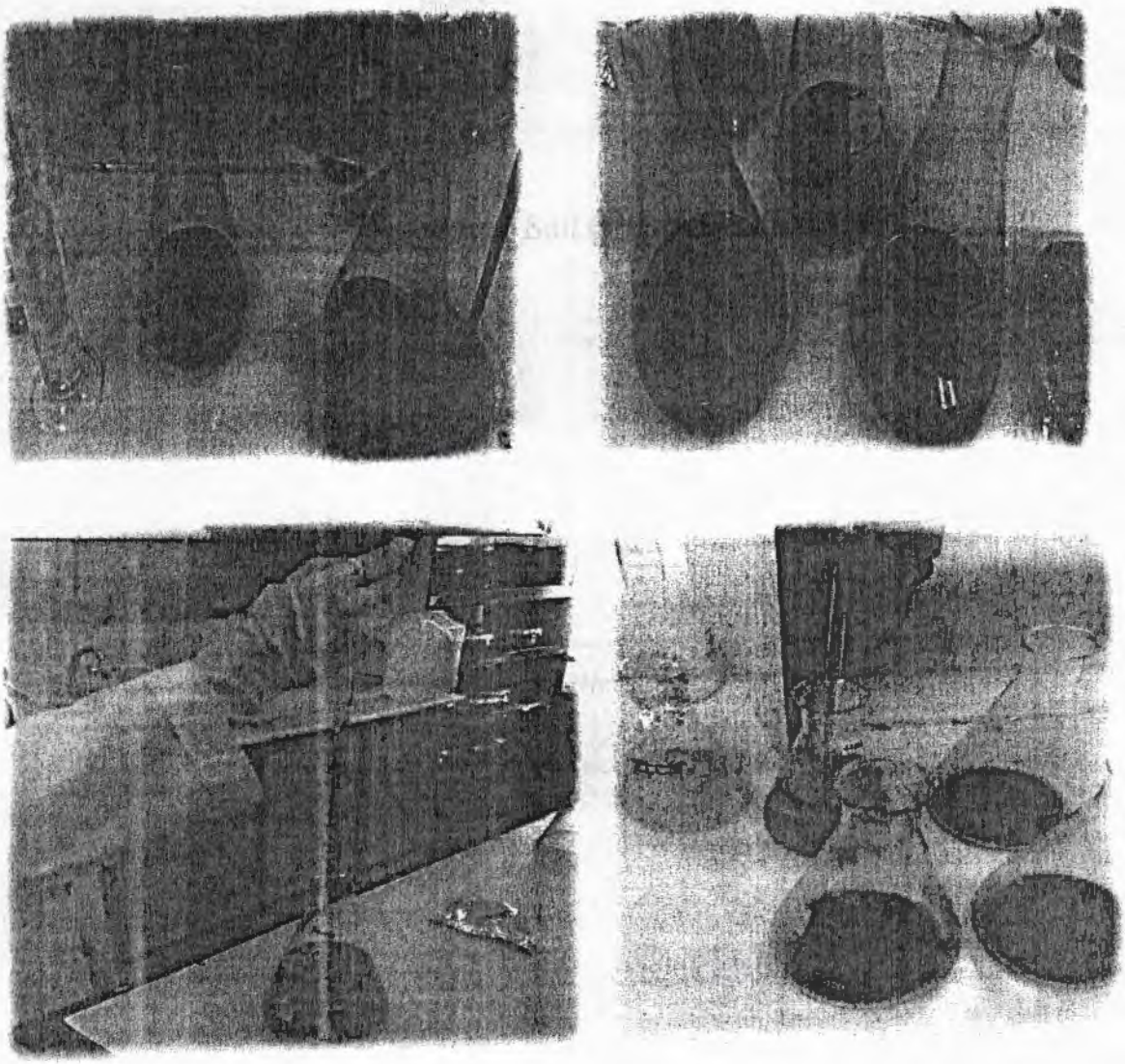
Annexure: F





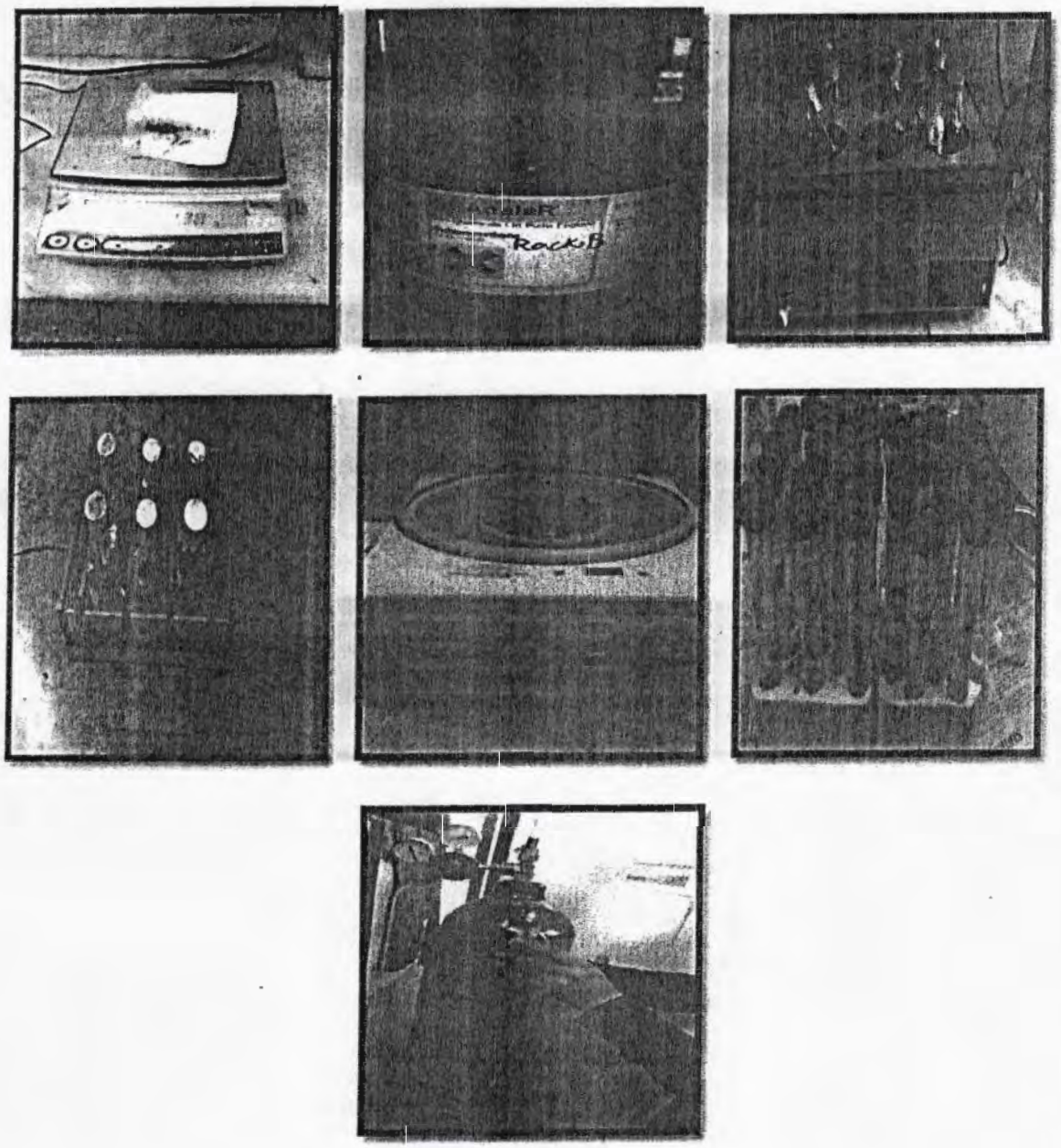
Analysis of Soil pH and Electrical Conductivity

Annexure: G



Analysis of Soil Organic Matter Content

Annexure: H



Preparation of Soil Samples for Heptachlor