Biodegradation of Dichlorvos using Indigenous Soil Microorganism

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2010-NUST-MSPhD-EnvS-03

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By

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I dedicate my thesis work to my beloved siblings

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LIST OF ABBREVIATIONS

AChE	Acetyl cholinesterase
API	Analytical profile index
COD	Chemical oxygen demand
C:N	Carbon:Nitrogen
C:P	Carbon:Phosphorus
DDT	Dichlorodiphenyltrichloroethane
DO	Dissolved oxygen
DEP	Diethyl phosphate
ECD	Electron capture detector
ETPI	Environmental technology program for industry
E.U.	European Union
FAO	Food and Agriculture Organization
FEN	Fenamiphos
FSH	Follicle stimulating hormone
GC	Gas chromatography
GC-MS	Gas chromatography-Mass spectrometry
JICA	Japan International Cooperation Agency
LC_{50}	Lethal concentration
LH	Leutinizing hormone
LC-MS/MS	Liquid chromatography–mass spectrometry/ mass spectrometry

MSM	Mineral salt medium
HPLC	High performance liquid chromatography
MR-VP	Methyl Red –Voges Proskauer
MCLs	Maximum contamination levels
MRL	Maximum residue limit
OD	Optical density
OPs	Organophosphates
OPH	Organophosphate hydrolase
OPFRs	Organophosphorus flame retardants
PARC	Pakistan Agriculture Research Council
PNP	Paranitrophenol
PPSGDP	Private sector groundwater development project
SPME	Solid phase microextraction
UNDP	United Nations Development Program
WHO	World Health Organization

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ABSTRACT

The objective of the research was to isolate dichlorvos (2,2-dichlorovinyl dimethyl phosphate) degrading strain of *Pseudomonas* sp. The bacterial culture isolated from soil was acclimatized and it was identified through morphological, physical and biochemical characterization. Characterization was performed upto species level using analytical profile index (API), where the isolated strain was identified as *Pseudomonas aeruginosa*. Degradation of dichlorvos by *Pseudomonas aeruginosa* was studied at different pH, temperature and at different dichlorvos concentrations of 100, 300 and 500 mg L^{-1}. The decrease in organic matter was measured after every 24 h, indirectly by Chemical Oxygen Demand (COD) while the degradation metabolites of dichlorvos were analyzed by GC-MS. The results showed that maximum degradation was achieved for 100 mg L^{-1} of dichlorvos after 120 h of incubation period at pH 7 and 30°C, whereas the COD removal was 80, 65 and 53% at varying pH while at varying temperature it was 86, 67 and 59% for 100, 300 and 500 mg/L, respectively. Quantitative analysis through gas chromatography showed percent degradation of 85, 64 and 60% at pH 7 for 100, 300 and 500 mg/L of dichlorvos, whereas at 30°C, the maximum degradation observed was 91, 73 and 68 %, respectively. The degradation metabolites of dichlorvos extracted with n-hexane were studied using retention time and fragmentation pattern by GC-MS analysis. Dimethylphosphate and dimethylmonochlorvos were major degradation by-products with m/z value of 109 and 185 respectively, indicating a 90% degradation rate.

Chapter1

INTRODUCTION

1.1 Background

According to estimates 2.5 million tons of pesticides are being used worldwide annually and their application keeps on increasing with the time (Pimentel, 1995 ; FAO, 2002) causing approximately three million poisoning cases and 200,000 deaths each year around the world where majority of them belongs to the developing countries (WHO, 1990 ; FAO, 2002). The dilemma is aggravated because of the pesticides, which developed countries have banned due to their toxic effects but are constantly being used in the developing countries such as Pakistan (Wilson and Tisdell, 2001 ; Sankararama Krishnan *et al.,* 2005). Improper use, lack of awareness and improper storage of pesticides has caused ground water and soil contamination in more than forty districts of Punjab only (Khan *et al.,* 2010).

The degradation of pesticides in the environment takes place by a variety of mechanisms, but still the parent compounds and their metabolites persist long enough to adversely impact upon soils and groundwater. Residues are not restricted to areas where they are applied and are transported by water and air, they may occur in small quantities in several rivers, and even in dust carried by the air. As a consequence, the contamination of groundwater by agrochemicals has received considerable attention (Iwamoto and Nasu, 2001). Overuse of pesticides and subsequent pollution is not only affecting human health but the ecological environment as well. It was reported by Tariq *et al*., (2007) that about 80% population of many bird species has declined or migrated to somewhere else due to the repellent smell of pesticides in Kasur, Bahawalnagar, Multan and Muzaffargarh.

Bioremediation is a managed and spontaneous process which involves the use of microbes to detoxify and degrade environmental contaminants. It has received increasing attention as an effective biotechnological approach to clean up a polluted environment in

comparison to “conventional” remediation technologies, such as excavation and disposal in landfills, excavation and thermal treatment, and pump and treat. In general, the approaches to bioremediation are environmental modification, such as through nutrient application and aeration, and the addition of appropriate degraders by seeding. Bioremediation offers several advantages over the conventional chemical and physical treatment technologies, especially for diluted and widely spread contaminants through environmental modification, such as nutrient application and aeration, and the addition of appropriate degraders by seeding. In-situ bioremediation enables us to remediate a contaminated site without transportation of contaminants and with minimum site disruption. Manufacturing and industrial use of the site can continue while the bioremediation process is being implemented (Stempvoort and Biggar, 2008).

Presence of a hydrocarbon-degrading microbial population, the chemical composition of the oil/petroleum source, availability of oxygen or other electron acceptors, nutrient supply, presence of toxic or inhibiting chemicals, and characteristics of the soil/geological conditions are the few factors which affect biodegradation process. Due to the enormous number of genera of microorganisms, numerous enzymatic pathways have been developed to metabolize pesticides. Microbial adaptation to degrade pesticides was recognized soon after their introduction into markets with the pioneer research on the biodegradation of 2,4-D but its aims were mainly academic (Arbeli and Fuentes, 2007). Microbial bioremediation of pesticide-contaminated sites is considered a safer, more efficient, and less expensive alternative to physical and chemical methods for pollution abatement and it has been recognized as the most important process controlling the dissipation of OPs in soil (Singh and Walker, 2006 ; Chanika *et al.,* 2011).

The microbial populations of soil or aquatic environments are composed of diverse, synergistic or antagonistic communities rather than a single strain. In the natural environments, biodegradation involves transferring the substrates and products within a well-coordinated microbial community, a process referred to as metabolic cooperation (Abraham *et al*., 2002).

Organophosphates make a major and mainly used group of pesticides currently being used in Pakistan. Although organophosphates are biodegradable in nature, their residues are found in the environment so research on their biodegradation is being carried all over the world (Kanekar *et al.,* 2003).To date not many scientific studies have been made related to pesticide monitoring and their decontamination except few basic researches related to their biodegradation but those too lack the engineering prospect of degradation. Keeping in view the shortcomings in this regard, this study is conducted to investigate the biodegradation of organophosphate pesticides using dichlorvos as representative pesticide. Dichlorvos has been identified in water and soil samples in Pakistan in various studies and its residues have been found in surface and ground water, in soil as well as in food residues (Tariq *et al.,* 2007). Indigenous soil culture was used to study mineralization of dichlorvos at various environmental factors such as its concentration, pH and temperature.

1.2 Objectives of Study

Bioremediation technology to treat hazardous waste has gained considerable attention as it is ecologically sound and economical as compared to other technologies and has been attempted successfully in many countries of the world (Hashmi *et al.,* 2006). It has been defined as a process that uses microbial catalysis, green plants or their enzymes to treat the polluted sites for regaining their original condition by acting on pollutant compounds, thereby remedying or eliminating environmental contamination (Stempvoort and Biggar, 2008 ; Getenga *et al.,* 2000).

The principal biological route is the microbial consumption of the pesticide as an energy, carbon and/or nitrogen source (Getenga *et al.,* 2000; Hashmi et *al.,* 2003). The acceleration of microbial turnover of chemical pollutants generally depends on the supply of carbon, nutrients temperature, available oxygen, soil pH, redox potential, and the type and concentration of organic pollutant itself (Carberry and Wik, 2001 ; Megharaj *et al.,* 2011). Microbial growth is highly dependent on abiotic factors as pH and temperature. An extreme change in temperature i.e. high temperature causes inactivation of necessary enzymatic systems of the cell while low temperature leads to poor growth (Tortora *et al.,*

2010). Similarly, variations in pH harms microorganisms by disrupting the plasma membrane or by inhibiting the membrane transport proteins (Prescott *et al.*, 2003).

The experimental study has been designed to evaluate the degradation potential of microorganism for various concentrations of dichlorvos in nutrient broth so that it can be used at a contaminated site in future. The concentration of the target pesticide, pH and temperature are optimized for achieving maximum microbial growth corresponding to subsequent degradation during the process.

The specific objectives of this study were:

1. Isolation, identification and chemical characterization of indigenous soil microorganism having dichlorvos degradation potential, using the enrichment technique.
2. Investigate the degradation potential of dichlorvos using orbital shaker technique.
3. Evaluation of degradation potential of microorganism through quantitative analysis using gas chromatography and gas chromatography mass spectrometry, for various concentrations of dichlorvos, under different pH and temperature.

The research work is a continuation of the organophosphate biodegradation studies in developing remediation methodologies by optimizing different abiotic factors.

Chapter 2

LITERATURE REVIEW

The use of agrochemicals in Pakistan started in mid-fifties with the import of 254 metric tons of formulation in 1954. At present, more than 108 types of insecticides, 30 types of fungicides, 39 types of weedicides, 5 types of acaricides, and 6 different types of rodenticides are being used in Pakistan (PPSGDP, 2002). The majority of these are used for cash crops where 80% of total pesticides are now being used on cotton plants, whereas the import of pesticides is increasing gradually every year by 6% (Economic Survey of Pakistan, 2005–2006). In Punjab and Sindh Provinces, it is estimated that 5000 tons of obsolete pesticides and 3000 tons of contaminated materials are stored in 1900 warehouses located in a thickly populated area, and residents have complained of foul smell, strange taste in the water and recurring headaches for some time (JICA, 1999).

High dependence on pesticides by untrained farmers has increased health hazards and polluted the rural environment. Pesticide use poses a threat to farmers, children, and women workers in fields who are at high risk of being poisoned (UNDP, 2001; Khooharo, 2008). It is recognized that the soil is also a potential pathway of pesticide transport to contaminate water, air, plants, food and ultimately in the human via, runoff and subsurface drainage; interflow and leaching; and the transfer of mineral nutrients and pesticides from soils into the plants and animals that constitute the human food chain (Abrahams, 2002). The sources of contamination are closely related to human activities, such as domestic and industrial discharges, agricultural chemical applications and soil erosion due to deforestation (Bhattacharya *et al*., 2003).

2.1 Pesticide Contamination

Numerous pesticide companies have entered the pesticide market in Pakistan due to windfall profits and liberal policies of the government for import and registration of pesticides since 1980. Ever-since the shift of pesticide import and sale to private sector, over reliance on synthetic pesticides for crop protection has increased (Khooharo, 2008). Overuse and improper storage of agriculture chemicals have not only disturbed the agro-

ecosystem by killing the non-target and environment friendly organisms but have also caused contamination in soil and water. Pesticide poisoning cases have been reported all over Pakistan where as many as 10,000 farmers are poisoned annually by indiscriminate use of pesticides in only cotton growing areas of Pakistan (PARC, 1999).

To increase crop yield and to control pest infestation, indiscriminate and intensive use of even the long persistence pesticides is in practice which are endangering animal and human health through contamination in food stuff and environment. A number of organophosphates, carbamates and organochlorines residues were found in food samples including cereals, vegetables, pulses and fruits in various cities like Jhelum, Bahawalpur, Gujrat, Mardan, Charsaddah and Quetta. Even the dairy products like mutton, beef, milk, butter and cheese were found contaminated by DDT and methyl parathion. Only in Karachi, results showed an alarming level of 72 % pesticide residual contamination and maximum residue limit (MRL) violation of 35 % in 148 samples of fruits and vegetables (Zia *et al*., 2009).

Khan *et al*., (2010) reported extensively the overall use of pesticides in Pakistan and the practices which are leading to increased pollution levels due to pesticides. Farmer's poor knowledge about pesticide application, lack of awareness about integrated pest management (IPM), popularized and indiscriminate use of pesticide as a result of government pesticide oriented policies, use of obsolete and banned pesticides, improper storage and reuse of empty pesticide containers for purposes other than pesticides have been regarded as the main causes of pesticide pollution in agroecosystem.

Ahad *et al*., (2006) detected pesticide residues in Rawal Lake, Islamabad through GC-ECD. Among the pesticides identified, monocrotophos (4.3 µg/L), parathion (0.38 µg/L), azinophos (3.3 µg/L) and α-cypermethrin (5.82 µg/L) formed the major group with detection frequency ranging from 8-44%. Another study on Rawal Lake and Simly Lake by Iram *et al*., 2009 revealed the presence of residues of 21 pesticides belonging to the group of organophosphates, organochlorines and pyrethroids, and many of them exceeded the standard of European Union for drinking water.

Similarly, ground water samples collected from different areas of Bahwalnagar, Muzafargarh, D.G. Khan and Rajan Pur districts of Punjab were analysed for bifenthrin, E-cyhalothrin, carbofuran, endosulfan, methyl parathion and monocrotophos with 13.5, 5.4, 59.4, 8, 5.4 and 35.1% detection percentage, respectively. Few of them reached the maximum contamination levels (MCLs) established by the U.S. Environmental Protection Agency (USEPA) for drinking water (Tariq *et al*., 2004).

In a very recent study conducted by Anwar *et al*., (2012), pesticide residues were found in soil samples collected from cotton growing areas of Nawabshah district, Sindh. All the samples analysed were found contaminated with used pesticides i.e. dichlorvos, methyl parathion, endosulfan and chlorpyriphos with varying degree of concentration and frequency.

2.2 Dichlorvos

Organophosphates, carbamates and pyrethroid are the major classes of insecticides used in Pakistan. Organophosphates (OPs) comprise a broad and heterogeneous group of chemical compounds in terms of organic substituents, vapour pressures, polarities and industrial applications (Garcia *et al.*, 2010). Over 6,986 tons of OP pesticides are applied in Pakistan annually (ETPI, 2001). Due to their extensive application, dichlorvos is selected as a representative pesticide for biodegradation studies and its physical and chemical properties are given in Table 2.1.

Table 2.1: Physical and chemical properties of dichlorvos

Molecular formula	$C_4H_7Cl_2O_4P$
Relative molecular mass	220.98
Common name	DDVP, dichlorvos
Physical appearance	Colorless to amber liquid
Melting point	1000 mg/L
Boiling point	180°C
Aqueous solubility	10mg/mL at 20°C
Non-aqueous solubility	Dichloromethane, acetone, n-hexane
Half-life in aqueous solution	61.5 days at 20°C

Dichlorvos (O,O-dimethyl-2,2-dichlorovinyl phosphate) is currently recognized as an efficient broad-spectrum organophosphorus pesticide with medium toxicity (Sun *et al*., 2009; Chanika *et al*., 2011). It is being used extensively in many countries for controlling insects on agricultural, commercial, domestic, and industrial sites (Gan *et al*., 2006). It is effective against aphids, cater- pillars, spider mites, thrips, and white flies in greenhouses and outdoor fruit and vegetable crops and bedbugs (Yarsan and Cakir, 2006). Dichlorvos is generally preferred because of its cost-effectiveness and wide range of bioactivity. Its annual worldwide sales in 2003 were about 40 million U.S. dollars (Zhang *et al*., 2008) and its usage is expected to increase since highly toxic organophosphate insecticides (e.g., parathion) are being banned gradually.

Public exposure to dichlorvos may occur via air, water, or food as it is readily absorbed through all routes of exposure (Raheja and Gill, 2002; Dirican *et al*., 2012). The LC50 value shows that dichlorvos has a high acute toxicity (U.S. Environmental Protection Agency, 2006). To reduce its potential threat to human beings via drinking water,

drinking water quality standards have been set by various states or organizations and the European Union (E.U.) drinking water standards regulate an MCL for any particular pesticide at 0.1 mg/L (E.U, 2007). Its residues have been reported in a wide variety of fruits, vegetables as well as in ground water and soil samples in different areas of Pakistan.

Adsorption to soil and volatilization of dichlorvos do not occur significantly. Biodegradation has proven to be important in eutrophic system (Hua *et al.*, 2006). Dichlorvos degrades in the atmosphere by vapor- phase reactions with hydroxyl radical and possibly ozone. Dichlorvos is hydrolyzed into dichlorethanol, dichloroacetaldehyde, dichloracetic acid, dimethyl phosphate and dimethyl phosphoric acid. The hydrolysis reaction is sensitive to pH and temperature. The half-life in the atmosphere is as low as 2 d and as high as 320 d where direct photolysis of dichlorvos in the atmosphere does not occur (Schramm and Hua, 2001).

Because, dichlorvos does not readily sorb to soil particles, spills, or other large amounts of the pesticide, especially when released in a liquid form or dissolved in solvent carriers, it may migrate through soil profiles or sediments and into groundwater showing little tendency to bioconcentrate in living tissues. Microorganisms found in sewage sludges apparently can biodegrade dichlorvos, but a period of acclimation may be needed and the rate of biodegradation may be much less than that from abiotic transformation processes.

2.3 Health Hazards of Dichlorvos

Studies have proved that extensive use of pesticides and insecticides brings high risks of severe health problems to human beings attributed to the presence of their residues in agriculture products and water. Along with occupational poisoning in pesticide production and agricultural application, toxic instances of acute organophosphate poisoning also include suicide, homicide, and accidental overdose (Kose *et al.*, 2010). The chronic poisoning due to pesticide can cause adverse immune functions, peripheral neuropathies and allergic reactions, particularly of skin. The acute poisoning may vary from skin irritation to complex systematic illness resulting in death. Accidental exposure

in homes from inappropriate storage of pesticides and poisoning caused due to the use of empty container of pesticides for carrying water, are quite common (Yasmin, 2003). The pesticide residual toxicity in neurotic and pathological pathways is exhibited in the form of eye pain, abdominal pain, convulsions, respiratory failure, paralysis and even death (Liu *et al.*, 2008).

The acute toxicological effects of organophosphates are achieved by inhibiting acetylcholinesterase (AChE), resulting in acetylcholine accumulation. Acetylcholinesterase is a degradative enzyme of acetylcholine and it is essential for normal nerve impulse transmission by the termination of cholinergic response (Cycon *et al.,* 2009). The inhibition of this enzyme causes over-stimulation of nicotinic and muscarinic acethylcholine receptors and without medical treatment the level of enzyme activity return to normal only after several days, weeks or even months. The clinical symptoms of mild poisoning include fatigue, headache, giddiness, blurred vision, weakness, nausea, cramps, convulsions, discomfort in breathing and dizziness. In severe cases there are risks of unconsciousness, severe constriction of pupils and muscle twitching ultimately resulting in death.

The typical symptoms of poisoning are agitation, muscle weakness, muscle fasciculations, miosis, hypersalivation, sweating. Severe poisonings may cause respiratory failure, unconsciousness, confusion, convulsions and/or death (Sogorb and Vilanova, 2002). In a study conducted by Azmi *et al*., (2009) people living in Multan were affected by dyspnea, cyanosis, vomiting, backache and burning sensation in urine with elevated levels of enzymes. It has been reported that women working in cotton field as compared to men, sustain a variety of symptoms such as skin irritation (27%), headache (26%), nausea (12%) and gastroenteritis (10%). The health problems are further aggravated by the fact when certain varieties of cotton bloom late are being sprayed, the women picking cotton in the adjacent fields are badly affected (Rizwan *et al*., 2005).

OPs exert other toxic effect on the central and peripheral nervous system. This toxic effect is called ‘organophosphorus induced delayed neuropathy’ and it is related to

phosphorylation and further modification ‘aging’ of an esterase of the nervous system called ‘neuropathy target esterase’. The symptoms of this neuropathy (paralysis and ataxia) appear between 14 and 24 days after the poisoning (Sogorb and Vilanova, 2002).

Dichlorvos is highly toxic by inhalation, dermal absorption, and ingestion. Because dichlorvos is volatile, inhalation is the most common route of exposure. As with all organophosphates, dichlorvos is readily absorbed through the skin. Acute illness from dichlorvos is limited to the effects of cholinesterase inhibition. Compared to poisoning by other organophosphates, dichlorvos causes a more rapid onset of symptoms, which is often followed by a similarly rapid recovery (Extoxnet, 1996).

Dichlorvos not only inhibits AChE but also affects other organs and systems such as respiratory system, reproductive system (Okamura *et al*., 2005; Oral *et al*., 2006), and liver (Ogutcu *et al*., 2008). In a research study conducted on rats, dichlorvos showed subacute and subchronic reproductive toxicity in male rats where body and testis weights, sperm morphology, follicle stimulating hormone (FSH), leutinizing hormone (LH) and testosterone levels were decreased significantly at the end of 4th and 7th weeks in the exposed groups (Dirican and Kalender, 2012).

Similarly in another study conducted on rats, the most common change of dichlorvos poisoning damage to liver was small lipid-like structures that could be seen throughout the liver structure. In kidney, dense bodies were seen. The most significant changes in lungs were loss of lamellar structure of lamellar bodies in type II alveolar epithelial cell (Na-Na *et al.,* 2010). Other studies provide an evidence of liver dysfunction after chronic exposure to dichlorvos (Binukumar *et al.,* 2010).

2.4 Treatment Techniques

It is now widely accepted and has been proved that pesticides use has improved food production through better growth and yield but their use has also caused many environmental problems (Hashmi *et al.,* 2006). .The contamination of soils, water and other matrices by pesticides is an environmental concern. Chemical, physical or

biological combination of techniques for remediation aims at reduction of pesticide concentration in soil or water or at a a contaminated site. The conventional techniques for remediation involve contaminated soil excavation, its transport to a storage area and application of incineration,thermal desorption or soil washing controlled with respect to mass,temperature,volume or other parameters.

Bioremediation can be stimulated with selective nutrients or fortified by bioaugmentation, but is subject to prevailing temperature, moisture and edaphic conditions by utilizing microbial activity stimulated in the rhizophere. Pesticide biodegradaion has been documented in a wide range of habitats, including soils, sediments, surface and ground waters and sewage sludges while almost all pesticides have been shown to be susceptible to one or more biotransformations. Environmental factors such as temperature, moisture, pH, which affect microbial activity in general also affect pesticide-degrading microorganisms. Decreasing temperature, soil water potential and pH tend to inhibit microbial activity but these affects in general are organism specific.

Remediation of polluted sites following the conventional engineering approaches based on physicochemical methods is both technically and economically challenging. Bioremediation that involves the capabilities of microorganisms in the removal of pollutants is the most promising, relatively efficient and cost-effective technology. As much as the diversity in sources and chemical complexities in organic pollutants exists, there is probably more diversity in microbial members and their capabilities to synthesize or degrade organic compounds (Ramakrishnan *et al*., 2010, 2011).

Microbiologists have now realized that natural microbial populations are much more diverse than those expected from the catalog of isolated microorganisms. This is also the case for pollutant-degrading microorganisms, implying that the natural environment harbors a wide range of unidentified pollutant-degrading microorganisms that have crucial roles in bioremediation (Watanabe, 2001). Microorganisms depends upon a wide array of variables and conditions, which often limit effective bioremediation, and might include oxygen and nutrient availability, pH, C:N ratio, presence, number and activity of organic contaminant degrading microorganisms, enzyme induction, temperature, toxic

levels of contaminants, presence of co-contaminants (determining added toxic effects or preferential degradation), and presence of terminal electron acceptors (Hickman and Reid, 2008).

A well-known example of bioremediation, which highlighted the usefulness of this treatment strategy and accelerated its development, was in the biological cleanup in the large accidental oil spill by the tanker Exxon Valdez in Alaska in March 1989. The accident spilled approximately 41,000 m^3 of crude oil and contaminated about 2,000 km of coastline. Bioremediation through nutrient addition was extensively used in coastal environments including beaches and marshes (Wright *et al*., 1997). Fertilizers were typically applied on the surfaces of sand and sediments contaminated with oil, but the application was not feasible for large areas of contamination because it required huge quantities of nutrients. The study of using fertilizers in one shoreline following the Valdez-spill resulted in a fivefold increase in oil degradation. Later Alaskan bio-augmentation projects suggest that commercially available fertilizers more effective than commercial bio-products (Zhong *et al*., 2009).

A test in Antarctic mineral soils showed promise after one year only in the fertilizer-treated plots (C:N 61:1, C:P 607:1), and the fertilized soils had the highest level of microbial activity relative to untreated plots (Margesin and Schinner, 2001).

In a recent experiment at a diesel fuel-contaminated site at Prudhoe Bay, Northern Alaska, with enhanced temperatures of 0.5–7.8 ◦C in the upper subsurface, the bioremediation activity was increased in that layer (Filler *et al.*, 2001). Contaminated media were land-farmed, amended with fertilizers, augmented with microbial products, and manipulated with engineered systems in Alaska (Filler *et al*., 2006).

Biofilter microcosms containing a mixture of straw, peat and soil and harboring micro-populations which use the herbicide linuron for growth were irrigated with linuron for 28 weeks with a stop in its supply between week 12 and 17. Matrix samples were regularly taken to assay linuron mineralization. A first-order approximation of the Monod model was used to simulate the observed mineralization data. Lag times in linuron mineralization decreased during the initial weeks of linuron irrigation but increased after

supply of linuron ceased. It was predicted that the population size decreased at a rate of 0.031 d^{-1} after pesticide supply ceased to reach its initial population size after 25 weeks (Sniegowski *et al.,* 2009).

2.5 Dichlovos Degradation

Bacterial degradation is one of the important transformation mechanisms for dichlorvos as it has low volatility, so a variety of microorganisms are capable of degrading it in different conditions. Most organophosphorus pesticides have similar general structure, containing three phosphoester linkages, and hydrolysis of one of the phosphor-ester bonds dramatically reduces the toxicity of the pesticides by eliminating their acetylcholinesterase inactivating properties (Horne *et al*., 2002).

Rate of dichlorvos degradation was evaluated by a natural microbial community on rape leaves and it was found that more dichlorvos was degraded on microbial population inhabited leaves than on surface sterilized leave. It was reported that microbial consortium degraded almost 55% of applied 400 mg/L concentration after incubation for 2 days at 30°C. Six dichlorvos degrading bacteria with 16S rRNA gene sequences that are most similar to those of members of the genera *Pseudomonas, Xanthomonas, Sphingomonas, Acidovorax, Agrobacterium and Chryseobacterium* were isolated from the natural community. It was reported for the first time that three of these epiphytic bacterial species, from the genera *Sphingomonas, Acidovorax* and *Chryseobacterium,* can degrade organophosphorus compounds (Ning *et al.,* 2010).

Naturally occurring soil bacteria have evolved the ability to degrade OPs with the help of an enzyme called organophosphate hydrolase (OPH). One approach is to exploit a microbial consortium with the required degradation pathways. A co-culture of engineered *E. coli* with ability to hydrolyze parathion and diethyl-thiophosphate and the natural para-nitrophenol (PNP) degrader *Pseudomonas putida* was used to successfully decompose parathion without accumulation of PNP (Gilbert *et al*., 2003)

A less complicated strategy utilizing either engineered *Moraxella sp.* or *Pseudomonas putida* that can naturally degrade PNP has been reported. In the former, OPH was

expressed on the cell surface and demonstrated to simultaneously degrade parathion, methyl parathion and paraoxon, and their hydrolysis product PNP (Mulchandani *et al*., 2006). In the later, a *P*. *putida* strain, containing a natural PNP degradation operon was used. A synthetic operon for expression of OPH, phosphodiesterase (Pde), and alkaline phosphatase (PhoA), was introduced to enable the hydrolysis of OPs and diethyl phosphate (DEP) mineralization. The resulting *P*. *putida* strain was able to completely degrade paraoxon, PNP, and DEP within 24 h, 78 h and 142 h, respectively (Singh *et al.*, 2008).

Cycon *et al*., (2009) suggested a strong correlation between microbial activity and chemical processes during diazinon degradation. An enrichment culture technique was used for the isolation of bacteria responsible for biodegradation of diazinon in soil. Three bacterial strains identified as *Serratia liquefaciens, Serratia marcescens* and *Pseudomonas* sp. were able to grow in mineral salt medium (MSM) supplemented with diazinon (50 mg/L) as a sole carbon source, and within 14 days, 80–92% of the initial dose of insecticide was degraded by the isolates and their consortium. Glucose addition in the medium accelerated the degradation of diazinon but it was attributed to the decrease of pH values, after glucose utilization. Studies on biodegradation in sterilized soil showed that isolates and their consortium exhibited efficient degradation of insecticide (100 mg/kg soil) with a rate constant of 0.032–0.085 d^{-1}.

Hashmi *et al.*, (2006), studied the degradation of methyl parathion by Pseudomonas strain isolated by enrichment technique. The degradation efficiency was evaluated by maintaining dissolved oxygen (DO) level at 4 mg/L. High performance liquid chromatography (HPLC) analysis showed 90 % malathion removal within 29 days with 78 % decrease in chemical oxygen demand (COD).

The observed bacterial utilization of malathion probably contributes greatly to the reduced persistence of malathion when employed as an insecticide to control mosquitoes. It is further anticipated that the present method, possibly slightly modified, could also be applied to several other pesticides possessing related chemical structures (Hashmi *et al*., 2003).

The lab scale research was conducted for remediation of a dichlorvos-contaminated soil through isolation of *Ochrobactrum sp.* Using dichlorvos as a sole carbon source, the isolated strain grew successfully at the optimal pH of 7 and temperature 30°C. In addition, the soil degradation test indicated that in soil spiked with 100 mg/L or 500 mg/L dichlorvos and inoculated with 0.5% or 1.0% (v/v) strain DDV-1, complete degradation of dichlorvos could be achieved in 24 h (Hua *et al.*, 2006).

Figure 2.1 shows the generalized biotic degradation pathway of dichlorvos as proposed in many scientific studies.

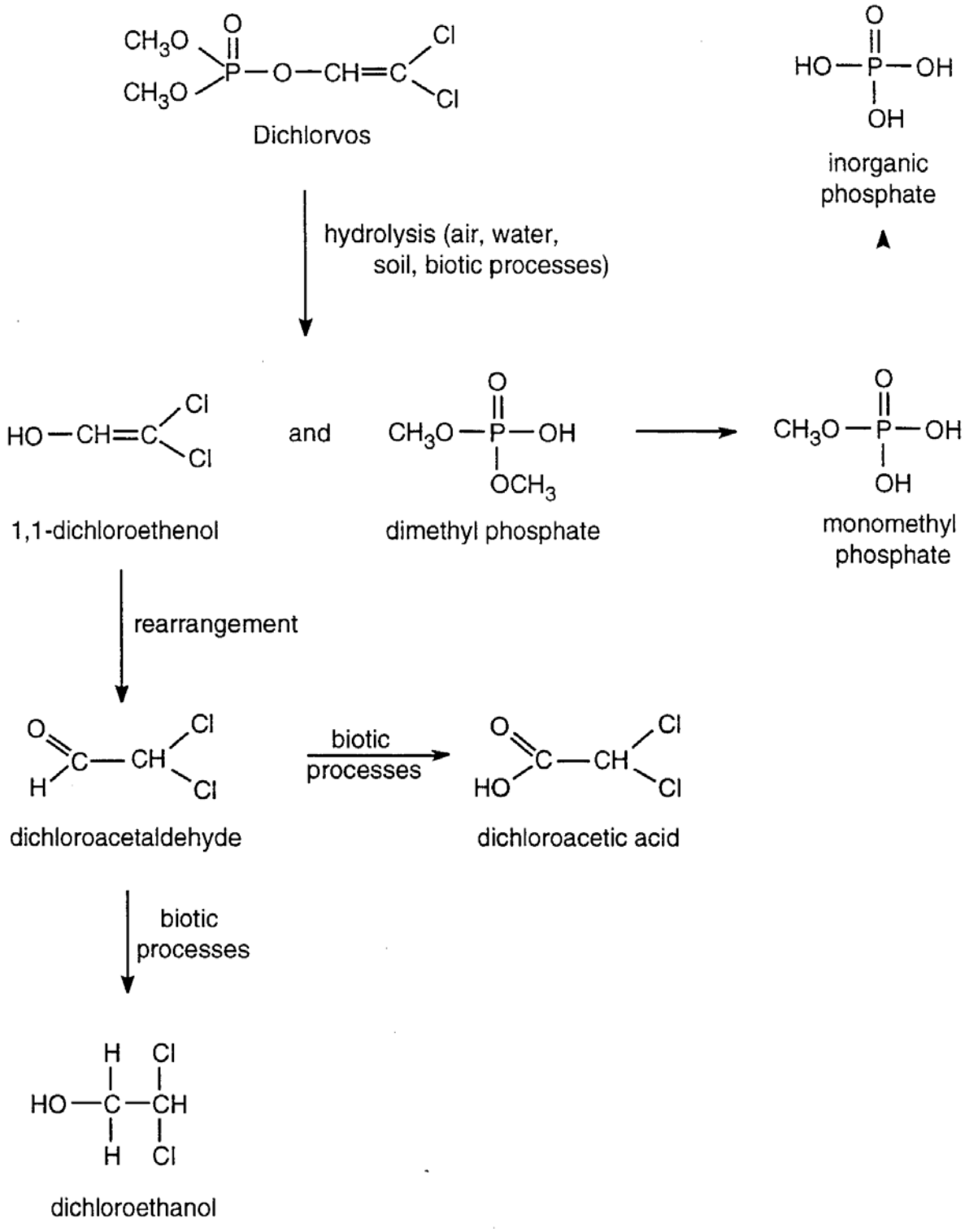

Figure 2.1: Biodegradation pathway of dichlorvos

Benimeli *et al*., (2007) investigated the lindane bioremediation abilities of *Streptomyces sp.* M7 in soil samples. A decrease of the residual lindane at a concentration of 100, 150, 200, and 300 µg/L was detected in soils samples in relation to the abiotic controls as 29.1, 78.03, 38.81 and 14.42%, respectively. The optimum *Streptomyces sp.* M7 inoculum was selected in sterile soil spiked with lindane 100 µg/L at a temperature of 30°C.

The susceptibility to bioremediation of the hydrocarbons contained in a waste from crude oil extraction was examined by Capelli *et al*., (2001). The total hydrocarbon content was reduced to 70% of its initial value through biodegradation after 45 days. Saturated and aromatic hydrocarbons were the most readily degraded fractions with, respectively, 70 and 60% of the fraction remaining at the end of the experiment. The substrate preferences of the natural population towards various fractions of the crude oil were determined by both the length of the lag phase and the slope of the exponential growth in a MSM containing either of the different hydrocarbon fraction as the sole source of carbon.

Two bacteria identified as *Pseudomonas putida* and *Acinetobacter rhizosphaerae* able to rapidly degrade the OP fenamiphos (FEN) were isolated being the dominant members of the enrichment culture. Both strains hydrolyzed FEN to fenamiphos phenol which was further transformed, only by P. *putida*. The two strains were using FEN as C and N source. Cross-feeding studies with other pesticides showed that *P. putida* degraded OPs with a P–O–C linkage. The same isolate exhibited high bioremediation potential against spillage-level concentrations of aged residues of FEN and its oxidized derivatives at 25°C at 150 rpm for a concentration of 200 mg/L, within 2 days (Chanika *et al*., 2011).

Organophosphates were detected by using liquid–liquid extraction and ultrasound-assisted solvent extraction followed by liquid chromatography coupled to tandem mass spectrometry detection (LC−MS/MS) in waste and surface water, as well as in sediment samples. Recoveries ranged from 63 to 94% in water and from 74 to 104% in sediment with estimated quantification limits between 2.6 and 7.9 ng/L in surface water, 4.1 and 13 ng/L in effluent waste water, and between 0.48 and 11 µg/Kg in sediment. The validated gas chromatographic method was also applied to determine the occurrence of the selected

organophosphorus flame retardants (OPFRs) and plasticizers in the aquatic environment (Carballo *et al.,* 2007).

Similarly gas chromatography (GC) coupled with electron capture detector (ECD) was used for pesticide detection in vegetable samples through extraction into an aqueous solution with the aid of microwave irradiation and then directly onto the SPME fiber in headspace. After being collected on to the SPME fiber and desorbed in the GC injection port, the pesticide (dichlorvos) was analyzed with a GC–electron-capture detection system. Experimental results indicated that the proposed gas chromatographic technique attained the best extraction efficiency of 106% recovery under the optimized conditions, i.e. irradiation of extraction solution (10% aqueous ethylene glycol) at pH 5.0 with medium microwave power for 10 min (Chen *et al.,* 2002).

A study was conducted from 2002 to 2003 to determine the levels of selected organochlorine and organophosphate pesticides in the Selangor River in Malaysia. A liquid–liquid extraction followed by gas chromatography–mass spectrometry technique was used to determine the trace levels of these pesticide residues in surface water samples (Leong *et al.,* 2007).

Chapter 3

MATERIALS AND METHODS

Lab scale biodegradation studies of dichlorvos were carried out in the Environmental Microbiology and Biotechnology Research Laboratory at Institute of Environmental Science and Engineering, National University of Sciences and Technology, Islamabad, Pakistan.

The biodegradation studies were conducted by using indigenous soil bacterial culture in orbital shaker. The growth increase of microorganisms is helpful in determining the decrease in organic matter thereby corresponding to degradation of target pesticide through its utilization. The concentration of dichlorvos, pH and temperature were studied as these affect the microbial growth leading to breakdown of pesticide. The degradation rates were analysed quantitatively using gas chromatography

3.1 Chemicals and Pesticide

The organophosphate pesticide used in this study was purchased from Awan Seed Center, Rawalpindi. Dichlorvos (50 EC) used was a commercial grade sample so as to maintain the field conditions to which microorganisms are exposed. However the standard HPLC grade analyte for gas chromatographic analysis was purchased from Merck, Germany. The n-hexane for GC extractions was also of analytical grade. The API 20E kit was used for biochemical identification (bioMerieux, Canada).

3.2 Concentration of Dichlorvos

Different concentrations of dichlorvos were used in nutrient broth for growth studies and to determine its biodegradation relevant to utilization potential of *Pseudomonas sp*. Concentrations of 100, 300, 500 and 700 mg/L were used in nutrient broth during degradation studies.

3.3 Preparation of Culture Media

3.3.1 Glassware Sterilization

For all experiments, all glassware was dipped into solution of 95% chromic acid for 24 hours, then it was thoroughly washed with tap water and finally rinsed minimum of three times by distilled water. For sterilization, they were wrapped in aluminum foil and autoclaved at 121°C and 15 lb/cm^2 pressure for 15 minutes. The sterilized glassware was then dried in oven at 171°C for two hours before use (Prescott *et al.,* 2003).

For gas chromatographic analysis, all glassware was sterilized just prior to sampling. The glassware was washed with phosphate free liquid detergent, then with chromic acid and after thorough rinsing with tap water and later on distilled water; they were dried in oven at 200°C for 30 minutes followed by rinsing with n-hexane.

3.3.2 Preparation of Medium

Nutrient agar and Nutrient broth (Oxiom) were prepared by mixing 8 gm and 27 gm of nutrient agar and nutrient broth, respectively, in I L of distilled water according to manufacturer's instruction and autoclaved at 121°C for 15 minutes. Nutrient broth medium after preparation was dispensed in 250 ml flasks for growth studies. Nutrient agar medium in 250 ml quantity was prepared in 500 ml conical flask for preparation of slants and media plates. These were autoclaved at 121°C and 15 lb/cm^2 pressure for 15 minutes. Nutrient agar plates and slants were prepared in sterile conditions of laminar flow hood. Sterility of media was checked for 48 hours by incubating them at 37°C and then used for the isolation of microorganism. Slants were used for preparation of inoculum to use in biodegradation studies (Hashmi *et al*., 2008).

3.4 Isolation of Dichlorvos Degrading Bacteria

3.4.1 Isolation of Bacteria

The *Pseudomonas* specie capable of degrading dichlorvos was isolated using soil enrichment technique. Wet un-sieved soil was collected from IESE Lawn in Islamabad and mixed with 250 ml of distilled water in 500 ml Erlenmeyer flask containing 5 mg/L

of dichlorvos. The flasks were incubated in an orbital shaker at 120 rpm for two weeks at ambient temperature ranged between 25-27°C. During experiment 5 mg/L of pesticide was added after every 24 hrs. Similarly, after every 24 hrs one loopful of enrichment culture from the flask was streaked on nutrient agar supplemented with 5 mg/L of dichlorvos and incubated at 37°C for 24-48 hrs. Individual colonies were sub-cultured on to nutrient agar.

3.4.2 Purification and Enrichment of Bacterial Culture

The isolated bacterium was purified by repeated streaking on nutrient agar plates. Growth was obtained after 24 hours by incubation at 37°C and culture was maintained at low temperature. The bacterial culture was sub cultured after every one week. Figure 3.1 shows the purification and identification of degrading bacterial culture.

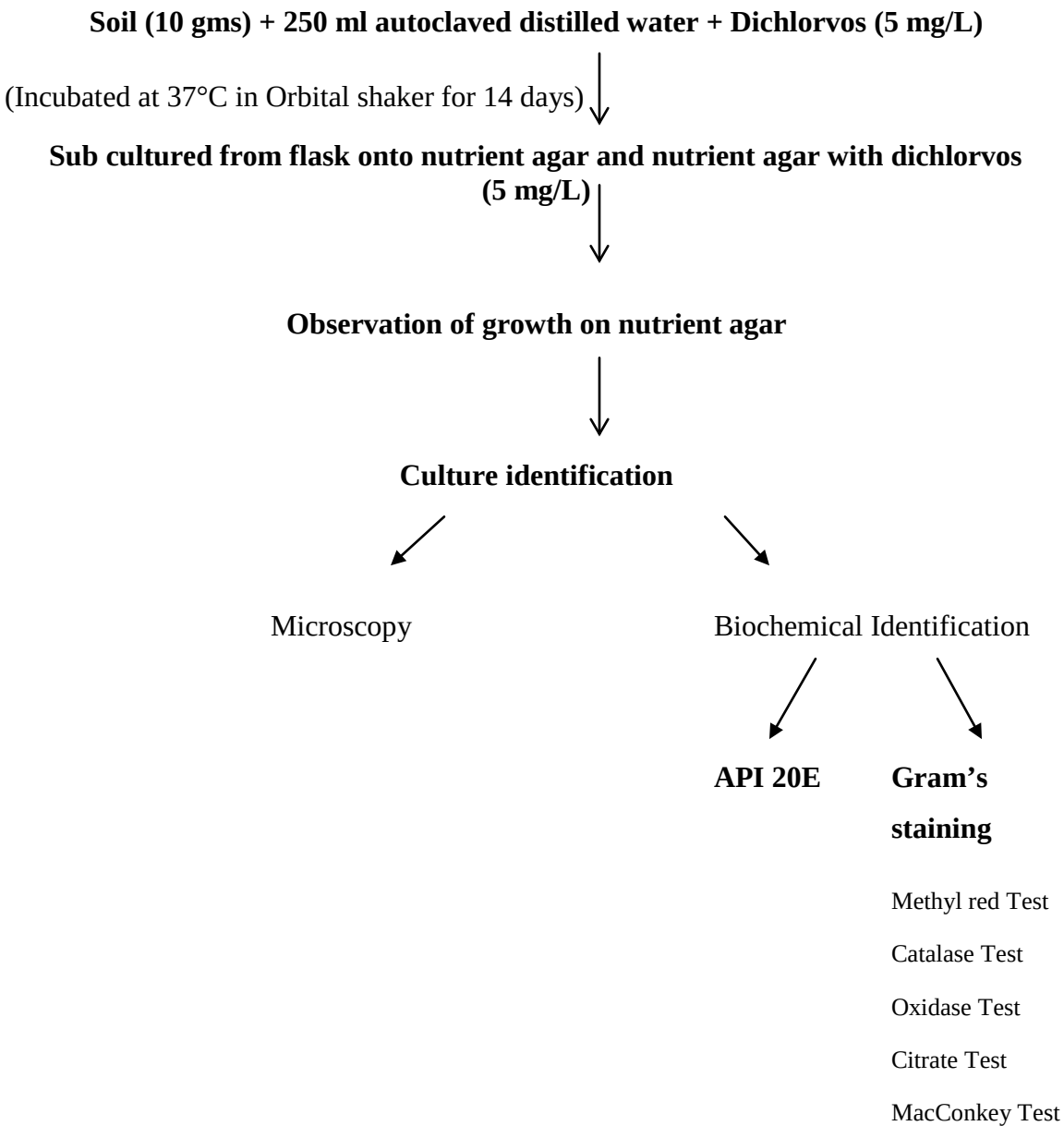

Figure 3.1: Schematics of isolation and identification of dichlorvos degrading bacterial culture

3.5 Identification and Characterization of Isolated Strain

Identification and characterization of isolated strain was done up to the specie level using Gram's staining, biochemical test (Collins and Lyne, 1985) and API 20E system.

3.5.1 Morphological Characterization

Morphological characterization was observed through Gram's staining method under oil immersion objective of microscope. Nutrient agar medium in media plates was used to study the colonial characters of bacterial culture.

3.5.2 Biochemical Characterization

Biochemical characterization and identification was conducted using different standard biochemical tests and API 20E identification kit as given below.

3.5.2.1 Methyl Red Test

Out of two MR-VP broths in sterile test tubes, one was inoculated with culture while other served as control. Both tubes were incubated at 37°C for 24-48 hours and after incubation a dropful of methyl red indicator was added to each broth. Non-acid formation gives yellow color after few minutes due to higher pH indicative of a negative test.

3.5.2.2 Citrate Utilization Test

Simmon's Citrate agar slants were streaked with the organism and incubated at 37°C for 24-48 hours. Change in color was observed for results of citrate utilization. Appearance of blue color shows citrate utilization as sole carbon source.

3.5.2.3 Catalase Test

Catalase test was performed by taking a loop full of 24-48 hours grown culture in a test tube. One drop of 3% H_2O_2 was dropped on it. Appearance of bubbles shows a positive reaction for catalase indicating evolution of oxygen.

3.5.2.4 Oxidase Test

A good amount of inoculum was taken from a plate culture and placed on a piece of filter paper. One drop of 1% N,N-dimethyl-p-phenylenediaminedihydrochloride solution was added. A blue or purple color formation after few seconds is indicative of oxidase positive showing the presence of enzyme cytochrome oxidase.

3.5.2.5 MacConkey Test

MacConkey agar slants were streaked with culture and incubated at 37°C for 24-48 hours. Appearance of pink colonies shows a positive result.

3.5.3 API 20E Identification System

API 20E system is used for identification of Gram negative bacterial cultures up to the specie level. It makes use of the labeled strips with twenty small cupules each responding to a biochemical test. The inoculum was distributed into test strips according to the manufacturer's instructions. After 24 hours incubation at 37°C API 20E strips were read. Biochemical reactions were read as positive or negative, translated into numerical profiles and interpreted with the software.

3.6 Biodegradation Studies

3.6.1 Preparation of inoculum for biodegradation studies

The inocula for the experiment of dichlorvos degradation study were prepared by growing acclimatized culture on nutrient agar slants, incubated at 37°C for 24 hours. The culture slants were washed with 10 ml sterilized phosphate buffer having pH 7 and used as inoculum for degradation studies. The turbidity of the culture was assessed against McFarland's Index (3×10^9 bacteria ml^{-1}) (Qiu *et al*., 2007).

3.6.2 Acclimitization of *Pseudomonas* sp. in nutrient broth using lower concentrations of dichlorvos

Early experiments were conducted with lower concentrations of pesticide to study the growth pattern of isolated *Pseudomonas* strain in relation to pesticide in nutrient broth.

Two weeks studies were conducted with initial concentration of 50 mg/L and subsequent streaking of acclimatized culture on nutrient agar medium plates having 50 mg/L concentration. All the experiments were conducted in duplicate.

3.6.3 Dichlorvos degradation in Orbital Shaker

The degradation potential of *Pseudomonas* for dichlorvos was performed using the orbital shaker. In 250 ml conical flasks, 200 ml nutrient broth with different concentrations of dichlorvos (100, 300, 500 and 700 mg/L) was inoculated with 10 ml of inoculum and incubated at orbital shaker at ambient temperature at 120 rpm. Control flasks with equal volumes of nutrient broth having bacterial culture but no pesticide was used with each set of experiments. Table 3.1 gives the technical data for orbital shaker.

Table 3.1 Technical data for Orbital Shaker

Orbital Shaker	**Labcon Spo-MP8**
Speed	120 rpm
Operating Temperature	Ambient
Volumetric load	250 ml
Retention time	120 Hours
Size of inoculum	10ml ($9X10^8$ CFU/mL)
Sample collection	24 hours

3.6.4 Sampling Procedure

17 ml sample was extracted from the experiment flasks after every 24 hours and filtered under sterilized conditions of laminar flow hood. 2 ml of these samples was diluted twenty times by pouring it in 38 ml of distilled water. The samples obtained were used for COD studies. In rest of the 15 ml samples, 2 to 3 drops of dilute H_2SO_4 were added to stop any further degradation process. Samples were then refrigerated.

3.7 Analytical Procedures

3.7.1 Optical density

The growth of isolated strain was expressed by measuring the optical density (OD) of nutrient broth containing the pesticide and the inoculum at 0 hr, 4 hrs, 8 hrs, 96 hrs and 120 hrs for each concentration of pesticide at 600 nm using the spectrophotometer (Spectronic Genesys 5). Nutrient broth was used as blank for calibration of instrument.

3.7.2 Chemical Oxygen demand

COD was determined by dichromate reflux, method as in Standard Methods (APHA, 2005). To perform COD of samples after every 24 hours, first the original sample was diluted twenty times and then 2.5 ml of sample was used for analysis in HACH-COD reactor for each concentration. In determination of COD, 3.5 ml of sulphuric acid reagent and 1.5 ml of potassium dichromate were used for digestion of the organic matter. Reading was obtained by titration against 0.1 M ferrous ammonium solution.

3.8 Quantitative Analysis

3.8.1 Gas Chromatography

A Shimadzu 2010 series gas chromatograph coupled with electron capture detector (ECD) was used for quantitative analysis. Chromatographic separation was achieved on a 30 m capillary column having 95% dimethyl polysiloxane, 5% diphenyl internal coating with an inner diameter of 0.32 mm and thickness of 0.5 µm. The splitless injection mode was used with a split ratio of 20. Helium was used as the carrier gas with a constant column flow of 2 ml/min. Linear velocity for the gas flow was 23.8 cm/sec while makeup flow was 30 mL/min.

3.8.2 Standard Dilutions Preparation

Dichlorvos dilutions were prepared by dissolving 142 µl of standard analyte in 100 ml of GC grade n-hexane. The subsequent dilutions of 0.1, 0.3, 0.5, 0.7 and 1 ppm for calibration curve were prepared from this stock solution.

3.8.3 GC Programming

Shimadzu GC 2010 with ECD was used for analysis. The separation column used was fused silica capillary column with 30 m length. Before running actual samples the instrument was optimized by altering various conditions such as injector temperature, column oven temperature, detector temperature, split ratio and column flow. 1 µl stock solution of each standard concentration was injected to check the signal and retention time of analyte. The final temperature program for maximum separation and resolution of solvent and analyte was developed and evaluated for signal resolution and noise level. Table 3.2 shows gas chromatographic conditions.

Table 3.2: Gas Chromatographic conditions

Parameters	Conditions
Column	95% dimethyl polysiloxane, 5%diphenyl (TRB-1), 30m,0.32mm,0.5µm
Carrier gas	Helium
Flow rate	2ml/min
Program	80°C for 1 min, 10°C/min-180°C, 180°C for 4 min.
Program run time	15 min
Injector temperature	180°C
Detector temperature	280°C

3.8.4 Gas Chromatography-Mass Spectrometry

GC–MS coupled technique was used for the separation and degradation study of commercial grade dichlorvos at ambient pH and temperature. Dynamic measurements were carried out in helium atmosphere. The analyses for detection of degradation metabolites were performed on a Shimadzu QP 5000 instrument, equipped with a DB 5-MS column (J & W Scientific) of 30 m length and 0.25 internal diameter, coated with 5% phenyl 95% methyl polysiloxane. The MS operated in electron ionization mode with a potential of 70 eV and the spectra were obtained at a scan range from *m*/*z* 50–450 (full scan mode). Separation of the by-products was conducted under the chromatographic conditions as given in table 3.3.

Table 3.3: Gas Chromatographic - Mass Spectrometric conditions

Parameters	Conditions
Column	5% phenyl 95% methylpolysiloxane (DB 5 MS), 30m,0.32mm,0.25 mm
Carrier gas	Helium
Flow rate	1ml/min
Program	55°C at 5°C/min to 200°C, 1°C/min-210°C for 2min, 270°C at 20°C/min for 3min.
Program run time	47 min
Injector temperature	240°C
Detector temperature	280°C

3.8.5 Liquid-Liquid Extraction

Liquid-liquid extraction, also known as solvent extraction and portioning, is a method to separate compounds based on their relative immiscibility in liquid, usually water and an organic solvent. The liquid containing the analyte is vigorously shaken with another liquid and is then allowed to settle till the separation of aqueous and non-aqueous layer.

The required layer containing analyte is removed separately. It is the extraction of a substance from one liquid to another using pear shaped separatory funnel.

The extraction procedure was carried out by liquid-liquid extraction method (USEPA, 1994). 100 ml separatory funnel was mounted on retort stand. Prior to use the funnel was sterilized using chromic acid, tap water, distilled water, dried in muffle furnace and finally rinsed with n-hexane after drying.

15 ml of nutrient broth to be extracted was transferred to the separatory funnel and 15 ml of n-hexane was added. After thorough mixing for 5 minutes, it was allowed to separate and settle. After 10 minutes the organic layer was removed and the process was repeated with aqueous layer by adding 10 ml of n-hexane. In the third extraction 5 ml of n-hexane was used. The organic solvent was then passed through hydrophilic syringe filters cartridges for removal of any water molecules left behind. The sample after extraction were kept in glass vials and stored at 4°C in refrigerator.

3.8.6 Sample Analysis

1 µl of the extracted samples were injected in to GC and GC-MS for analysis. The gas chromatogram showed the peaks of solvent and dichlorvos while mass spectrum showed the relative presence of dichlorvos degradation metabolites.

3.9 Experimental Design

To study the effect of concentration, experiments were performed using 200 ml nutrient broth inoculated with 10 ml inoculum and dichorvos with various concentrations i.e. 100, 300, 500 and 700 mg/L along with control for 96 hours. Optical density (OD) was measured after every 24 hours to check the performance of bacteria. The concentrations with significant increase in OD were selected for further experiments at varying pH and temperature. Experiments were conducted at 20, 25 and 30, and at pH 4, 7 and 9 to study the growth of *Pseudomonas* and degradation of dichlorvos. Media were prepared using buffers to examine the effect of pH. During each experiment COD was measured after every 24 hrs to determine the decrease in organic matter. All experiments were performed in duplicate and results were reported as their average. The samples were analyzed

through gas chromatography and gas chromatography-mass spectrometry after liquid-liquid extraction with n-hexane.

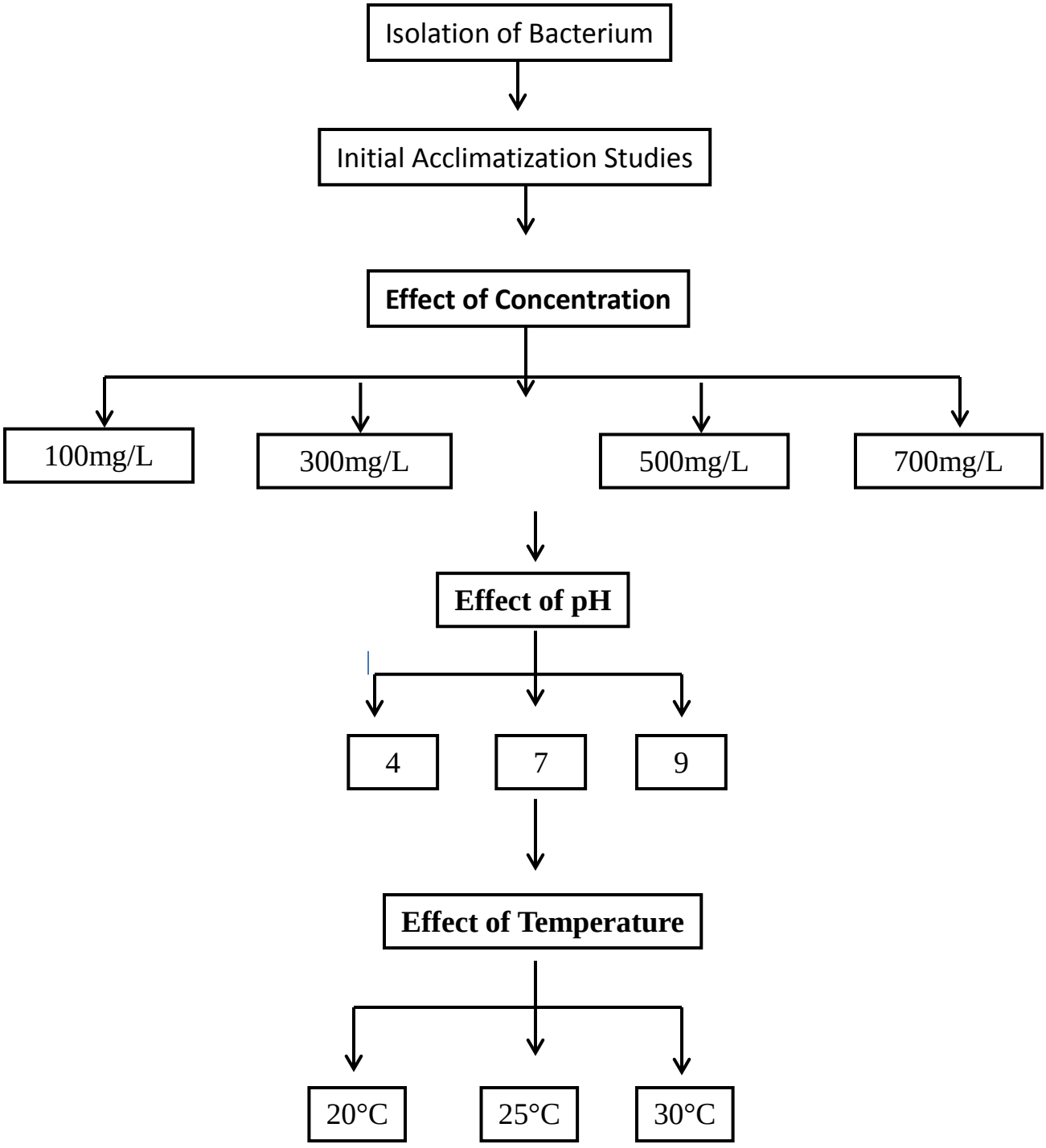

Fig 3.2: Flow sheet of Experimental design

Chapter 4

RESULTS AND DISCUSSION

Biodegradation studies were conducted to study the removal of dichlorvos as a model organophosphate, in nutrient broth medium, by *Pseudomonas* culture and to determine the influence of pesticide concentration, pH and temperature on bacterial growth and subsequent removal of organic matter in the medium.

4.1 Isolation of Dichlorvos Degrading Bacteria

Enrichment culture technique was used for the isolation of dichlorvos degrading bacteria from soil under sterilized conditions. Bacterial colonies were observed on nutrient agar with dichlorvos. Microscopic observations revealed these colonies as Gram negative.

4.2 Identification and Chemical Characterization of Bacterial Strain

In the present study Gram staining technique was carried out to study the morphological characteristics and MR, Citrate, Catalase, Oxidase and MacConkey tests were performed for chemical identification. On the basis of morphological, cultural and biochemical characteristics, the isolated strain was identified as a member of genus *Pseudomonas* according to "Bergeys Manual of Determinative Bacteriology" (1994) as shown in Annexure 1. The characteristics of *Pseudomonas* are listed in Table 4.1.

Table 4.1: Characteristics of *Pseudomonas* sp.

Tests	***Pseudomonas aeruginosa***
Shape	Thin short rods
Gram Stain	Gram negative
Growth on nutrient agar	Round, smooth and pale colour colonies
Motility	Motile
MR reaction	Negative
Citrate Utilization	Positive
Oxidase and Catalase	Positive
MacConkey	Positive

4.2.1 API 20E

API identified the strain to specie level. The strip results after as interpreted by software confirmed the strain to be *Pseudomonas aeruginosa.*

4.3 Bacterial Acclimitization to Dichlorvos

The isolated organism was designated as *Pseudomonas aeruginosa*, and its ability ti grow on dichlorvos was tested. Initial experiments were carried out to study the adaptation and acclimatization of bacterial culture in dichlorvos. Different low concentrations of dichlorvos (30, 50 and 80 mg/L) and control was used to study the growth of inoculated culture for two weeks. The *Pseudomonas* strain showed positive growth in the presence of dichlorvos.

After acclimatization the isolated strain was compared with pure culture of *Pseudomonas aeruginosa* to evaluate the acclimatization and subsequent degradation potential as shown in Figure 4.9.

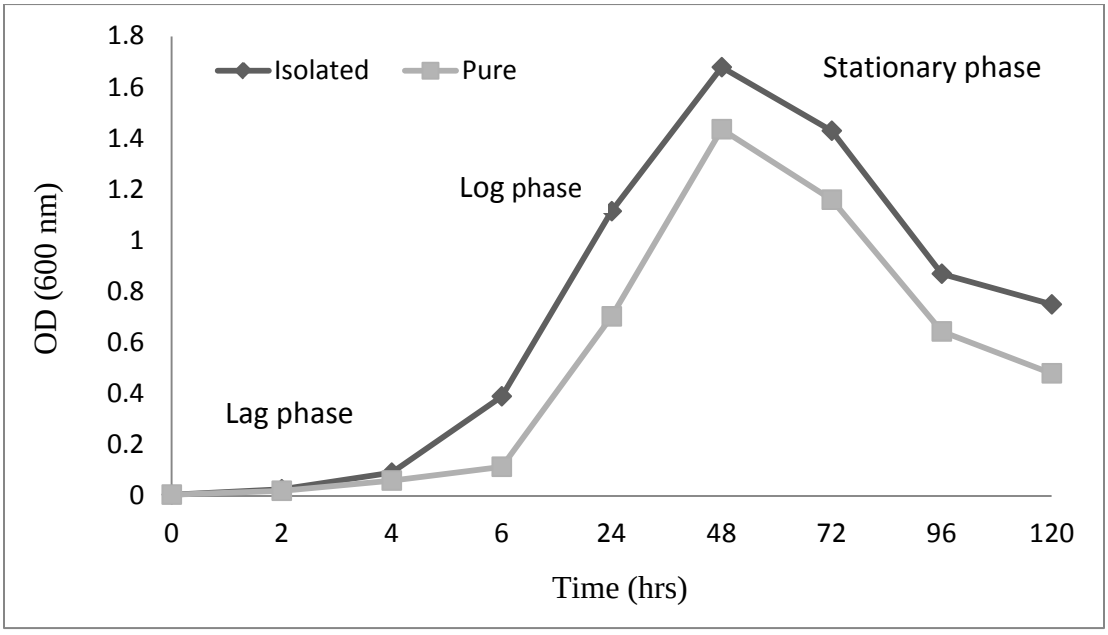

Figure 4.9: Acclimatization of *Pseudomonas* strain as compared to pure culture

The isolated specie was compared to its pure culture showing a lag phase of 4 hrs and entering and staying into log phase for a period of 44 hrs which extended over a period of 48 hrs, finally declining to stationary phase.

The results were found in accordance with work done by previous researchers. The study conducted by Jilani and Khan (2006) concentrated on biodegradation of cypermethrin using *Pseudomonas* strain and found out that with 40 and 60 mg/L of cypermethrin the culture showed a marked increase in viable counts for 24 hrs, indicating the utilization of the organic matter and degradation of pesticide.

Based on the results of present study, and the those of other researchers, it is concluded that *Pseudomonas aeruginosa* is able to grow in the presence dichlorvos.

4.4 Growth Studies using Orbital Shaker

4.4.1 Effect of different concentration of dichlorvos

In the present study growth for four different concentrations of dichlorvos in nutrient were observed by measuring the optical density at 600 nm. One set of control experiment, without pesticide, was also run in parallel, to study the bacterial growth curve. The growth curve produced for the control experiment showed initial acclimatization curve for 24 hrs with OD values of 1.115, followed by log phase for 50 hrs with OD value of 1.698 nm, which ends at stationary phase after 72 hrs with OD values of 1.43 ending at 0.7 nm at 120 hrs.

For 100, 300, 500 mg/L of dichlorvos concentration in nutrient broth, bacteria showed a significant increase in OD at 600 nm, which resulted due to increase in bacterial population. The growth curve for these three concentrations has a lag phase of 24 hrs and a log phase of 50 hrs which is similar to growth curve exhibited by control experiment. These results clearly indicated that the inoculated culture was well acclimatized to utilize dichlorvos.

Among 4 different concentrations of dichlorvos, very little increase in absorbance was observed in 700 mg/ L of dichlorvos concentration during 120 hrs which might be due to

increased level of toxicity at higher concentrations of dichlorvos hindering bacterial growth (Figure 4.10).

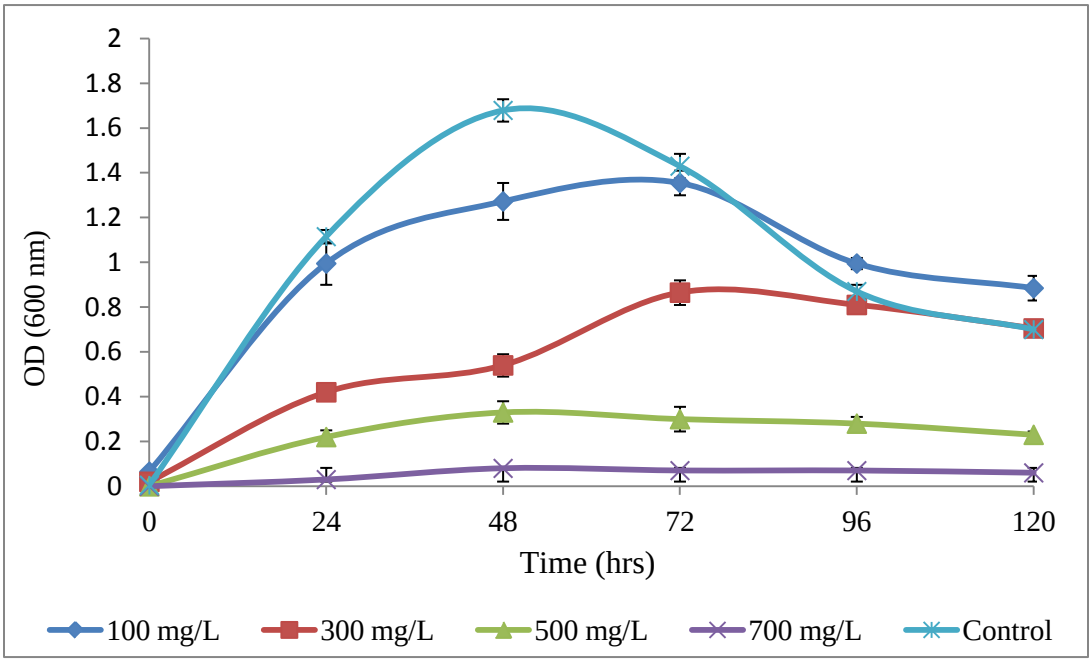

Figure 4.10: Growth of *Pseudomonas* at different concentrations of dichlorvos

4.4.2 Standardization of COD Technique

Hach COD reactor was calibrated using solution of potassium hydrogen phthalate as shown in Table 4.2. The removal of organic content was determined by measuring COD removal after every 24 hrs.

Table 4.2: Standardization of COD Technique

Dilution	COD (mg/L)
Original	512
1:100	32
1:200	16

Krishna *et al*., (2008) investigated the biodegradation of lindane, methyl parathion and carbofuran by various enriched bacterial isolates and used COD as an indirect measure of the mineralization of the compounds. Their results showed significant decrease in COD under aerobic and anaerobic conditions for breakdown of these pesticides indicating that COD can be taken as indirect measure of mineralization and pesticide degradation.

4.4.3 Effect of pH on COD removal

Microbial growth and activity are readily affected by pH, temperature and moisture. It is essential to achieve optimal environmental conditions for microbial growth as they cannot tolerate extreme environments in most cases. To explore the effect of pH, nutrient agar medium was adjusted separately to pH 4, 7 and 9. Control experiments were conducted under selected pH conditions. The removal of organic matter was indirectly measured through COD. It was found that bacteria can grow well at pH 7 and 9 as compared to pH 4. The results showed that maximum COD removal was achieved at neutral pH, followed by pH 9 and lowest removal at acidic pH (4). The COD percent reduction at pH value of 4 was 19 which increased to 69 percent after 120 hrs (Figure 4.11). At pH value of 9, the initial percent removal at 24 hrs was 29 which increased to almost 82 percent by the end of experiment. However, maximum COD percent reduction for control experiment was observed at pH value of 7, which ranged from 35 to 87 percent. It was concluded that *Pseudomonas aeruginosa* grew well at neutral pH giving maximum removal of organic matter.

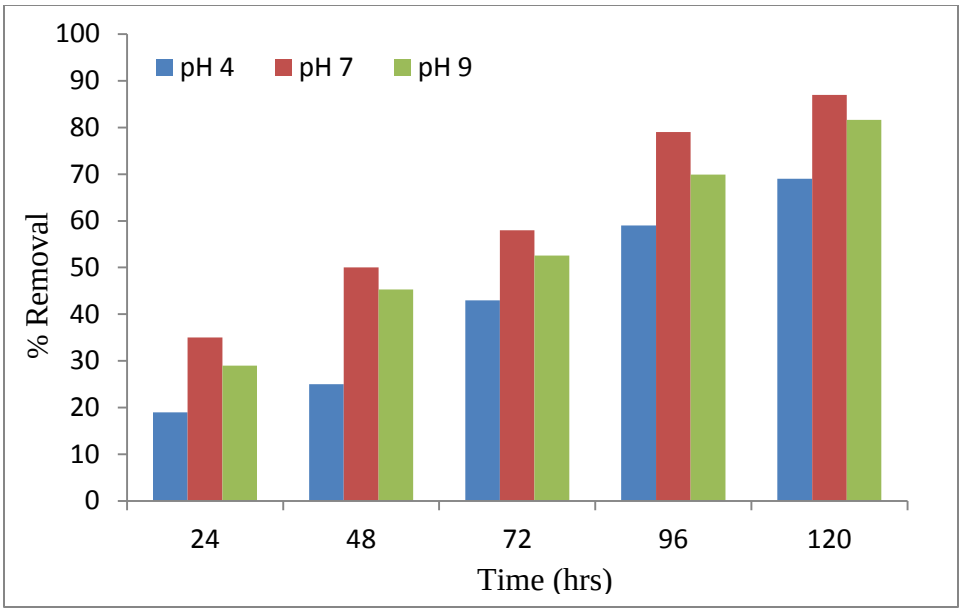

Figure 4.11: Effect of pH on percent reduction of COD in control experiment

The first batch experiment was conducted using 100 mg/L of dichlorvos under different pH values i.e., 4, 7 and 9. At pH value of 4 showed an initial percent COD removal of 20 at 24 hrs which increased to 64 after 120 hrs of incubation (Figure 4.12).

At pH value of 9, the initial percent removal was 28 which increased to 75.4 by the end of experiment. However, at the pH value of 7, the COD removal percentage increased to 80 starting from an initial value of 38. Therefore the maximum percent reduction in COD for 100 mg/L dichlorvos in nutrient broth was achieved at pH 7 whereas acidic and alkaline pH resulted in less removal of organic matter. It was observed that the COD percent removal values for 100 mg/L of dichlorvos were quite relative to that of control experiment. The relevance can be attributed to acclimatization studies where the *Pseudomonas sp.* was exposed to lower concentrations of dichlorvos successfully degrading the organic matter.

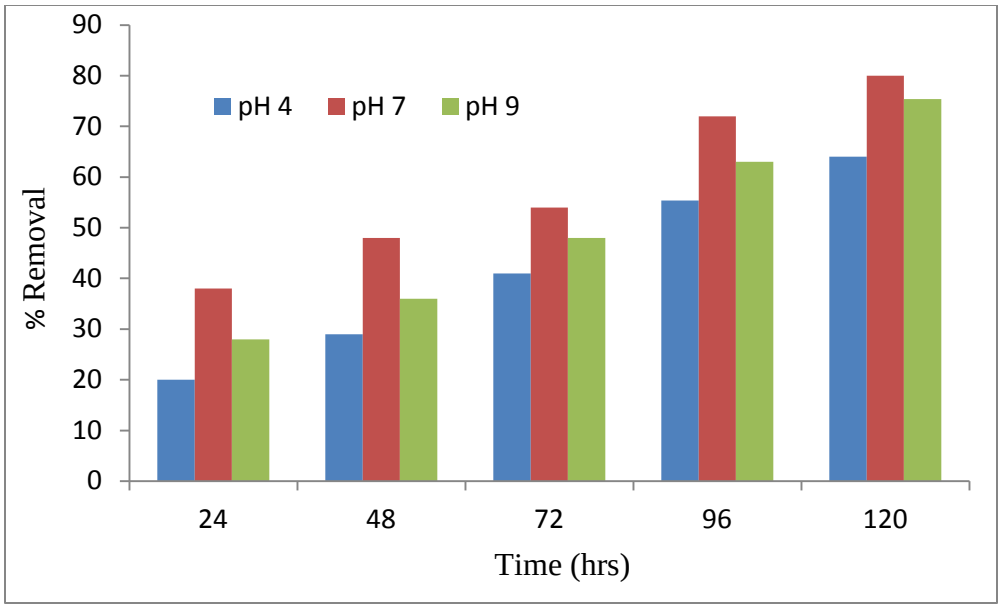

Figure 4.12: Effect of pH on percent reduction of COD in 100 mg/L of dichlorvos

The results obtained for 300 mg/L dichlorvos concentration in nutrient broth medium followed the same trends as shown by 100 mg/L. The growth of *Pseudomonas aeruginosa* in 300 mg/L concentration gave highest percent removal of 65.

At pH 4, the initial percent removal was 11 and showed an increase percent of 50 after 120hrs of incubation. At pH 9 the COD removal percentage increased from 16 to 52. At pH value of 7, maximum COD percent removal was observed starting at 28 at 24 hrs and ending at 65 after 120hrs (Figure 4.13).

Therefore the results show maximum reduction in COD for 300 mg/L dichlorvos at pH 7 as compared to acidic and alkaline range.

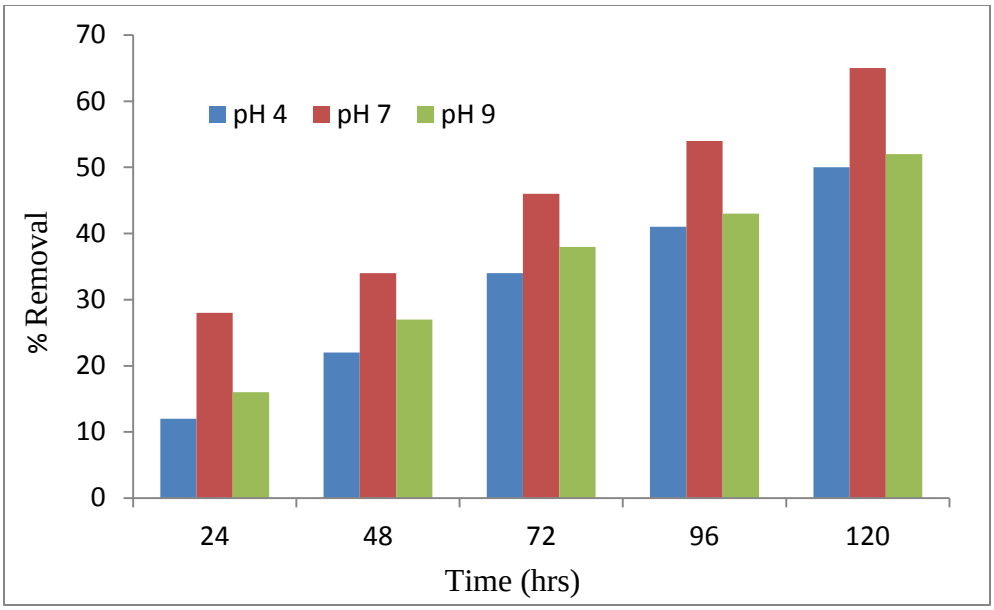

Figure 4.13: Effect of pH on percent reduction of COD in 300 mg/L of dichlorvos

In case of 500 mg/L, the value of COD percent removal at pH 4 increased from 6 to 29. The initial value of COD removal at pH 9 was 11 which increased to 49 percent after 120 hrs. Finally, pH 7 showed the percent removal of 15 after 24 hrs increasing to 53 after 120 hrs showing maximum COD removal as compared to other pH values (Figure 4.14).

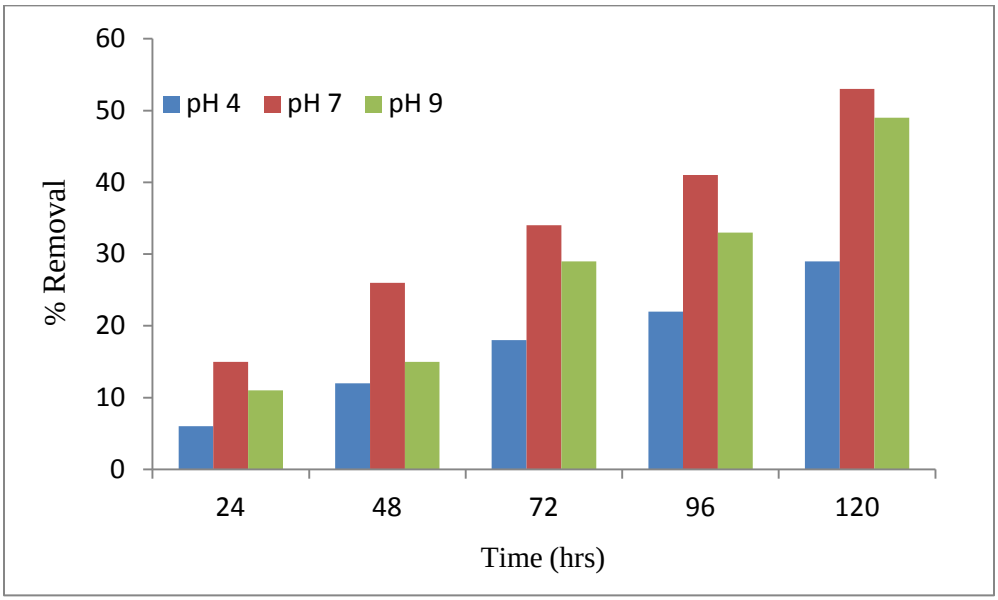

Figure 4.14: Effect of pH on percent reduction of COD in 500 mg/L of dichlorvos

4.4.4 Effect of Temperature on COD removal

Temperature is known to affect the biochemical reaction rates, as the rates of many reactions double after each 10°C rise in temperature (Prescott *et al*., 2003). The cells die above a certain temperature, therefore it is always critical to determine the optimum temperature for bacterial growth. In the present study experiments were carried out to determine the optimal temperature for the growth of culture that resulted in maximum removal of organic content. Results showed that growth can occur over a range of temperature i.e. 20°C to 30°C.

For the control experiment, the value of COD showed the increased reduction in organic content with time at higher temperature. At 20°C, 14 percent decrease in COD content was observed after 24 hrs which increased to 50 percent by the end of experiment after 120 hrs. The initial value of COD percent removal was 35 at 25°C which increased to 87 after 120 hrs. Maximum COD percent removal was observed at 30°C where it increased from 45 to 91 percent (Figure 4.15).

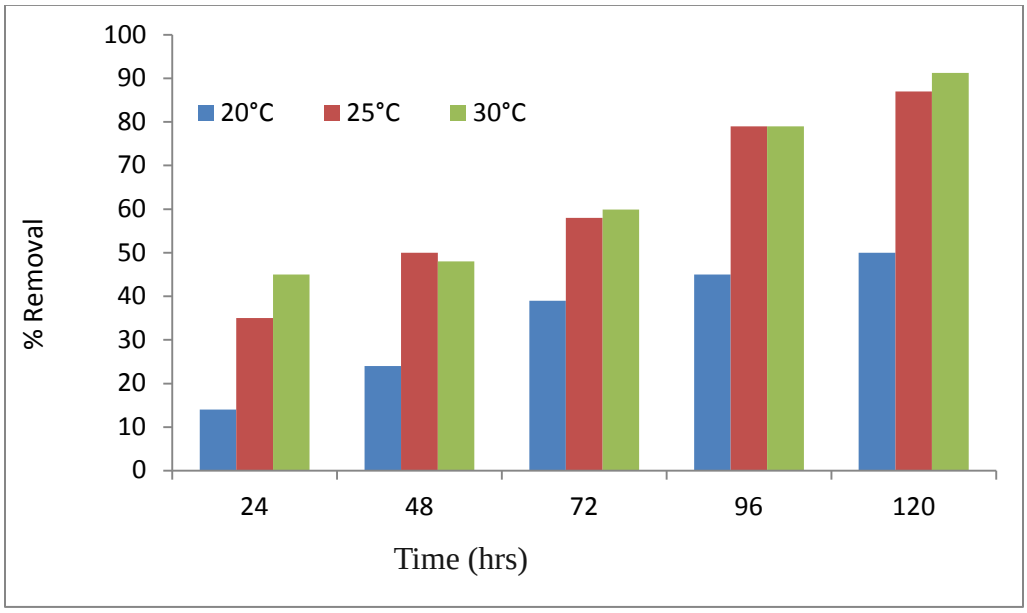

Figure 4.15: Effect of temperature on percent reduction of COD in control experiment

In case of 100 mg/L, the percent removal value of COD at 20°C was 12 increasing to 46 percent showing a marked increase in COD reduction. Similarly, the initial COD percent removal at 25°C was 38 which increased to 80 after 96 hrs. At temperature of 30°C, the initial COD percent removal was 43 which increased to 86 showing a maximum COD removal at this temperature (Figure 4.16).

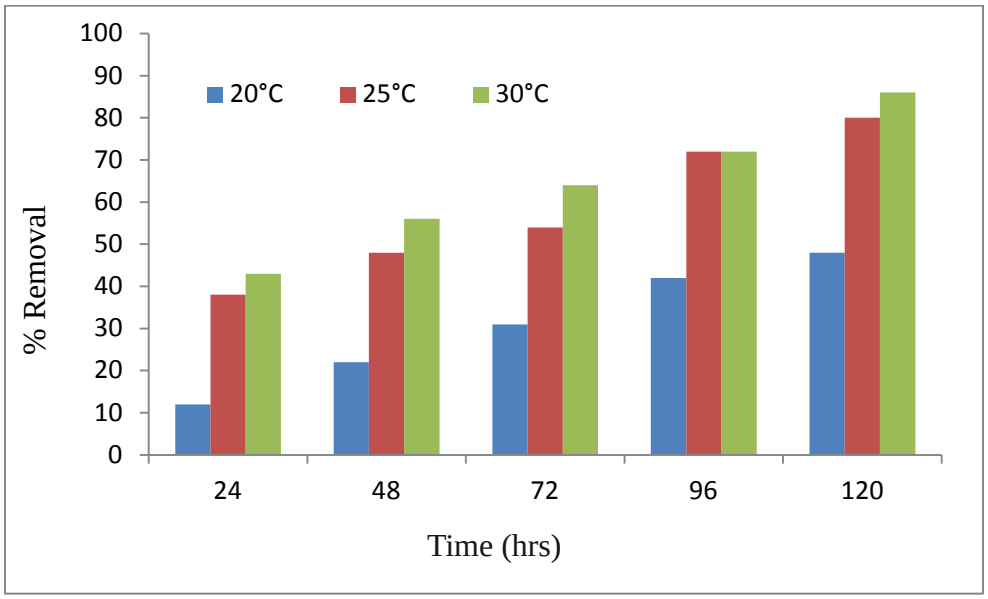

Figure 4.16: Effect of temperature on percent reduction of COD in 100 mg/L of dichlorvos

For 300 mg/L, the value of COD percent removal at 20°C was 8 which increased to 42. The initial value of COD percent removal at 25 °C was 28 and it increased to 65 percent. Similarly, at 30°C, the value for organic matter removal increased from 32 to 67 (Figure 4.17).

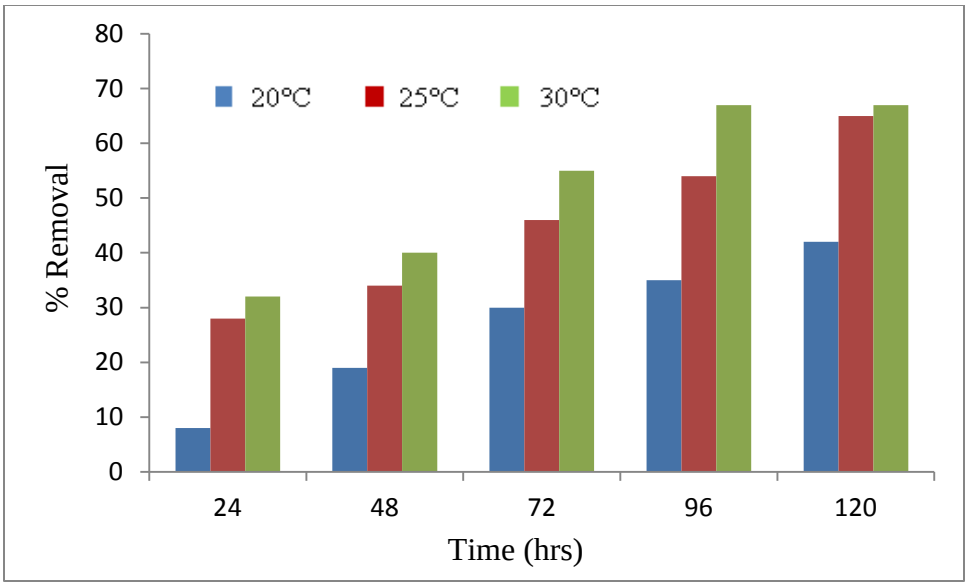

Figure 4.17: Effect of temperature on percent reduction of COD in 300 mg/L of dichlorvos

In case of 500 mg/L, the initial COD percent removal at 20°C was 3 which increased up to 30 percent after 120hrs of incubation. At 25°C, the removal of 12 to 53 percent was achieved. The COD percent removal of 59 was observed at 30°C (Figure 4.18).

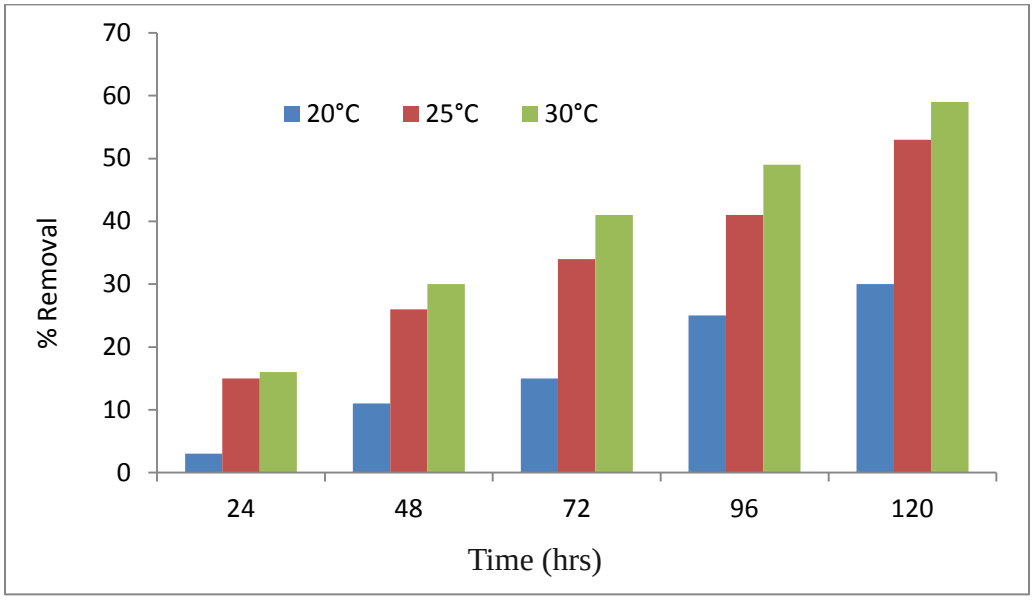

Figure 4.18: Effect of temperature on percent reduction of COD in 500 mg/L of dichlorvos

These results showed that higher concentration of pesticide resulted in less removal of organic matter by the culture even at same temperature: however maximum removal of organic matter was achieved at 30°C which was determined as optimal temperature for the growth of *Pseudomonas aeruginosa* culture.

4.5 Gas Chromatographic analysis

4.5.1 Calibration Set

Standard analyte for dichlorvos was purchased from Merck, Germany. Stock solutions (100 mg L^{-1}) for the standard analyte were prepared by dissolving in GC grade n-hexane. From this 2 mg L^{-1} standard pesticide solution was prepared. From this mixed solution, five calibration solutions (from 0.1 to 1.0 mg L^{-1}) were prepared in n-hexane. All solutions were stored frozen in the dark at -20 °C until use. The stock solutions were run on gas chromatograph to detect their signal and retention time. Signals were observed by injecting 1 µL of stock solution into the injection port. Figure 4.19 shows the calibration curve for different standard solutions of dichlorvos.

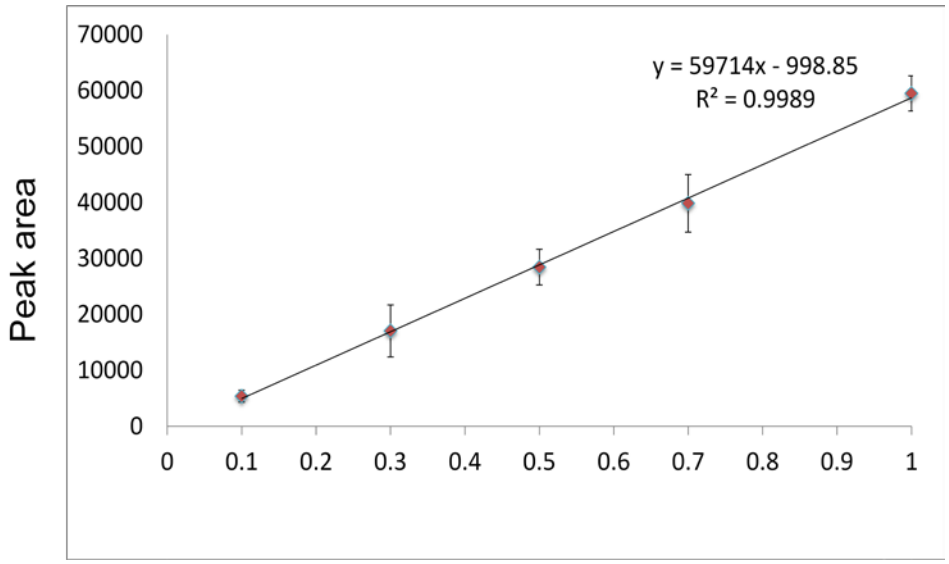

Figure 4.19: Calibration curve using different standard dilutions

4.5.2 Retention time

Temperature of injector, column and detector were also adjusted according to the signals. A temperature ramp for the column was designed to get well resolved and separate peaks for the analyte and solvent. The temperature of injector was adjusted to 180°C after trial and error. Detector temperature was 280°C. The reason for the high temperature was to make the detector (ECD) more sensitive for analyte to be studied. If the detector is more sensitive it will detect the analyte in ppb or even ppt concentration (Saba and Hashmi, 2010).

The initial temperature of the column oven was 80°C with ahold time of 1 minute. Then a temperature ramp of 10°C rise per minute was given to the column. Final temperature of the column was 180°C with 4 minute hold time. The hold time is given to flush out the remaining traces so that the column may become ready for the next injection.

Helium gas was used as carrier gas for the analysis at a flow rate of 2 mL/min. Nitrogen gas was used only at the time of sample analysis. Table 4.3 shows the retention time of solvent and analyte.

Table 4.3: Retention time of solvent and analyte

Analyte	**Retention time (min)**
Solvent (n-hexane)	4.63
Dichlorvos	11.17

The relative retention time of n-hexane (solvent) is 4.63 minutes while for dichlorvos it is 11.17 minutes. This is in contrast to 5.7 minutes reported by Wei *et al*., (2011) and 8.97 minutes reported by Hongxia *et al*., (2010). This variation in retention time can be attributed to different conditions of temperature and flow rate optimized in GC program. Moreover, the analyte being studied was a commercially used formulation in comparison to standards used in above mentioned researches. The test analyte was easily dissolved in n-hexane making it a better choice as a solvent. Figure 4.20 in Annexure 2 shows the gas chromatogram with distinct peak of dichlorvos along with its retention time.

4.6 Reproducibility graphs

The quantitative analysis for the percent degradation of dichorvos at varying pH and temperature was conducted. Reproducibility of analyte was calculated. Each sample was injected at-least three times and the mean was used to calculate the average retention time.

4.6.1 Effect of pH

For 100 mg/L of dichlorvos, the gas chromatographic analysis showed degradation of 27 and 80 for pH 4 and 9, respectively while highest degradation was observed at pH 7 to be 85 % (Figure 4.21).

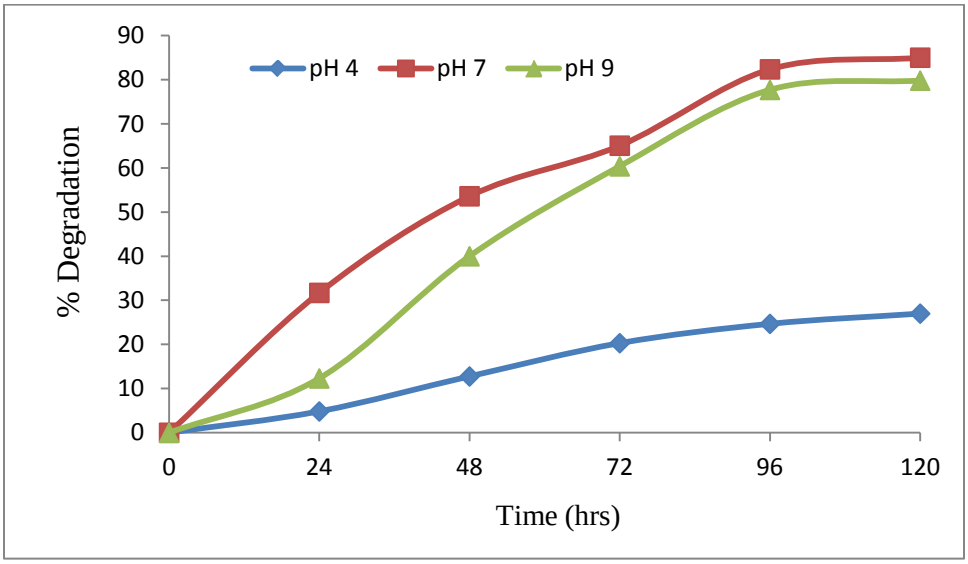

Figure 4.21: Biodegradation of dichlorvos 100 mg/L at different pH

Similarly, in case of 300 mg/L percent degradation at pH 4 and 9 was obtained as 23.5 and 51 while for pH 7 it was observed to be 64 percent as shown in Figure 4.22.

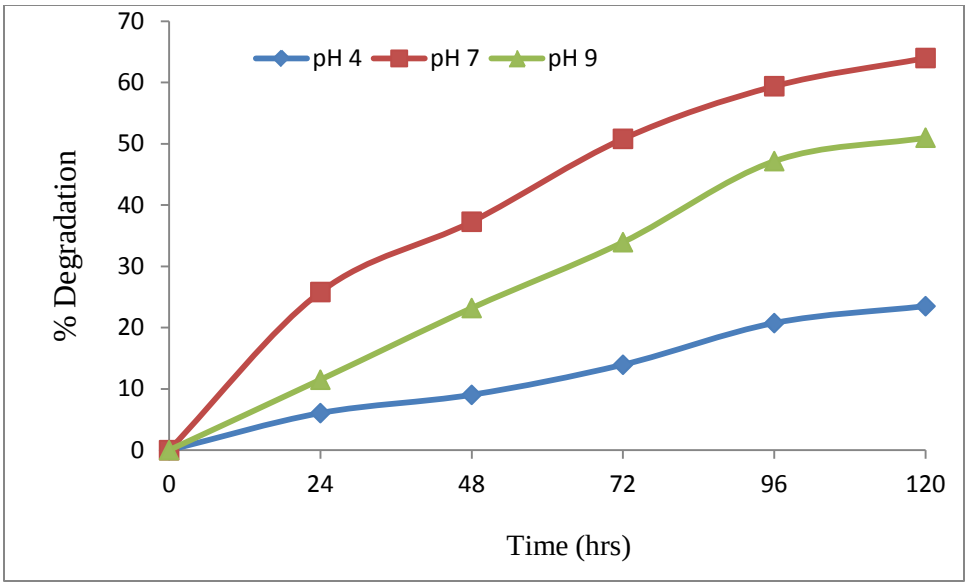

Figure 4.22: Biodegradation of dichlorvos 300 mg/L at different pH

Percent degradation for 500 mg/L at varying pH was observed to be 23 and 53 for pH 4 and 9. But at pH 7 maximum degradation was achieved as 60% (Figure 4.23).

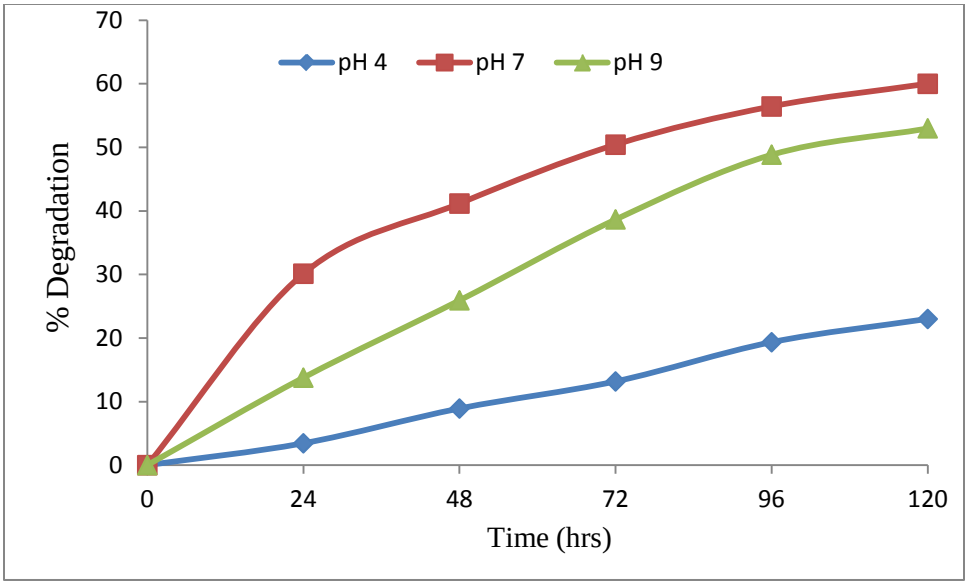

Figure 4.23: Biodegradation of dichlorvos 500 mg/L at different pH

4.6.2 Effect of Temperature

For 100 mg/L, the percent degradation at 20°C , 25°C and 30°C was achieved as 50,85 and 95.32% where and 30°C gave maximum degradation.

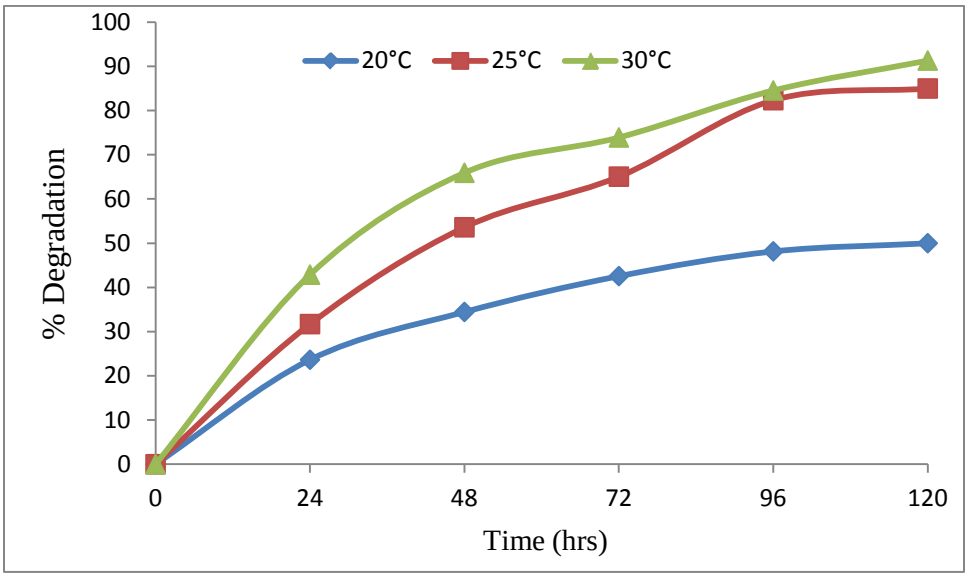

Figure 4.24: Biodegradation of dichlorvos 100 mg/L at different temperature

In case of 300 mg/L, highest degradation was observed at 30°C as 73% while for 20 and 25°C, it was 50 and 64%, respectively.

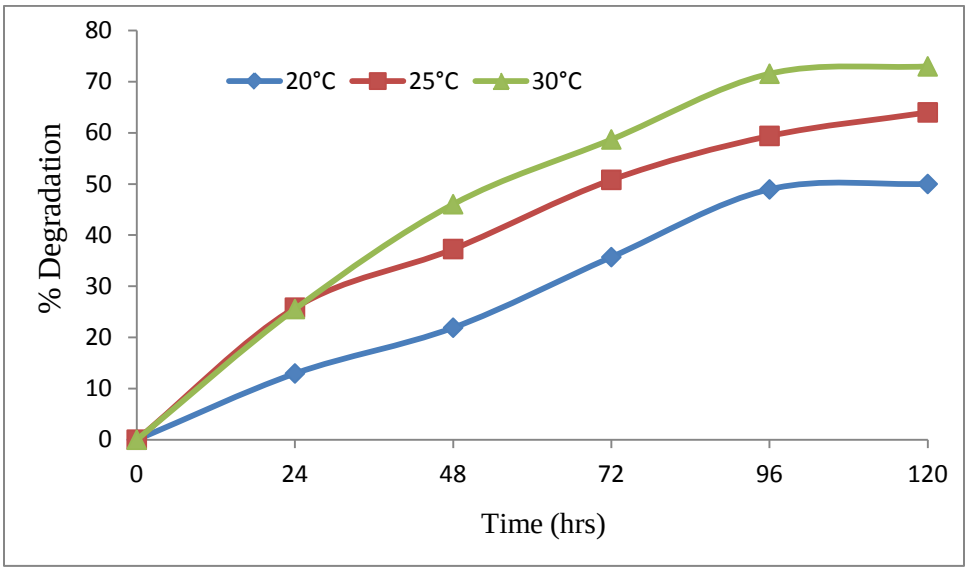

Figure 4.25: Biodegradation of dichlorvos 300 mg/L at different temperature

For 500 mg/L, dichlorvos showed a percent degradation of 33 and 60 % at a temperature of 20 and 25°C while at 30°C, degradation of 68% was observed.

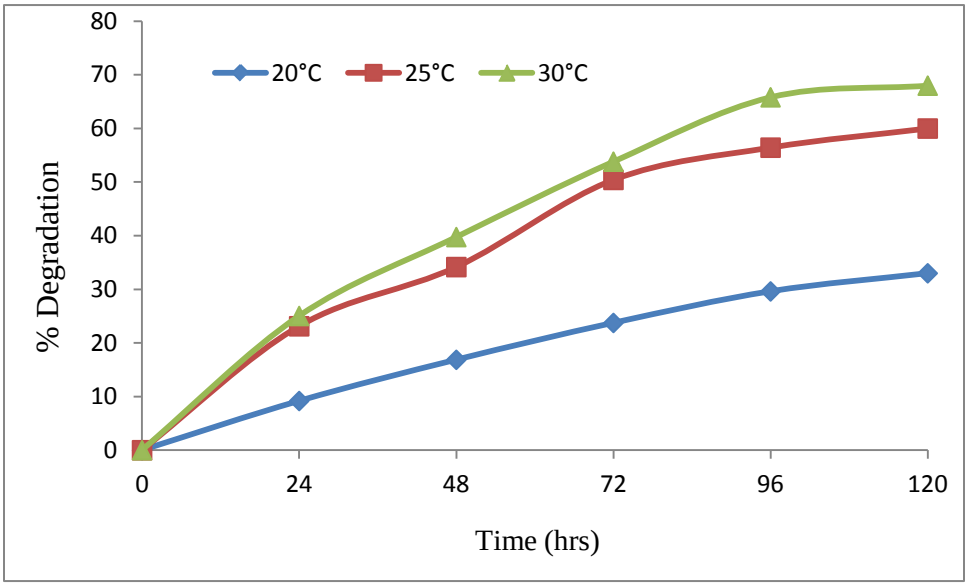

Figure 4.26: Biodegradation of dichlorvos 500 mg/L at different temperature

Madhuri and Rangaswamy (2009) studied biodegradation of dichlorvos in soil through gas chromatography. The bacterial cultures were isolated by selective enrichment technique and identified as species of *Bacillus* and *Pseudomonas*. 50% of dichlorvos was degraded in 7 days by the formation of one unidentified metabolite while complete degradation was observed at the end of 14 days.

Experiments conducted in Houston Black clay to determine the factors responsible for degradation of dichlorvos were related directly to the presence of *Bacillus cereus.* Gas liquid chromatographic analyses showed 50% degradation after 10 days in perfusion system and 4 days in mineral salt medium. Chemical hydrolysis of dichlorvos in aqueous, soil-free systems showed that hydrolysis did not occur in very acid systems where pH is below 4, but increased with increasing pH values (26% in 4 days at pH 6.9), and was rapid at pH 9.3 with 100% degradation in 2 days) (Lamoreaux and Newland, 2003).

The results showed that higher concentrations of pesticide resulted in less removal of organic matter. However, maximum removal of organic matter was achieved at higher temperature of 30°C with neutral pH value.

4.7 Gas Chromatography- Mass Spectrometry

GC–MS analysis was used to identify the intermediates formed during the biodegradation of commercial grade dichlorvos. The identification of fragments was confirmed by matching retention times of standard and by the presence of major ions. GC–MS analysis of commercial grade dichlorvos showed 4 different peaks, including dichlorvos peak. Analysis of solvent extracted samples during biodegradation process reaction showed three distinct peaks for one unidentified metabolite with m/z 79, dimethyl phosphate with m/z 109 and dimethylmonochlorvos with m/z 185 an. The small peak with m/z 220 was identified as dichlorvos. Figure 4.27 and 4.28 show the GC-MS chromatogram for dichlorvos as well as structure of two major identified peaks for intermediates.

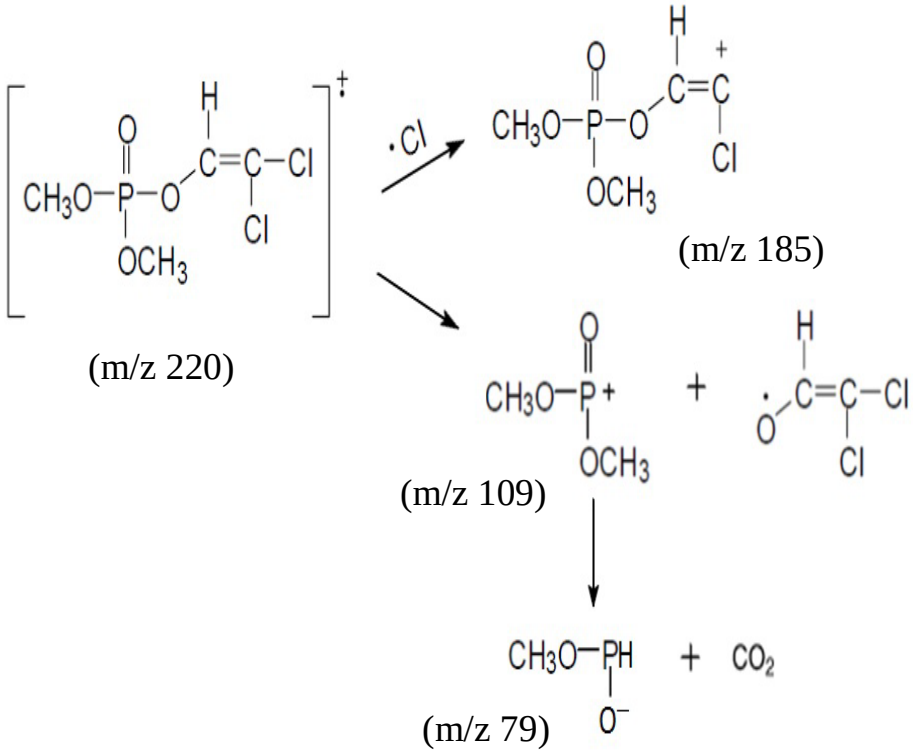

Figure 4.28: Major intermediates with m/z values as given by GC-MS

Physico-chemical methods such as photodegradation using titania or reduced sulphur species, ultrasonic irradiation and photocatalytic oxidation have been used to study the kinetics and mechanism of dichlorvos degradation but very few are related to biodegradation.

Total organic carbon and ion chromatographic analyses were employed by Schramm and Hua (2001), to determine and quantify major degradation products of dichlorvos through ultrasonic irradiation , including dimethyl phosphate, formate, carbon dioxide, chloride, and phosphate. A pathway for dichlorvos decomposition was proposed, based upon formation rates of the various intermediates and products and the rate of decrease of the total organic carbon in the system. The limiting steps in the mineralization pathway appeared to be transformation of dimethyl phosphate and formate due to their further oxidation.

Similarly, Rahman and Muneer (2005), investigated photocatalysed degradation of dichlorvos in aqueous suspensions of titanium dioxide. The degradation was monitored by the change in substrate concentration employing UV spectroscopic analysis as a function of irradiation time. The degradation kinetics was studied under different conditions such as pH, catalyst concentration and substrate concentration. The pesticide derivative of dichlorvos was found to degrade faster as compared to other pesticides. Intermediate products of dichlorvos were identified through GC/MS analysis technique and probable pathway for the decomposition of dichlorovos was proposed.

Gan *et al.,* 2006 studied the reactions of dichlorvos with five reduced sulfur species in anoxic aqueous solutions to investigate their role in its degradation. Reactions were monitored at varying concentrations of reduced sulfur species over pH range to obtain the second order reaction rate constants at 25°C. Predicted half-lives of dichlorvos ranged from hours to days with intermediates identified as dimethylphosphate and dichloroacetaldehyde.

Chapter 5

CONCLUSIONS

Organophosphates represent one of the major classes of pesticides with a bulk use in Pakistan. Their presence as well as application has caused many environmental problems including contamination of soil and water as well as their residual presence in food chain. The overall goal of this research was to study biodegradation of organophosphate, using dichlorvos as model pesticide, with emphasis on optimization of abiotic parameters such as temperature and pH.

The outcomes of this lab scale study are listed below.

1. Pure culture isolated from soil by enrichment technique was identified as *Pseudomonas aeruginosa.*

2. The isolated strain showed growth at different concentrations of dichlorvos where increase in concentration decreased the degradation rate.

3. The 100, 300 and 500 mg/L of dichlorvos showed COD percent removal of 80, 65 and 53% respectively at pH 7 as compared to other acidic and alkaline pH. While maximum COD percent removal at 30°C was observed to be 80, 67 and 59 % for the same concentrations of dichlorvos.

4. Quantitative analysis through gas chromatography showed percent degradation of 85, 64 and 60% at pH 7 for 100, 300 and 500 mg/L of dichlorvos, whereas at 30°C, the maximum degradation observed was 91,73 and 68 %, respectively.

5. GC-MS analysis of dichlorvos degradation showed two distinct peaks for dimethyl phosphate and dimethyl monochlorvos with being the major metabolites with m/z value of 109 and 185, respectively.

Chapter 6

RECOMMENDATIONS FOR FUTURE WORK

- Studies may be conducted on the interaction between different bacterial species, mixture of pesticides and effect of different environmental factors.
- Ambient conditions as studied in experiments may be tested in field.
- Quantitative analysis may be done in detail to identify the toxicity and percentage presence of biodegradation metabolites.

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Isolation, Morphological and Chemical Characterization of *Pseudomonas aeruginosa*

Figure 4.1: Isolation of dichlorvos degrading bacteria

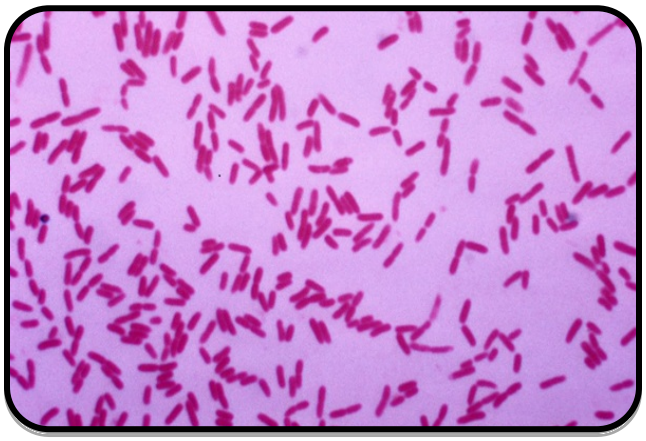

Figure 4.2:Dichlorvos degrading bacteria, stained with Gram's method

Figure 4.3:MR test for *Pseudomonas aeruginosa*

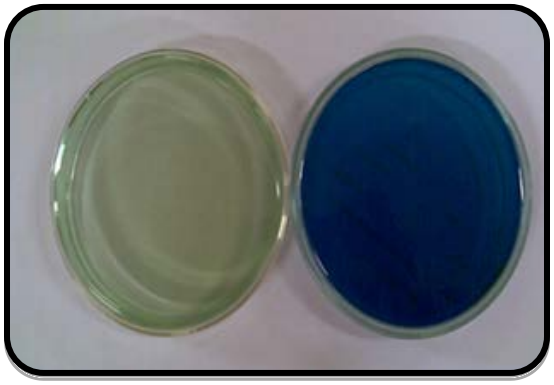

Figure 4.4:Citrate test for *Pseudomonas aeruginosa*

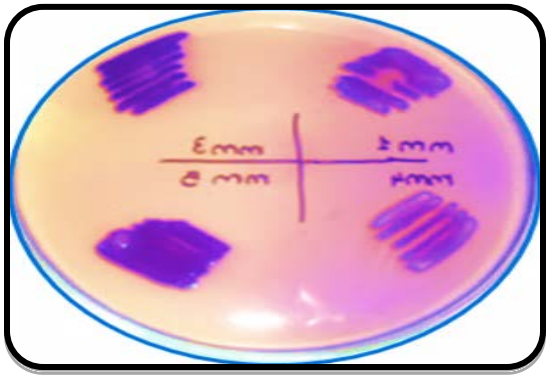

Figure 4.5:Oxidase test for *Pseudomonas aeruginosa*

Figure 4.6:Catalase test for *Pseudomonas aeruginosa*

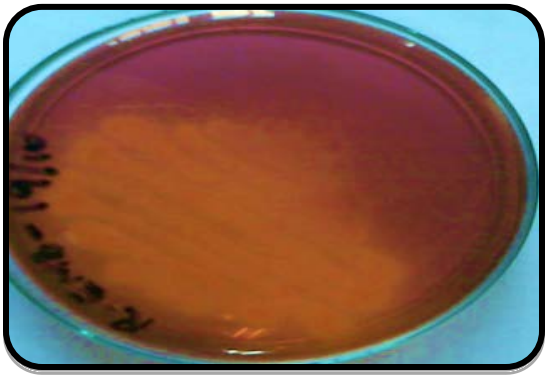

Figure 4.7: MacConkey test for *Pseudomonas aeruginosa*

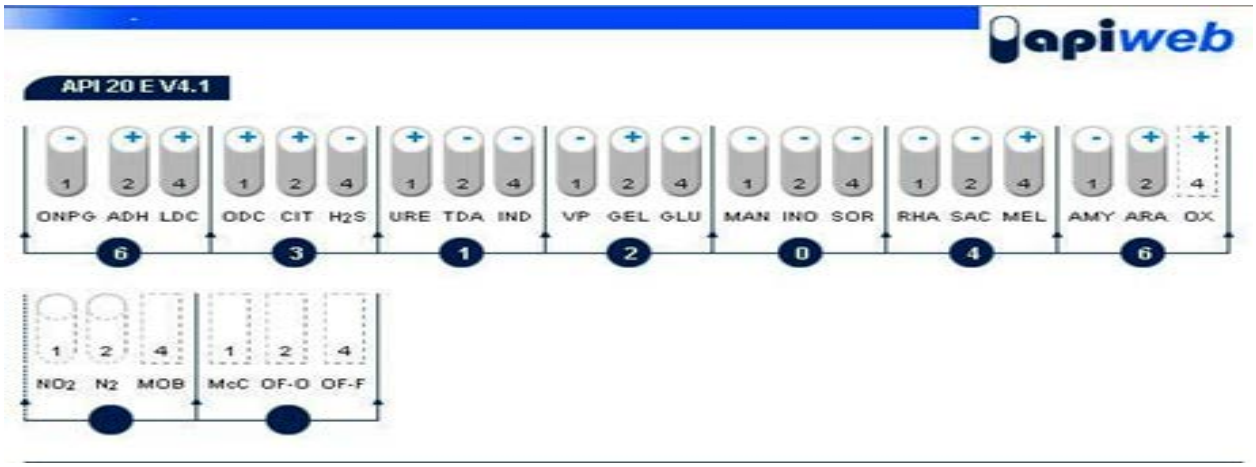

Figure 4.8: API 20E identification for *Pseudomonas aeruginosa*

Gas Chromtograms for Dichlorvos

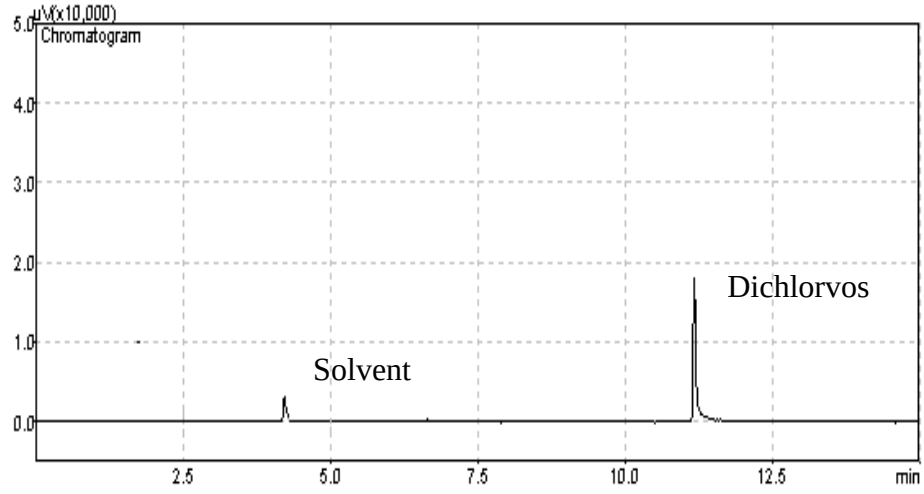

Figure 4.20: Gas chromtogram showing dichlorvos peak (11.17 min)

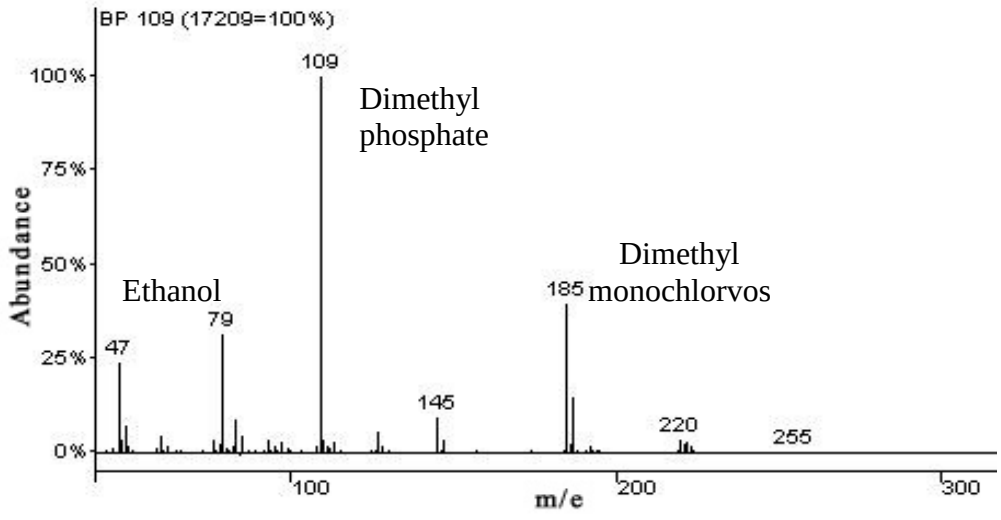

Figure 4.27: Chromatogam for GC-MS analysis of Dichlorvos