DEGRADATION OF IMIDACLOPRID BY BACTERIA ISOLATED FROM CONTAMINATED SITES

A Thesis

Submitted in the partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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CERTIFICATE

This is to certify that the thesis entitled "Degradation of imidacloprid by bacteria isolated from contaminated site" submitted by Rabia Arora (Roll No. 301001019) as a part of curriculum for degree of Master of Science, Department of Biotechnology and Environmental Science, Thapar University, Patiala, is a record of student's own work under our guidance and supervision. This report has not been submitted for the award of any other degree or certificate in this or any other university.

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Candidate's Declaration

I, hereby declare that the work which is being presented in the dissertation entitled "Degradation of imidacloprid in broth culture and soil microcosm by an isolated soil organism", in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab; is an authentic record of my own work during a period of six months from January 2012 to June 2012, under the supervision of Dr. M. Sudhakara Reddy, Head of Department of Biotechnology and Environmental Sciences, Thapar University. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any other degree.

Place: Patiala RABIA ARORA

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DATE : RABIA ARORA

Abbrevations

K_{oc}	Adsorption coefficient
K_{ow}	Water partition coefficient
LD	Lethal dose
FAV	Final accute value
Rfd	Reference dose
LC	Lethal concentration
SPE	Solid phase extract
NOAEL	No adverse effects level
nAChR	Nicotinic acetylcholine receptors
HPLC	High performance liquid chromatography
MS	Mass Spectroscopy
LC	Liquid chromatography
UF	Uncertainity factor
FOPM	Federal oofice of public health
NA	Nutrient agar
PDA	Potato dextrose agar
MUB	Modified Unversal Buffer
a.i.	Active ingredient
ACN	Acetonitrile
%	Pecentage
bp	Base pair
DNA	Deoxyribonucleic acid
DNTP	2’-deoxynucleoside-5’-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
g	Gram

IPTG	Isopropyl-β-thiogalactoside
kb	Kilo base
M	Molar
mg	Milligram
ml	Milliliter
mM	Milli molar
P	Phosphorus
mg/l	Milligram per litre
w/v	Weight by volume
H_2O_2	Hydrogen peroxide
NaOCl	Sodium hypochlorite
CO_2	Carbon dioxide
H_2O	Water
PCR	Polymerase chain reaction
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
µg	Microgram
µM	Micromolar
OD	Optical density
EtBr	Ethidium bromide
rpm	Revolution per minute
ppm	Parts per million
CFU	Colony forming unit
PSMs	Phosphate solubilzing microorganisms

Abstract

After analyzing the effect of pesticides on the physiochemical properties and enzymatic activities in the soil, the need of pesticide removal seems to be mandatory in order to maintain soil fertility and nature's equilibrium. Thus imidacloprid, one of the insecticide needs to be degraded to prevent the ground water from contamination. For this bacterial strains C-5 and C-9, isolated from pesticide contaminated soil, were isolated and grown in minimal medium and screened for one of the insecticide i.e., imidacloprid degradation. The strain, which utilized imidacloprid and showed maximum growth, was selected for detail study. The maximum degradation of 86% was observed. Similarly, degradation was also reported in soil microcosm experiment in order to mimic natural environmental conditions. However maximum degradation of 90% was observed in the nursery plantation experiment where the intense microbial activity in the rhizospheric soil leads to more degradation. Both the strains isolated were phosphate solubilzers results in plant growth and soil fertility and thus improving crop economy.

Chapter 1

Introduction

Imidacloprid 1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine shown in Figure 1. is a systemic chloronicotinyl insecticide with a novel mode of action, that acts as an agonist of the nicotinyl receptor (Bai *et al*., 1991; Mullins, 1993). Imidacloprid ($C_9H_{10}ClN_5O_2$) is sold under the trade name Premise® by Bayer Environmental Science (Research Triangle Park, NC). Imidacloprid was first synthesized in 1985 and was registered in France as an agricultural pesticide to be used on sucking insects attacking sugar beets (Sur and Stork, 2006).

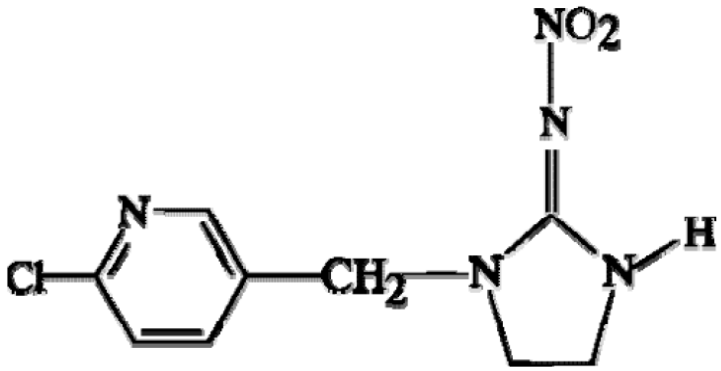


Figure 1: Chemical structure of imidacloprid (adapted from Fernandez-Perez *et al*., 1998)

Imidacloprid, the first neonicotinoid registered for use as an insecticide, is used worldwide against a wide diversity of piercing-sucking and chewing insects in a variety of crops and environments (Elbert *et al*., 1991; Leicht, 1993; Mullins, 1993; Krohn and Hellpointner, 2002). This systemic compound is structurally and functionally related to the tobacco toxin nicotine and has a unique mode of action, interfering with the chemical signal transmission mediated by the nicotinic acetylcholine receptors (nAChR) of insects (Abbink, 1991; Bai *et al*., 1991; Tomizawa *et al*., 1992; Buckingham *et al*., 1997)**.** Recent studies have suggested that imidacloprid can elicit induced systemic resistance pathways in plants, stimulate faster growth, enhance recovery from abiotic stresses, and increase yield when applied to plants growing under sub-optimal environmental conditions (Oosterhuis *et al*., 2003; Gonias *et al*., 2003; Thielert, 2006).Although these studies have mainly used agronomic and vegetables

plants for examining these non insecticidal properties, few studies have addressed these effects on woody species.

Mode of action

1.1.1 Target Organisms

Imidacloprid is designed to be effective by contact or ingestion. It is a systemic insecticide that translocates rapidly through plant tissues following application (Tomlin, 2006; Fossen, 2006).

Imidacloprid acts on several types of post-synaptic nicotinic acetylcholine receptors in the nervous system (Buckingham *et al*., 1997). In insects, these receptors are located only within the central nervous system. Following binding to the nicotinic receptor, nerve impulses are spontaneously discharged at first, followed by failure of the neuron to propagate any signal (Schroeder and Flattum, 1984; Sheets *et al*., 2001). Sustained activation of the receptor results from the inability of acetylcholinesterases to break down the pesticide (Matsuda and Sattelle, 2005). This binding process is irreversible.

1.1.2 Non-Target Organisms

Imidacloprid's mode of action is similar on target and non-target beneficial insects including honeybees, predatory ground beetles and parasitoid wasps (Fossen, 2006). However, imidacloprid is ineffective against spider mites and nematodes (Tomlin, 2006).

Mammalian nicotinic receptors are made up of a number of subtypes (Sheets *et al*., 2001). In contrast to insects, these receptors are present at neuromuscular junctions as well as in the central nervous system. However, the binding affinity of imidacloprid at the nicotinic receptors in mammals is much less than that of insect nicotinic receptors (Tomizawa and Casida, 1999). This appears to be true of other vertebrate groups including birds. The blood-brain barrier in vertebrates blocks access of imidacloprid to the central nervous system, reducing its toxicity (Sheets *et al*., 2001).

1.2 Fate and effect on the environment

The low K_{oc} of 132 to 310, combined with a high water solubility of 514 ppm, suggests a potential to leach to ground water, although earlier field studies, under normal weather conditions, have found imidacloprid to be relatively immobile in silt loam soils (Rouchaud *et al.,* 1994; Miles Inc., 1993). The moderate K_{ow} value of 3.7, combined with its rapid photodegradation in water, (half-life ($_{t1/2}$) < 3 hours) and on soil ($t_{1/2}$ 39days), suggests a low potential for bioaccumulation.

1.2.1 Fate in Air

Imidacloprid's low vapour pressure results in a very low potential for volatilization. Therefore, imidacloprid is most likely to be found in air during and immediately after spraying on crops, where it will exist primarily as an aerosol, with very little occurring in a gaseous state. Imidacloprid is rapidly photodegraded and transformed by photochemical radicals and therefore is not likely to persist in air (Krohn and Hellpointner, 2002).

1.2.2 Water

There is a potential for imidacloprid to enter streams and ponds via drift during application or in runoff water. Rouchaud *et al* (1994) and Miles Inc. (1993) found that imidacloprid did not leach to ground water in their field studies. Bayer Corporation found imidacloprid in ground water, 18feet below ground surface (sandy loam soil). Concentrations ranged from < 0.1 ppb to 1ppb. Hydrolysis of this pesticide is greater than 30 days at pH 7 and 25^{o}C. Sarkar *et al*. (1999) reported that the hydrolysis half-life varies from 33 to 44 days at the same pH and temperature. Imidacloprid was found to be stable in acidic and neutral water, but more readily hydrolyzed in alkaline water (Zheng *et al*., 1999). The formulation of the insecticide can affect the half-life. In wettable powder formulations persistence increased by 3 to 6 days compared to liquid formulations (Sarkar *et al*., 1999).

1.2.3 Soil

The high water solubility and low K_{oc}, indicate a low tendency to be adsorbed to soil particles. Field studies show that imidacloprid can persist in soil, with a half-life ranging from 27 to 229 days (Miles Inc., 1993). Half-life in soil varies depending on soil type, use of organic fertilizers, and presence or absence of ground cover. Scholz *et al* (1992**)** found that imidacloprid degraded more rapidly under vegetation, $t_{1/2}$ 48 days, versus 190 days without vegetation. Degradation on soil via photolysis has a $t_{1/2}$ of 39 days. In the absence of light, the longest half-life of imidacloprid was 229 days in field studies and 997 days in laboratory studies (Miles Inc., 1993). This persistence in soil, without the presence of light, makes imidacloprid suitable for seed treatment and incorporated soil applications because it allows continual availability for uptake by roots (Mullins, 1993). In field experiments, low application rates showed high sorption (Cox *et al*., 1998). The sorption level of imidacloprid is also affected by soil properties such as organic carbon and minerals. As the organic carbon levels and laminar silicate clay content in the soil increases, the potential for imidacloprid to leach would decrease (Cox *et al*., 1997). Organic fertilizers, such as chicken and cow manure, increased the pesticide adsorption to the organic matter and also increased its half-life. Half-

lives ranged from 40 days when no organic fertilizers were used to 124 days when cow manure was used. However, residual insecticide soil concentrations were low at the time of harvest, to those not treated with organic fertilizers (Rouchaud *et al*., 1996). Plants readily absorb imidacloprid through the roots, and metabolize it. No correlation was found between K_{oc} and the soil carbon content (Rouchaud *et al*., 1996).

1.2.4 Biota

Even with imidacloprid's potential to persist in soil, the high photodegradation tendency and high water solubility indicate there is low potential for bioaccumulation in the environment. Plants readily absorb imidacloprid through the roots. In one study, at 97 days after sowing, the metabolites in sugar-beet leaves represented 44.5% of the applied parent compound. In the same study the main metabolites in plants were found to be 6- hydroxynicotinic acid, an olefinic compound and an unidentified metabolite. Koester (1992) found the main metabolites to be 1-[(6-chloro-3-pyridinyl)methyl]-5-hydroxy-4,5-dihydro-N-nitro-1H-imidazol-2-amine and 1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-1H-imidazol-2-amine. If imidacloprid were to enter surface water, the photodegradation half-life in water is less than 3 hours. Without light, hydrolysis can range from 33 to 44 days. When applied level of 211 ppm for the rainbow trout. No studies have been done to determine or identify imidacloprid in fish.

1.3 Toxicity

While acute toxicity to adult fish occurs at relatively higher concentrations of imidacloprid (over 80,000 μg/l), the early life stages of fish exhibit much higher sensitivity (Cox, 2001). In mammals, imidacloprid is rapidly absorbed from the gastrointestinal tract and within 48 hours of administration is effectively eliminated through urine (70 to 80%) and faeces (20 to 30%) (Tomlin, 1994; PMRA 2001).

In mammalian systems, imidacloprid is hydroxylated and hydrolyzed to the critical metabolic product, 6-chloronicotinic acid. This metabolite is further conjugated and eliminated or reduced to guanidine (Tomlin, 1996). Imidacloprid is moderately toxic to mammals via the oral route of exposure (PMRA 2001). Acute toxicity of imidacloprid to rats varies, depending on the route of exposure, with oral dosing posing the greatest toxic threat (Mulye, 1996). Imidacloprid has been classified as a 'Group E' chemical, one for which no evidence of carcinogenicity exists (U.S. EPA 1995). Tomlin (2000) states that it is neither mutagenic nor teratogenic.

1.4 Remediation of imidacloprid

The serious concern of the agricultural community is the increase of insecticide residues because the application of these xenobiotics in soil can cause damage to the ecosystem. They may also influence microbial processes that are an essential part of carbon, sulphur and nitrogen cycles (Somich *et al*., 1990). Leaching of these insecticides through the soil to the ground water is also a concern since these chemicals may affect the quality of drinking water supplies and surface ecosystem (Koterba *et al*., 1993; Cox, 1994).

Thus, the frequency and widespread use of man-made "xenobiotic" chemicals has led to a remarkable effort to implement new technologies to reduce or eliminate these contaminants from the environment. Commonly used pollution treatment methods (e.g. land-filling, recycling, pyrolysis and incineration) for the remediation of contaminated sites have also had adverse effects on the environment, which can lead to the formation of toxic intermediates (Debarati *et al*., 2005). Furthermore, these methods are more expensive and sometimes difficult to execute, especially in extensive agricultural areas, as for instance pesticides (Jain *et al*., 2005). One promising treatment method is to exploit the ability of microorganisms to remove pollutants from contaminated sites, an alternative treatment strategy that is effective, minimally hazardous, economical, versatile and environment-friendly, is the process known as bioremediation (Finley *et al*., 2010). Bioremediation is considered as an efficient and cheap biotechnological approach to clean up the polluted environment (Xu *et al*., 2008; Karpouzas and Singh, 2006).

Hence, biotransformation of organic contaminants in the natural environment has been extensively studied to understand microbial ecology, physiology and evolution due to their bioremediation potential (Mishra *et al*., 2005).

The biochemical and genetic basis of microbial degradation has received considerable attention. Several genes/enzymes, which provide microorganisms with the ability to degrade organo pesticides, have been identified and characterized. Thus, microorganisms provide a potential wealth in biodegradation. The ability of these organisms to reduce the concentration of xenobiotics is directly linked to their long-term adaptation to environments where these compounds exist. Moreover, genetic engineering may be used to enhance the performance of such microorganisms that have the preferred properties, essential for biodegradation (Schroll *et al*., 2004).

About 30% of agricultural produce is lost due to pests and insects. Hence, the use of pesticides, insecticides has become indispensable in agriculture. The abusive use of pesticides for pest control has been widely used in agriculture. However, the indiscriminate use of

pesticides has inflicted serious harm and problems to humans as well as to the biodiversity (Gavrilescu, 2005; Hussain *et al*., 2009). The problem of environmental contamination by pesticides goes beyond the locality where it is used. The agricultural pesticides that are exhaustively applied to the land surface travel long distances and can move downward until reaching the water table at detectable concentrations, reaching aquatic environments at significantly longer distances. Therefore, the fate of pesticides is often uncertain; they can contaminate other areas that are distant from where they were originally used. Thus, decontaminating pesticide-polluted areas is a very complex task (Gavrilescu, 2005).

Organochloride pesticides are synthetic and were widely used in the 1970s, mainly in the United States (http://www.epa.gov/history/topics/ddt/02.htm, accessed in May 2011). Although their use has been banished in many countries, they are still used in developing countries. Organochloride pesticides are cumulative in the organisms and pose chronic health effects, such as cancer and neurological and teratogenic effects (Vaccari *et al.,* 2006). Many xenobiotic compounds are recalcitrant and resistant to biodegradation, especially the organochloride pesticides (Diaz, 2004; Dua *et al*., 2002; Chaudhry and Chapalamadugu, 1991). In general, these highly toxic and carcinogenic compounds persist in the environment for many years.

These pollutants i.e, insecticides and pesticides are removed from the soil by endogenous microorganisms including bacteria for their extrordinary ability to use wide variety of these chemicals as sole energy and carbon source (Hemmingson, 1993; Parsek *et al*., 1995; Thomas *et al*., 1996; Kato et al., 2000; Poelarends *et al*., 2000; Parekh *et al*., 1994; Cabras *et al*., 1995; Shakoori *et al*., 1999; Sutherland *et al*., 2002 ; Siddique *et al*., 2003). Microorganisms are termed as nature's biodegraders. They are scavangers in nature, responsible for recycling most natural harmful waste into harmless compounds, when faced with an increasing array of synthetic compounds. Microorganisms are highly adaptive and develop the capability of degrading such recalcitrant compounds, through evolution of new genes which encode enzymes that can use these compounds as their primary substrate (Parsek *et al*., 1995; Suenaga *et al*., 2001)

The survival of organism under insecticidal stress can provide efficient, cheaper and environmental friendly for bioremediation of xenobiotic contaminated soil

The biological methods are advantageous to decontaminate areas that have been polluted by pesticides. These methods consider the thousands of microorganisms in the environment that in order to survive seek for alternatives to eliminate the pesticides that were sprayed. Many native microorganisms develop complex and effective metabolic pathways that permit the

biodegradation of toxic substances that are released into the environment. Although the metabolic process is lengthy, it is a more viable alternative for removing the sources of xenobiotic compounds and pollution created by the use of pesticides and insecticides (Diaz, 2004; Schoefs *et al*., 2004; Finley *et al*., 2010).

Objectives

1. Physiochemical properties of soil in the pesticide treated sites
2. Isolation and characterization of imidacloprid degradaing bacteria
3. Field studies on the degradation of imidacloprid in contaminated sites

Chapter 2

Review of literature

All modern chemical pesticides, in addition to being effective against the targeted organism, must fulfill further criteria to obtain market admission and, furthermore, to prevail on the market.

Therein, special emphasis is laid on the exclusion of possible dangers to humans and animals, and on the minimization of undesirable environmental side effects. From an economical point of view, the use of a new active agent should be cost effective, i.e., plant protection measures should require less application of the agent, or resistant harmful insects should be better controlled by new active mechanism (Schroeder and Flattum, 1984).

All these demands required are fulfilled by 1-[(6-chloro-3-)methyl]-N-nitro-2-imidazolidinimin, or imidacloprid as the trivial name. The enormous economical value of imidacloprid in particular, and of the chloronicotinyl insecticide classes in general, for present and future chemical phyto sanitary uses can be recognized when considering the sales volume, which was 1.28 billion$ in 2008 as shown in Table 2.1 (Hocker *et al*., 1995).

Imidacloprid was first synthesized in 1985 and was registered in France as an agricultural pesticide to be used on sucking insects attacking sugar beets (Sur and Stork, 2006). The invention of imidacloprid, the most important neonicotinoid insecticide, was initiated by replacement of the framework of nithiazine with an imidazolidine ring. Through the finding of 1-(6-chloro-3-pyridylmethyl)-2-nitromethyleneimidazolidine, imidacloprid was invented. These products possess pronounced systemic properties and improved photostability in addition to supreme insecticidal ability. Imidacloprid is placed in the chemical family known as the chloronicotinyls (Abbink 1991, Gahlhoff and Koehler, 2001).

Table 2.1: Sales of imidacloprid and its economical importance :

Brand	Active Ingredient	Company	Application	Sales 2008	
				Billion$	MT
Round Up	Glyphosate	Monsanto	Herbicide	8.30	620,000
Admire, Gaucho	Imidacloprid	Bayer Crop Science.	Insecticide	1.28	5450
Heritage	Azoxystrobin	Syngenta	Fungicide	1.16	7000
F 500	Pyraclostrobin	BASF	Herbicide	1.10	7200
Flagship	Thiemethoxam	Syngenta	Insecticide	0.73	1895
Callisto	Mesotrione	Syngenta	Herbicide	0.62	2040
Grammoxone	Paraquat-dichloride	Syngenta	Herbicide	0.60	26000
Flint	Trifloxystrobin	Bayer Crop Science.	Fungicide	0.60	3405
Horizon , Folicur	Tebuconazole	Bayer Crop Science	Fungicide	0.55	2860
Regent MG,FRONTLINE	Fipronil	BASF	Insecticide	0.53	1375

Source: Cropnosis Ltd- Agranova

2.1 Physical and chemical properties

Imidacloprid has the molecular formula $C_9H_{10}ClN_5O_2$ (Figure 1), with a molecular weight of 255.7 g/mol (Table 1). In appearance, it consists of colourless crystals. The insecticide is quite water soluble even at the lowest solubility value reported (510 mg/L, Table 2.2; Krohn 1989, reviewed in Mulye, 1995) and could potentially leach to groundwater (Cohen *et al.*, 1984, cited in Mulye, 1995) or be transported in runoff (Mulye, 1995). However, according to the comparatively low vapour pressure values, imidacloprid would be relatively non-volatile under field conditions (U.S. EPA, 1975b, cited in Mulye, 1995). Imidacloprid did not dissociate when titrated with either acid or base (Wohlers, 1988) as reviewed in Mulye (1995). The octanol/water partition coefficient (log K_{ow}) of imidacloprid is 0.57 (Tomlin, 2000), suggesting that it would not accumulate much in aquatic biota (Krohn and Hellpointner, 2002).

Table 2.2: Physiochemical properties of imidacloprid

Physiochemical property	Imidacloprid	Reference
Appearance	Colourless crystals	Tomlin, 2000
Chemical name	IUPAC-1-(6chloro-3-pyridylmethyl)-N-nitroimidazolin-2-ylideneamine; CAS: 1-[6-chloro-3-pyridinyl)methyl] –N-nitro-2-imidazolidinimine	Tomlin, 2000
Chemical formula and	$C_9H_{10}ClN_5O_2$	Tomlin, 2000
CAS number	138261-41-3	
Soil adsorption coefficient (K_{oc})	262.0	Orme and kegley, 2003
	210	Nemeth konda *et al*., 2002
Molecular weight	255.7 g/mole	Tomlin, 2000
Water solubility	0.510g/L @ 20°C	Mulye, 1995;
	0.61 g/L @ 20°C	Tomlin, 2000
Melting point	144°C	Tomlin, 2000
	143.8°C	Byrtus *et al.*, 2003
Vapour pressure	4×10^{-10} Pa @ 20°C	Tomlin, 2000
	9×10^{-10} Pa @ 25°C	
	2×10^{-7} Pa @ 20°C	EXTOXNET, 1998
Henry's Law constant (h)	1.0025×10^{-7} Pa m^3 mol^{-1}@ 20°C	Mulye, 1995
		Tomlin, 2000
	2.002×10^{-10} Pa m^3 mol^{-1}@ 20°C	
Partition coefficient (K_{ow})	log P = 0.57@ 21°C	Tomlin, 2000
Ultraviolet absorption	Maxima at : 1. 211nm (extinction coefficient=1.378×10^4) 2. 269nm (extinction coefficient=2.0545×10^4)	Mulye, 1995

2.2 Isolation and enrichment of microorganism

Microbial diversity offers immense environmental friendly options for mineralization of contaminants or their transformation into less harmful non hazardous compounds. There is a general interest in studying the diversity of indigenous microorganisms capable of degrading different pollutants due to their varied effects on the environment. Efforts have been made to characterize bacterial communities, isolate potential degraders and identify the genes involved in degradation process (Watanabe *et al*., 2002)

Cultures of predominant heterotrophic bacteria efficient in degrading the selected insecticides were isolated by enrichment culture technique. Isolates can then be obtained on culture medium containing the compound as carbon source (Jaya Madhuri and V Rangaswamy, 2003). Several imidacloprid (nicotinoid) degrading bacteria have been isolated so far such as *Acinetobacter* sp., *Sphingomonas* sp., based on their physiology, morphological and biochemical tests and 16S rDNA sequencing

2.3. Molecular characterization

Early traditional characterization of the microorganism depend upon phenotype, biochemical and serological tests. But these are not fully reliable as mutation and environmental conditions can affect the physiological traits. Ribosomal RNA (particularly 16S rRNA) is considered to be most reliable candidate molecule for identification-classification study. The analysis of 16S rRNA genes, aided by using PCR to amplify target sequences in environmental samples, has enabled microbial ecologists to identify and characterize micro-organism in a natural community. The taxonomic position of an organism can be determined by comparing the sequence with those of other bacteria (Amann *et al*., 1995).

Analysis of 16S rRNA genes is now widely used for analysis of bacterial population. The macromolecules that are most suitable for this purpose would require the following prerequisites: (1) generally present (2) functionally homologous in all organism, and (3) the sequence in the molecule should equally change during evolutionary process. Ribosomal RNA is one of the best candidates and it has been used for the studies on bacterial evolution. Major properties of rRNA are: (1) present as old molecules in the ribosomes, (2) functionally constant, (3) wide distribution, (4) well conserved over large phylogenetic distances, and (5) occurrence in large number of cells (10^4-10^5/cells). In the sedimentation rates: 5S rRNA (about 120 nucleotides), 16S rRNA (about 1600 nucleotides) and 23S rRNA (about 3000 nucleotides). The 5S molecule is too small and only suitable to distinguish major

phylogenetic groups. 23S rRNA is an excellent candidate but very few phylogenetic studies are available. 16S rRNA has been given most attention (Woese *et al*., 1990)

2.4. Analytical methods

There are a number of different analytical methods that are currently being used for detection and measurement of imidacloprid. The methods differ in their applicability to different types of environmental media, and also in the detection levels that can be achieved

Concentrations of imidacloprid in water and soil can be measured using a gas chromatography-mass spectrometry (GC-MS) technique (Vilchez *et al*., 1996). Samples of imidacloprid are transformed into a volatile compound through hydrolysis in a basic medium. Using a liquid-liquid extraction with chloroform will allow for sufficient extraction and pre-concentration of the hydrolysis product. The detection limits using this technique have been reported as 0.16 μg/l for water and 1 μg/kg for soil (Vilchez *et al*., 1996).

The GC-MS technique has also been utilized to determine the photocatalytic degradation of imidacloprid in industrial water (Aguera *et al*., 1998). Coupling GC-MS with liquid chromatographic atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) allowed Aguera (1998) to detect five degradation products, three of which were identified [i.e., chloronicotinic acid, chloronicotinic aldehyde, and 1-(6-chloro-3-pyridilmethyl)-imidazolidin-2-ona)] (Aguera *et al*., 1998). Overall, the LC-APCI-MS technique was considered to be a good and complementary method for monitoring imidacloprid but did not provide enough information for structural elucidation (Aguera *et al*., 1998).

A second technique for determining concentrations in water uses a photochemical-fluorimetric method (Vilchez *et al*., 1998). This methodology is based on the conversion of imidacloprid to the fluorophore 1-(6-chloro-3-pyridyl-methyl)-2-(hydroxyimino)-3,4-didehydroimidalozolidene through photodegradation. Using this methodology, the detection limit was reported to be 0.7 μg/l with a linear concentration range of 2.5-100 μg/l (Vilchez *et al*., 1998).

Quantification of imidacloprid and its metabolites has been mostly done by HPLC-MS/MS, and, in some cases, with radioactive labelled compounds. HPLC is very sensitive and has been used most frequently. A HPLC protocol has been developed to quantify imidacloprid with a detection limit of 0.0075 and 0.0060 mg/kg respectively (Mandic *et. al.,* 2004).

Fernández-Alba *et al*. (1998) proposed an HPLC-diode array detection method for the determination of imidacloprid residues extracted from vegetables involving acetone and C18

reverse-phase cartridges. Also, Ruizde Erenchun *et al*. (1999) have developed an HPLC-pulse reductive amperometric detection method for the determination of imidacloprid in soil and water.

High-performance liquid chromatography (HPLC) has also been used to measure levels of imidacloprid residues in water and soil (Baskaran *et al*., 1997)**.** Baskaran *et al*. (1997) suggest that levels of imidacloprid cannot be determined directly using gas chromatography as a result of its thermolabile and polar N-nitroguanidinyl moiety. Volatility may also be increased as a result of the substitution of the acidic hydrogen of the NH at the 3-position of the imidazolidine ring (Baskaran *et al*., 1997). HPLC allows for the separation and detection of analytes using conditions that are milder than those utilized by gas chromatography. Using a reversed-phase HPLC with UV detection with a mobile phase of acetonitrile-water and either a solid-phase extraction (SPE) or liquid-liquid extraction method, the extract can be collected and evaporated using a rotary evaporator (Baskaran *et al.,* 1997). By re-dissolving the extract in acetonitrile-water, the extract can be concentrated and then analyzed. Using this methodology, detection limits of 0.5 μg/l and 5 μg/kg were reported for imidacloprid in water and soil respectively (Baskaran *et al*., 1997).

2.5. Production, uses and source to the environment

Imidacloprid, produced by Bayer Crop Science Inc., is an insecticide active ingredient used to control sucking insects, such as aphids, leafhoppers, psyllids, thrips, whiteflies and beetles in agricultural crops, to control white grubs in lawns and turfgrass, as well as to control domestic pests such as fleas and cockroaches. Trade names for imidacloprid include Admire, Advantage, Confidor, Gaucho, Genesis, Impower, Intercept, Maxforce IC, and Merit (PMRA EDDENet Labels search, 2005). It is most commonly applied as a soil and foliage treatment, and as a seed dressing (Tomlin, 2000).

Imidacloprid (in the form of *Admire 240 Flowable*) was sold and used for the first time in Canada in 1995 for the control of the Colorado potato beetle in eastern Canada (PMRA, 2001). It is also approved for use on the Colorado potato beetle in tomato crops, to control the Spotted Tentiform Leafminer in apple crops, aphids in field lettuce, and aphid and whitefly control in greenhouse-grown plants. In addition, it is used as a seed treatment in Canola, rape, mustard and corn (PMRA, 2001). It was first registered for use in the United States in 1994 (Cox, 2001).

Crops to which imidacloprid is applied include: grains, maize, fruits, vegetables, potatoes, hops and turf (EXTOXNET, 1998; Tomlin, 2000). Typical application rates range from

approximately 50 to 320 g/ha (PMRA EDDENet Labels search 2005). Application rates vary with plant type. For instance, the recommended application rate for the formulation Admire 240F to potato crops is 1.3 L Admire/ha (i.e., 312 g imidacloprid/ha) to soil, or 26-39 ml Admire/100 kg seed pieces (i.e., 6.2-9.4 g imidacloprid/100 kg seed pieces) (PMRA EDDENet Labels search, 2005). For tomatoes, the application rate is 200 ml Admire/ha (i.e., 48 g imidacloprid/ha) to foliage, or 7-10ml Admire/100 m row (i.e., 1.7-2.4 g imidacloprid/100 m row) to soil. Admire 240F may be applied to field lettuce foliage at the rate of 200 mL/ha (i.e., 48 g imidacloprid/ha) and to soil at the rate of 650 ml/ha (i.e., 156 g imidacloprid/ha).

Imidacloprid is also used in urban areas to control turf pests in household lawns, parks, athletic fields, golf courses, etc., and this type of use appears to be increasing. For example, in Ontario, licensed pesticide applicators have started using imidacloprid on lawns and turf as a replacement for diazinon, which was taken off the market for lawncare use in 2004 (John Struger, Environment Canada, personal communication, October 2006; Struger *et al*., 2002). For treatment of turf grass to control white grubs, the recommended application rate is approximately 280 g a.i./ha (PMRA EDDENet Labels search, 2005). Formulations of imidacloprid are available as: a slurry for seed treatments, flowable concentrate for seed treatment, granule, wettable powder, soluble concentrate, suspension concentrate (flowable concentrate), water dispersible granules, and dustable powder (Tomlin, 2000). Formulations of imidacloprid include other chemicals such as crystalline quartz silica (e.g., Merit 0.5G) and naphthalene (e.g., Leverage 2.7) (Cox, 2001).

Imidacloprid is also used for flea control on domestic pets (Tomlin, 2000). It is typically available as a solution that can be applied topically once a month to dogs and cats (PMRA EDDENet Labels search, 2005). Products contain varying percentages of active ingredient depending on the weight of the animal to which it is intended to be applied.

Recently, imidacloprid has been investigated for potential use in controlling emerald ash borer, an exotic insect pest on ash trees in North America, through either direct stem injections or soil injections around the tree (Kreutzweiser *et al*., 2007b).

2.6.1 Acute Toxicity

Imidacloprid is moderately toxic if ingested. Oral LD_{50} values in rats were estimated to be 450 mg/kg for both sexes in one study and 500 and 380 mg/kg in males and females, respectively in another study (Tomlin, 2006). In mice, LD_{50} values were estimated at 130 mg/kg for males and 170 mg/kg for females (Yamamoto *et al*., 1999). Imidacloprid is very

low in toxicity via dermal exposure. The dermal LD_{50} in rats was estimated at greater than 5000 mg/kg (Tomlin, 2006).

Researchers did not observe eye or skin irritation in rabbits (Yamamoto *et al*., 1999). Imidacloprid is not considered a skin sensitizer although reports of hypersensitivity in skin following exposure to imidacloprid have been reported in companion animals (Plumlee *et al*., 2004).

Imidacloprid is variable in toxicity if inhaled. The inhalation LC_{50} was estimated to be greater than 5323 mg/m^3 for dust and 69 mg/m^3 for aerosol exposure in rats. Imidacloprid dust is considered slightly toxic but the aerosol form is highly toxic (Tomlin, 2006; Thyssen, *et al*., 1999)

Salivation and vomiting have been reported following oral (Hovda *et al*., 2002; Wismer, 2004). Very high oral exposures may lead to lethargy, vomiting, diarrhea, salivation, muscle weakness and ataxia, which are all indicative of imidacloprid's action on nicotinic receptors (Wismer, 2004). Other signs of exposure at high doses are uncoordinated gait, tremors, and reduced activity (Thyssen *et al.,* 1999).

Three case reports of attempted suicides described signs of toxicity including drowsiness, dizziness, vomiting, disorientation and fever (Wu *et al*., 2001; Shadnia *et al*., 2007). Pet owners have reported contact dermatitis following the use of veterinary products containing imidacloprid on their pets (Tomlin, 2006)

2.6.2 Chronic Toxicity

Rats consumed imidacloprid in their diet for three months at doses of 14, 61, and 300 mg/kg/day for males and 20, 83, and 420 mg/kg/day for females. Researchers noted reductions in body weight gain, liver damage, and reduced blood clotting function and platelet counts at 61 mg/kg/day in males and 420 mg/kg/day in females. Liver damage disappeared after exposure ended, but abnormalities in the blood were not entirely reversible. (Eiben and Rinke, 1989). No studies were found involving human subjects chronically exposed to imidacloprid. No data were found evaluating the potential of imidacloprid to disrupt endocrine function.

Although signs of toxicity were noted, researchers concluded that imidacloprid showed no evidence of carcinogenic potential (Thyssen *et al*., 1999). A range of studies using both *in vitro* and *in vivo* techniques concluded that imidacloprid did not damage DNA. The U.S. EPA has classified imidacloprid into Group E, no evidence of carcinogenicity, based on studies

with rats and mice. No human data were found on the reproductive effects of imidacloprid (Thyssen *et al.*, 1999).

2.7. Guidelines from other jurisdictions

Quebec, in 1998, derived interim guidelines for imidacloprid, in accordance with the Ministère de l'Environnement du Quebec protocol for water quality criteria (MENVIQ, 1990), which largely follows the U.S. EPA protocol (Stephan *et al*., 1985). This method utilizes the Final Acute Value (FAV) as an estimate of the concentration corresponding to a cumulative probability of 0.05 of the LC_{50} values for genera with acceptable acute data (Stephan *et al*., 1985). Where the geometric mean of the acute toxicity values for a sensitive species of concern results in a lower value than this estimate, the Species Mean Acute Value is used instead. The FAV for imidacloprid was determined as 85,090 μg/l, based on the geometric mean of two *Daphnia magna* LC_{50} values. The acute guideline was calculated by dividing the FAV by an interspecies sensitivity factor of 5 (because salmonids are represented), and by a factor of 2 to move from a 50% mortality to a low percentage of mortality. The acute guideline is 8509 μg/l. Studies that reported chronic values were not readily available, so the Chronic Aquatic Life Criteria was determined through application of an interspecies sensitivity factor of 5 and a standard acute/chronic ratio of 45 to the FAV, resulting in an interim chronic criteria value of 378 μg/l (Guay, 1998). It should be noted that data for more sensitive species such as insects (e.g., chironomids, mayflies) or ostracods were not available in the dataset used to derive the Quebec criteria.

The U.S. EPA (1995) has established a chronic reference dose (RfD) of 0.057 mg/kg/day, for use in human health risk assessment. This RfD is generated from a study that determined the no adverse effects level (NOAEL) for male rats exposed to dietary intake of imidacloprid. The RfD was calculated by dividing the NOAEL by an uncertainty factor (UF) of 100.

In Switzerland, imidacloprid was registered by the Federal Office of Public Health (FOPH) as a Toxic Substance Class 3 (SFOPH, 2005) within the Information System for Dangerous and Ecologically Relevant Substances (IGS). Switzerland's Toxic Substances classification is based on acute oral threshold levels, usually determined on rats. The acute oral threshold for a Toxic Substance Class 3 is 50-500 mg/kg (SFOPH, 2005).

2.8. Degradation of imidacloprid in water

Mobay (1989) found the two major metabolites via hydrolysis were 1-[(6-chloro-3pyridinyl)methyl]-4,5-dihydro-1H-imidazol-2-amine] (imidacloprid guanidine) and 6-chloro-3-pyridyl-methylethylendiamine. Zheng et al. (1999) found the only main metabolite was 1-[(6-chloro-3pyridinyl)methyl]-2-imidazolidone.

The aqueous photolysis half-life is less than 3 hours (Wamhoff et al., 1999; Moza, 1998). The prime degradation products resulting from photolysis in water (figure 2.1).

• 1-[(6-chloro-3-pyridinyl)methyl]-2-imidazolidinoe (imidacloprid urea)

• 6-Chloro-nicotinaldehyde

• N-methylnicotinacidamide

• 6-chloro-3-pyridyl-methylethylendiamine

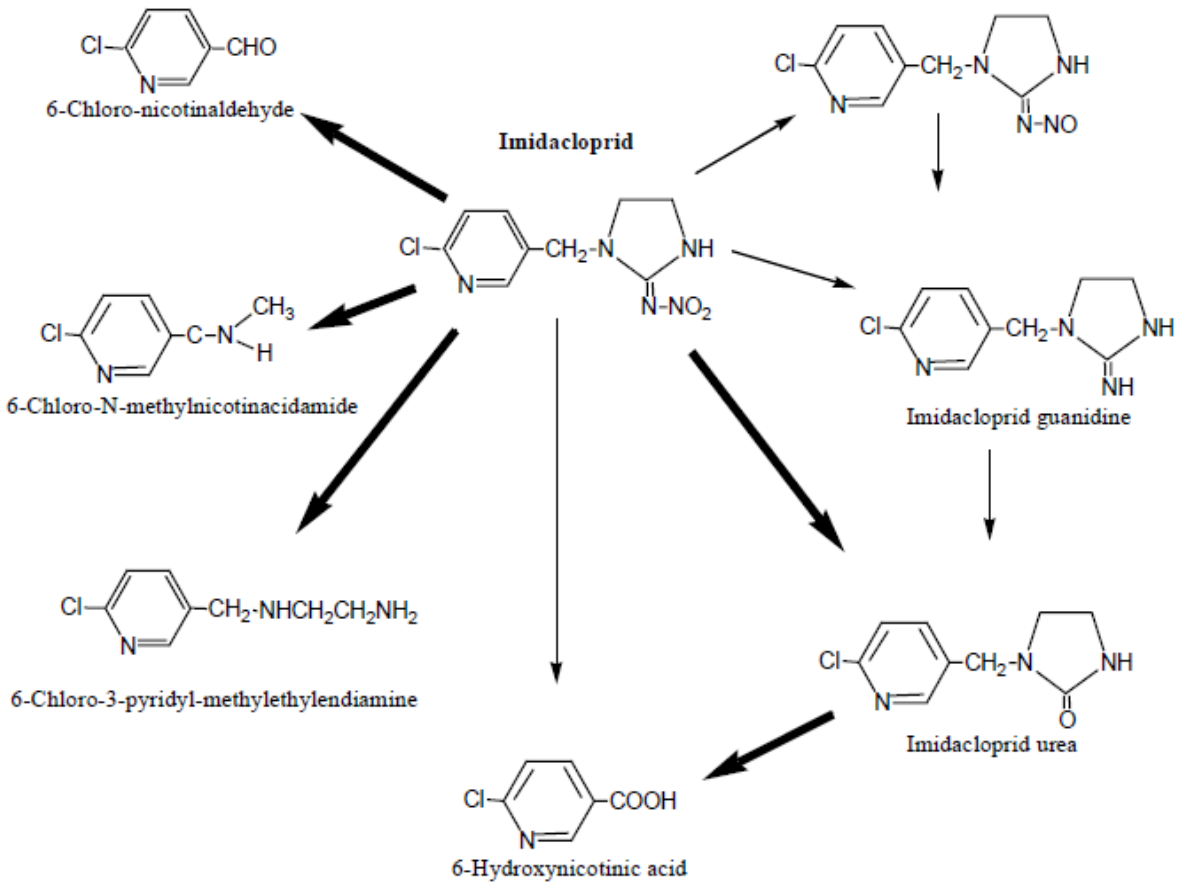


Figure 2.1: Proposed pathway of the degradation of imidacloprid in water (Bacey, 1995)

2.9. Degradation of imidacloprid in soil

The prime breakdown products from imidacloprid in soil include 3 metabolites (figure 2.2).

- 1-[(6-chloro-3-pyridinyl)methyl]-2-imidazolidinoe (imidacloprid urea)
- 6-chloronicotinic acid
- 6-hydroxynicotinic acid

CO_2 is then formed from 6-chloronicotinic acid (Miles Inc., 1993)

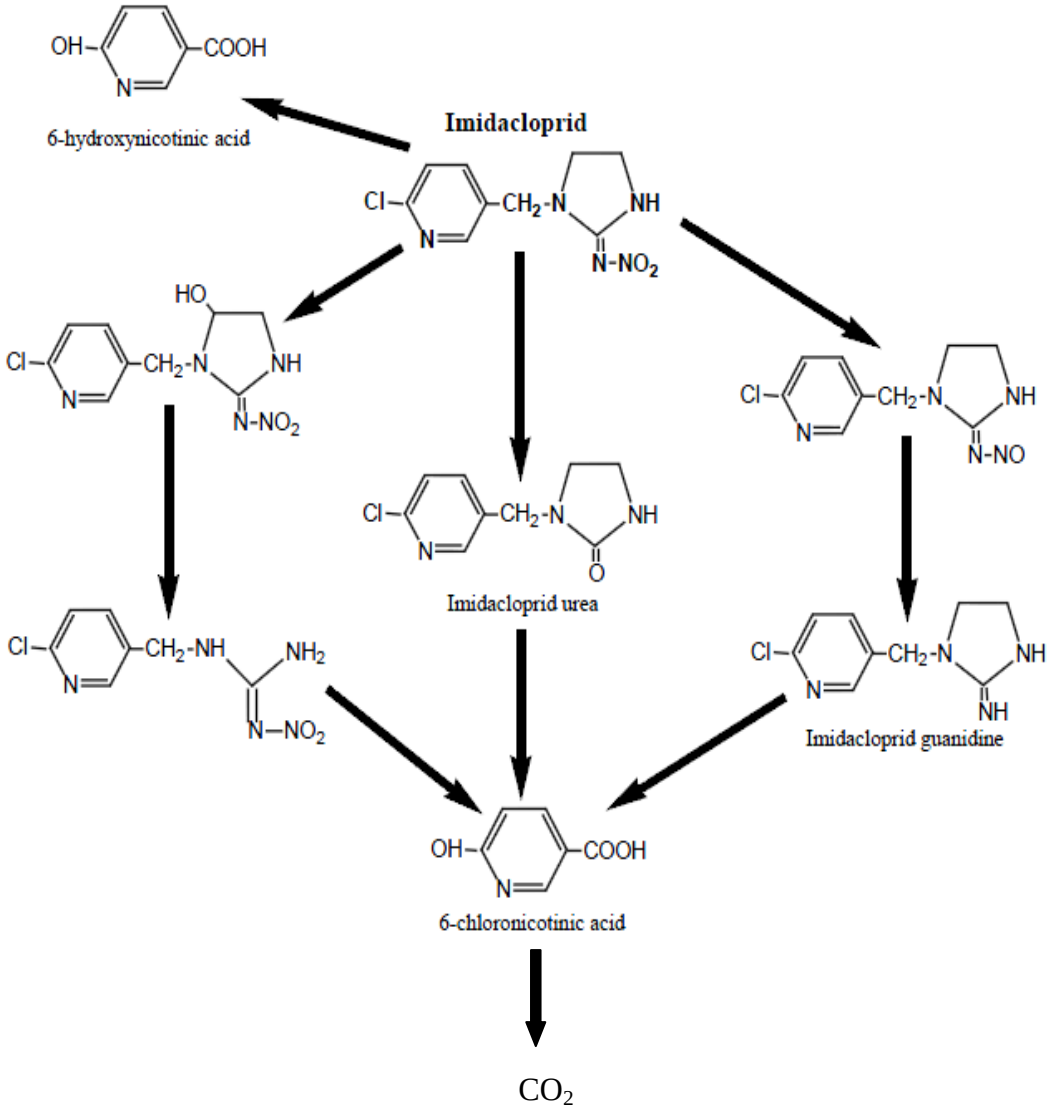


Figure 2.2: Proposed pathway of the degradation of imidacloprid in the soil (Scholz, 1992)

Chapter 3

Physiochemical analysis of the pesticide treated sites

3.1. Collection of soil samples

Soil samples were collected from imidacloprid and other pesticides treated areas (Site 1 and Site 2) where the potato plants *Solanum tuberosum* of variety ATL and FC-1 were grown (pH 8.3 and 8.1 respectively) and one normal soil without any pesticide treatment (pH 8.5) from fields of Thapar University, Patiala, Punjab and stored at 4ºC.

3.2 Total microbial count in soil samples

Total microbial flora of these three soil samples (Site 1, Site 2 and normal soil) were determined by serial dilution method (10^{-5}, 10^{-6}, 10^{-7} for bacteria, 10^{-4}, 10^{-5}, 10^{-6} for actinomycetes and 10^{-3}, 10^{-4}, 10^{-5} for fungi) followed by spread plate method in which 100 µl of these diluted samples were spread on nutrient agar (NA) and potato dextrose agar (PDA) plates. NA plates were incubated at 37ºC for 24 hrs in order to count microbial flora (bacteria, actinomycetes). PDA plates were incubated at 28ºC for 48 hours to check fungal growth.

3.3 Physiochemical analysis of soil

Prior to analysis, the soil samples (Site 1, Site 2 and normal soil) were air-dried and sieved through a 2 mm mesh sieve for physical analysis. Further, the 2 mm-sieved soil was crushed to pass through 0.2 mm sieve for chemical analysis.

3.3.1 Determination of pH

pH of soil was measured potentiometrically in a 1:2 or 1:5 soil water suspension or in a saturated soil plates.

Procedure

1. 25g of air dried soil samples were weighed and taken in a 100 ml beaker.
2. Added 50 ml of distilled water and thoroughly stirred for 2-3 min using a glass rod.
3. Further, it was kept in shaking condition (120 rpm) for 2 h.
4. Suspension was allowed to settle down for 30 min.

5. Mean while, pH meter was switched on and checked with two buffer solutions of known pH viz. one acidic and other alkaline with the help of standardization knob.
6. The pH of sample was measured by immersing the electrode in supernatant solution and recorded when the reading was stabilized (usually after 30 sec).
7. The electrode was rinsed with distilled water and carefully wiped with filter paper for every sample.

3.3.2 Available phosphorus (P)

Reagents for the estimation of available phosphorus

1. **0.5M $NaHCO_3$ extracting solution –** 84g of sodium bicarbonate was added in distilled water and volume was made upto 2 litre. The pH was adjusted to 8.5 with 1M or 1N NaOH
2. **Reagent A -** 12.0g ammonium molybdate in 250ml distilled water and 0.2908g antimony potassium tartarate in 100ml distilled water was added to 1000ml of 2.5M H_2SO_4 ,mixed thoroughly and volume was made upto 2 litre with distilled water
3. **Reagent B (freshly prepared) –** 1.058 g of ascorbic acid was added in 200ml of reagent A and mixed
4. **Sulphuric acid (2.5M) –** 140 ml of conc. H_2SO_4 was diluted to 1 litre
5. **Stock standard P solution (50 ppm) –** 0.2917 KH_2PO_4 was dissolved in distilled water to a final volume of 1 litre
6. **Working standard P solution(1 ppm) –** 20ml of 50 ppm solution was diluted to 1 litre

Procedure for estimation of available phosphorus (Olsen *et al*., 1954)

1. 2.5g soil was weighed and 50 ml of extracting solution was added to it.
2. Kept on a shaker for 30 minutes and was filtered through whatman filter paper no. 42
3. 10ml aliquot of filterate was transferred to a 100ml beaker
4. 1ml of 2.5M H_2SO_4, 15.5ml distilled water, 8ml reagent B and again 15.5ml of distilled water was added.
5. After 10 minutes, the intensity of the colour was measured at 882 nm against blank
6. Blank was prepared as above without the soil
7. To prepare standard curve, 0, 2, 5,10, 15 and 20 ml of 50 ppm standard stock solution was measured in 50 ml volumetric flask separately and followed the steps as above.
8. The P concentration of these solutions were 0.04, 0.1, 0.2 ,0.3 and 0.4 ppm respectively. After 10 min read the P concentration at 882 nm.

Calculation

Available P in soil (ppm): P in extract (ppm) × 20 (standard soil to solution ratio)

3.3.3 Total phosphorus (P)

Reagents for the estimation of total phosphorus

Vanadomolybdate solution –

1. **Solution A –**25 g ammonium molybdate [$(NH_4)_6 Mo_7O_{24}.4H_2O$] was dissolved in 300 ml water in a 500 ml beaker
2. **Solution B –**1.25 g ammonium vanadate (NH_4VO_3) was dissolved in 300 ml boiling water, cooled and 250 ml concentrated HNO_3 was added and cooled again. Solution A was added to solution B and was made upto 1000ml in a volumetric flask.
3. **Phosphorus stock standard solution (50 mg/l) –**0.2195 g of dried KH_2PO_4 was dissolved in distilled water and mixed thoroughly. Acidified with 25 ml of 7N H_2SO_4 and made the volume upto 1 litre to get 50 mg/ml P solution. 4 to 5 drops of toluene was added to prevent microbial activity. (KH_2PO_4 was dried to 100°C for 1 hour and cooled

Sample preparation for elemental analysis

For the release of mineral elements from soil and sediments, di acid (HNO_3- $HClO_4$) oxidation of sample was carried out.

HNO_3/ $HCLO_4$ digestion

1. 1 g sample of air dried soil was weighed in digestion tube and added 10 ml concentrated HNO_3 digest on electric heater for 1hr at 145°C in acid proof digestion chamber having fume exhaust system
2. Allowed to cool it and 5 ml $HClO_4$ was added and heated to about 100°C for the first one and then raised the temperature to about 200°C
3. Continued the digestion until the contents become colourless and only white fumes appeared
4. Reduced the acid contents till white matter remains left in the digestion tube
5. After this removed from the heating mental and cooled and 50% diluted HCl was added and filtered through whatman filter paper no. 42
6. 2 or 3 washings with 50% diluted HCl was given and final volume made was 50 ml with diluted 50% HCl

Procedure for the estimation of total phosphorus in soil and plant samples (Kitson and Mellon, 1944)

Ammonium molybdate reacts under acidic conditions to form a heteropoly acid and molybdophosphoric acid. In the presence of vanadium, yellow vanadomolybdate acid is formed. The intensity of colour is propotional to phosphorus concentration.

1. 10 ml of acid digests of soil sample was placed in 50ml volumetric flask, 10 ml of the vanadate molybdate reagent was added and diluted to 50 ml
2. Mixed well and read the phosphorus concentration after 10 minutes using spectrophotometer at 420 nm.
3. Blank was prepared by taking 10 ml of distilled water in place of 10 ml of acid digests of soil sample
4. For standard readings, 0, 1, 2, 3, 4 and 5 ml of 100 mg per litre stock phosphorus solution was taken in 50 ml volumetric flask and the colour was developed as mentioned above
5. Calibrated the spectrophotometer with known phosphorus concentration and read the concentration of the sample.

Calculation

P (mg/kg):

$$\frac{Volume makeup\ after\ acid\ digestion}{Weight\ of\ sample(s)\ to\ develop colour\ (ml)} \times \frac{50}{volume of\ digest\ used} \times P(mg)\ in\ 50ml\ solution$$

Where volume make up after acid digest was 50 ml

3.3.4 Organic carbon

Reagents for the estimation of organic carbon

1. **1N potassium dichromate –** 49.04 g was added in distilled water and volume was made upto 1 litre
2. **0.5N ferrous ammonium sulphate –** 198 g was added in distilled water and volume was made upto 1 litre
3. **Diphenyl amine indicator –** 0.5 g of diphenyl amine indicator (DPA) was dissolved in a mixture of 200ml water and 100ml concentrated H_2SO_4

Procedure for estimation of organic carbon and organic matter (Walkley and Black, 1934)

1. 1 g of soil was taken in 500 ml conical flask and 10 ml of 1 N $K_2Cr_2O_7$ was added.

2. The flask was swirled for mixing the soil and reagent.
3. 20 ml of concentrated H_2SO_4 was added and the flask was allowed to stand undisturbed for 30 minutes after which 200ml of distilled water was added
4. 1 ml of diphenylamine indicator was then added
5. Ultimately the contents were titrated with freshly prepared 0.5N ferrous ammonium sulphate till the end point is observed from blue violet to green.
6. Run a blank without soil sample and followed the steps as above

Calculation

Organic carbon (%): $\frac{10(B-T)\times 0.003\times 100}{B\times weight\,of\ soil(g)}$

Where

B is volume of ferrous ammonium sulphate solution for blank titration

T is volume of ferrous ammonium sulphate solution for soil sample

Because organic matter contains 58% carbon, so

Organic matter (%): Organic carbon (%) × 17.24 (van bemmelen factor)

3.3.5 Total nitrogen

Reagents for the estimation of total nitrogen in soil

1. **Concentrated H_2SO_4**
2. **0.02 N H_2SO_4**
3. **Sulphuric salicylic acid –** 1g salicylic acid was mixed with 30 ml sulphuric acid
4. **Sodium thiosulphate**
5. **4% boric acid –** 4g of boric acid was dissolved in 100 ml of distilled water
6. **Mixed indicator –** 0.066 g of methyl red and 0.099 g of bromo cresol green was dissolved in 100 ml of ethyl alcohol
7. **50 % NaOH**
8. **Digestion mixture –** 10 g HgO , 5 g $CuSO_4$ and 100 g K_2SO_4 (2:1:20)

Procedure for estimation of total nitrogen in soil (Kjeldahl method given by Piper, 1960)

1. 5 g soil was mixed thoroughly with sulphuric salicylic acid and followed by 5 g of sodium thiosulphate. Heating was carried out for 5minutes followed by cooling and addition of 10 g of digestion mixture. The contents were mixed well in a kjeldahl flask
2. The flask was kept in a digestion chamber at 100°C for two hours

3. The colour change was monitored from dark brown to greenish white after which the contents were cooled and 300 ml distilled water was added.
4. 20 ml of the digested sample, 15-20 ml NaOH and glass beads were added to the distillation flasks through the open end of the condenser attachment and stoppered. Water flow was maintained through the condenser.
5. The distillate was collected through a receiver tube in a beaker containing 15 ml boric acid and 2 drops of mixed indicator was added till the end point colour changes from pink to green.
6. The distillate was titrated against 0.02 N H_2SO_4 until the colour changed from green to pink.

Calculation

Total N (%): $\dfrac{(T-B)\times normality of\ H_2SO_4 \times 1.4 \times 300}{Weight\ of\ sample(g)}$

T is titre value for sample

B is for blank

3.4 Enzyme activities of soil

3.4.1 Acid phosphatase activity

Reagents for estimation of acid phophatase activity

1. **p– nitrophenyl phosphate solution (0.115 M) –** 4.268 g of p- nitrophenyl disodium salt hexahydrate was dissolved in 100 ml of modified universal buffer(MUB) pH 5
2. **NaOH (0.5N)** – 20 g of NaOH was dissolved in distilled water and the volume was made up to 1 litre
3. **p-nitrophenol –** 1mg per ml solution in modified universal buffer(pH 5)
4. **5X modified universal buffer (pH 5) (Skujins *et al*., 1962)**

Tris(hydroxyl methyl) amino methane	12.10g
Maleic acid	11.60g
Citric acid	14.00g
Boric acid	06.28g
NaOH	488 ml

   Volume was made upto 1000ml with distilled water

Procedure for the estimation of acid phosphatise activity (Tabatabai and Bremner, 1969)

Phosphatase activity was indicated as the amount of p-nitrophenol released in thefilterate from the p- nitrophenyl phosphate substrate per gram of soil.

The p- nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol.

1. 1 g of air dried soil was weighed and transferred to flask
2. 4 ml of modified universal buffer (pH 5) and 1 ml of filter sterlized 0.115M p-nitrophenyl phosphate solution was added to the flask
3. The flask was swirled for few seconds to mix the contents
4. The flasks were stoppered and incubated at 37°C for 1hour in dark
5. 4 ml of 0.5M NaOH was added to stop the reaction
6. Mixture was swirled and filtered through whatman filter paper no.2
7. Filterate was transferred to the glass cuvettes
8. The intensity of the yellow colour formed was measured at 410 nm
9. To perform control the above procedure was followed without the soil sample and 1 ml p-nitrophenyl phosphate was added after 0.5N NaOH

Calculation :

Phosphatase activity (µM PNP/g /hour): $\frac{Concentraton\ of\ PNP(\mu M)}{2\times Weight\ of\ sample(g)}$

3.4.2 Alkaline phosphatise activity

Reagents for the estimation of alkaline phosphatise activity

1. **p–nitrophenyl phosphate solution (0.115 M)** –4.268 g of p-nitrophenyl disodium salt hexahydrate was dissolved in 100 ml of modified universal buffer (MUB) pH 9
2. **NaOH (0.5N)** – 20 g of NaOH was dissolved in 100 ml distilled water
3. **p-nitrophenol solution (1 mg/ml)–** in modified universal buffer (pH 9)

5X modified universal buffer (pH 9) **(Skujins *et al*., 1962)**

Tris(hydroxyl methyl) amino methane	12.10g
Maleic acid	11.60g
Citric acid	14.00g
Boric acid	06.28g
NaOH	488ml

Procedure for the estimation of alkaline phophatase activity (Tabatabai and Bremner, 1969)

Phosphatase activity was indicated as the amount of p-nitrophenol released inthe filterate from the p- nitrophenyl phosphate substrate per gram of soil. The p- nitrophenol content wascalculated with reference to a calibration graphplotted from the results obtained by standards containing 0,10,20,30,40 and 50µg of p-nitrophenol.

1. 1 g of air dried soil was weighed and transferred to flask
2. 4 ml of modified universal buffer (pH 9) and 1 ml of filter sterlized 0.115 M p-nitrophenyl phosphate solution was added to the flask
3. Flask was swirled for few seconds to mix the contents
4. The flasks were stoppered and incubated at 37°C for 1hour in dark
5. 4 ml of 0.5M NaOH was added to stop the reaction
6. Mixture was swirled and filtered through whatman filter paper no.2
7. Filterate ws transferred to the glass cuvettes
8. The intensity of the yellow colour formed was measured at 410 nm
9. To perform control followed the above procedure without the soil sample and the addition of 1 ml p- nitrophenyl phosphate was made after 0.5N NaOH
10. Phosphate activity was indicated as amount of p-nitrophenol released in the filterate from p- nitrophenyl phosphate substrate per gram of the soil.
11. Data was recorded as microgram p-nitrophenol per gram of the soil.
12. Phosphatase activity was calculated in the unit of µM PNP/g /hour.

Calculation

$$\textit{Phosphatase activity } (\mu M\ PNP/g\ /hour) : \frac{Concentraion\ of\ PNP(\mu M)}{2 \times Weight\ of\ sample(g)}$$

3.4.3 Soil dehydrogenase activity

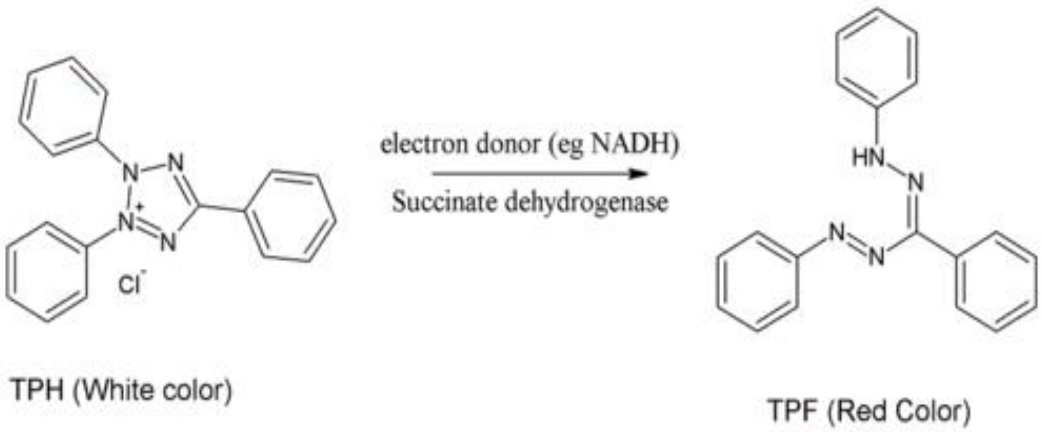
Reagents for the estimation of soil dehydrogenase activity

1. **1,3,5 triphenyl tetrazolium formazan stock solution(1mg/ml) –** 1mg of tetrphenyl tetrazolium formazan (TPF) was dissolved in 1 ml of methanol
2. **1,3,5 triphenyl tetrazolium formazan working solution (100µg/ml) –** added 2 ml of stock solution and volume was made upto 20 ml with methanol
3. **2,3,5 triphenyl tetrazolim chloride (3%) –**3 g of triphenyl tetrazolium chloride was dissolved in 100 ml of distilled water

4. **0.1% and 0.2% yeast extract** - 0.1 g and 0.2 g of yeast extract was dissolved in 100 ml distilled water respectively.

Procedure for the estimation of soil dehydrogenase activity (Cassida, 1977)

TTC (2,3,5- triphenyltetrazolium chloride) is a redox indicator used to differentiate between metabolically active and inactive tissues. The white compound is enzymatically reduced to red TPF (1,3,5- triphenylformazan) in living tissues due to the activity of various dehydrogenases (enzymes important in oxidation of organic compounds and thus cellular metabolism).



TPH (White color) TPF (Red Color)

1. 10 g of soil sample was weighed and calcium carbonate was mixed in the ratio of 100:1
2. 3g of each sample was dispensed in screw cap glass vials and 0.5 ml sterile water was added followed by 1 hour incubation at 28°C
3. 0.25 ml of single strength substrate solution was added (0.1 % yeast extract) followed by 0.25 ml distilled wate . The vials were incubated for 8 hours at 28°C
4. 0.5 ml of 3% aqueous TTC (2,3,5 triphenyl tetrazolium chloride) and 0.25 ml double strength (0.2%) yeast extract was added and mixed thoroughly with sterile glass rod.
5. This was followed by 6 hours incubation at 37°C followed by immediate extraction with 25 ml methanol and subsequent filteration through whatman no. 1 filter paper.
6. The methanol extract containing red coloured formazan was read at 480 nm
7. In 100 ml volumetric flasks 2, 5, 10, 15 and 20 ml of working standard of 100 µg/ml 1, 3, 5 triphenyl tetrzolium formazan was added (diluted from stock solution of 1mg/ml TPF) and final volume was adjusted with methanol.
8. The absorbance was read at 480 nm.

3.4.4 Urease activity

Reagents for the estimation of urease activity

1. **0.1 M potassium phosphate buffer (pH 8) –** 16 ml of 0.2mM KH_2PO_4 and 84ml of 0.2 mM K_2HPO_4 was dissolved and mix them well
2. **Urea solution(0.1 M) –** 6g of urea was dissolved in 1000ml of distilled water
3. **Phenolate solution –** 9.4g phenol and 12 mg of sodium nitroprusside was dissolved in 200 ml distilled water and made the volume to 500 ml
4. **NaHClO –** 4g NaOH was dissolved in 200ml distilled water and then 15 ml NaClO was added and the volume was made to 500 ml.

Procedure for the estimation of urease activity (Mc Garity and Myers 1967)

1. 10 gm of air dried soil was placed in 100 ml volumetric flask and treated with 1 ml toluene.
2. Therefore, 10 ml buffer (pH 7.0) and 5 ml 10% urea solution were added.
3. The flasks were shaken and incubated at 37°C for 3 hour.
4. For control 10 ml urea solution were replaced by 10 ml distilled water.
5. After incubation , volume of each flask was made upto 100 ml by adding distilled water
6. Flasks were then thoroughly shaken and their contents were filtered through whatman no.5 filter paper
7. NH_3 released as a result of urease measured by the indophenol blue method. Half a ml filterate was placed in 25 ml volumetric flask and 5 ml distilled water was added.
8. The mixture was treated with 2 ml phenolate solution and 1.5 ml NaHClO and volume of flask was made upto 25 ml by adding distilled water
9. The appearance of blue colour that developed as a result of urease activities was read spectrophotometrically at 630 nm.
10. One unit of urease is defined as the amount of enzyme hydrolyzing one µmole urea per min.

Calculation

Enzyme activity (µM/g/hr):

$$\frac{NH_3 \text{ concentration} \times \text{volume of enzyme used} \times \text{dilution factor}}{\text{Enzyme reaction period} \times \text{weight of sample(g)}}$$

3.5 Enumeration and arbuscular mycorrhizal colonization

Root samples of the potato plants, *Solanum tuberosum* of variety ATL and FC-1, contaminated with imidacloprid were collected from fields of Thapar University, Patiala. Vareity ATL and FC-1 were named as Site 1 and Site 2. Third root sample was collected from normal soil without prior imidacloprid treatment. All the respective samples (soil and roots) of each plant species were mixed thoroughly and preserved with proper labelling at 4 °C until processing. Root samples were stored in formyl-acetic acid [FAA: aquatic solution of 6.0% formaldehyde, 2.3% glacial acetic acid and 45.8% ethanol (all v/v)] prior to root percent colonization analyses.

Reagents for arbuscular mycorrhizal colonization

1. 0.1% teepol
2. 10% w/v KOH
3. 2% HCL solution(4mL v/v glacial HCl in 196 ml dH_20)
4. 0.05% Tryphan blue stain (10 ml glacial acetic acid, 200ml glycerol, 0.2g trypan blue, 190ml distilled water, *to accelerate, warm the solution)
5. Lactophenol

Ingredients	In 100 ml aqueous solution
Methyl Blue (Cotton Blue, Aniline Blue)	50 mg
Phenol	25 g
L(+)-Lactic Acid	25 g
Glycerol	50 l

6. mounting medium - typically Polyvinyl-Lacto-Glycerol (PVLG)
7. compound microscope with a 20x optical lens, a movable stage, and one eye piece fit with either a micrometer or crosshairs counter with 4 parts (or combination of a couple of counters to total 4 parts)

   The percentage of root length colonized by AM fungi was calculated by the gridline intersect method (Giovannetti and Mosse, 1980) after staining with trypan blue (Phillips and Hayman, 1970).

Procedure for AM mycorrhizal colonization

1. The roots samples collected from the potato plants of two different varieties ATL and FC-1 growing in the fields of Thapar University, Patiala were washed in 0.1% ‘Teepol’

followed by distilled water. Some amount of washed samples of roots were chopped to 1cm and simmered in 10% KOH at 90°C for 1-2 h.

2. The roots were rinsed 3-4 times in tap water and acidified by immersing them in 2% hydrochloric acid for 5 min.
3. The acid was drained off, 0.05% trypan blue in lactophenol was added and boiled again in stain for three min.
4. Drained off the stain, added lactophenol and left for 30 min to destain the tissues.
5. The roots were examined by mounting in glycerol.
6. Stained root samples were examined microscopically between 10 and 20x magnification to assess the percent root length colonization of AM fungi using the gridline intersects.

$$\%\ root\ length\ colonization\ of\ AM\ fungi = \frac{\text{No. of horizontal and vertical intersects with root}}{\text{No. of root pieces of infected with AM fungi}}$$

Isolation and characterization of imidacloprid degrading bacteria

3.6 Procurement of imidacloprid

Technical grade imidacloprid (97.8% purity) was obtained from Crop Science Limited, Gujarat. All organic solvents used (methanol, acetonitrile) Certified or Optima grade, were purchased from E Merck, India.

3.7 Media used

The media used for the microbial degradation of imidacloprid was Kaufmans and Kearney's minimal media (Table 3.1) in which microorganisms used imidacloprid as their carbon source.

Table 3.1: Composition of Kaufmans and Kearney's minimal media (1965)

Ingredients	**Quantity (g/l)**
K_2HPO_4	0.8
KH_2PO_4	0.2
$MgSO_4$	0.2
$(NH4)_2SO_4$	5.0
$(NH_4)_6Mo_7O_{24}$	0.001

Volume was made upto 1000 ml with distilled water

pH adjusted to 7.2 with 5N NaOH and HCl, strelized bt autoclaving at 15 lbs pressure (121ºC) for 15 minutes

And 1 ml of the trace metal solution which includes following ingredients:

Ingredients	**Quantity (ml)**
$FeSO_4.7H_2O$	5
$ZnSO_4.7H_2O$	4
$MnSO_4.4H_2O$	0.2
$NiCl_2.H_2O$	0.1
H_3BO_3	0.15
$CoCl_2.6H_2O$	0.5
$ZnCl_2$	0.25
EDTA	2.5

3.8 Enrichment, isolation and identification of microorganisms

Surface soil (0-15 cm) from repeatedly imidacloprid contaminated fields (since 10 years) in which potato plants were grown was being used for the isolation of imidacloprid degrading bacteria. Two soil samples were collected from two varieties of potato plant, namely ATL and FC-1 (Site 1 and Site 2) and the other from normal soil without imidacloprid treatment. All the three soil samples (5 g) in duplicates were taken in 250 ml Erlenmeyer flasks containing 100 ml of the minimal broth (Kaufman and Kearneys minimal media, 1965) and imidacloprid at a concentration of 50, 100, 150 mg/l incubated at 30°C with continuous shaking (150 rpm). After 20 days, 5 ml of broth culture from each flask was re-inoculated to 100 ml of fresh media with concentration of 50, 100, 150 mg/l imidacloprid and cultured under same conditions using imidacloprid as a sole carbon source. This process was repeated two more times. After that 0.2 ml of culture broth was spread on minimal agar plates (minimal media and 1.5% agar) for isolation of single colony. Each colony was considered as a different and streaked more than 4 times for screening of imidacloprid degrading bacteria before using for subsequent study.

3.9 Screening of efficient bacterial strain for degradation of imidacloprid

Screening was done by growing the bacterial isolates in the minimal media containing formulated imidacloprid (50, 100 and 150 mg/l) as a sole carbon source. Twelve bacterial isolates have shown the ability to grow on plates with minimal media and agar (1.5%). These isolates were then grown in minimal media containing imidacloprid of technical grade (98% purity) with the same concentration of 50,100 and 150 mg/l where two bacterial isolates (C-5 and C-9) were able to grow on plates with minimal media and agar (1.5%). These two bacterial strains were identified on the basis of morphological and biochemical tests like gram staining, motility, antibiotic resistance, nitrate and oxidase test.

3.10 Physiological characterization of bacteria

Morphological and biochemical studies of bacterial isolates

To characterize all the bacterial isolates conventional physiological and biochemical characterization tests were carried out as described in Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 1994).

3.10.1 Gram staining

Bacterial smear from actively growing cells were spread on a glass slide and heat fixed. Smear was flooded with filtered crystal violet for 10 sec and then washed briefly in water to remove excess crystal violet. Later it was flooded with Gram's iodine for10 sec and washed briefly in water. Smear was decolourised with acetone until the moving dye front has passed the lower edge of the section and washed immediately in tap water. Counterstaining was done with safranin for 15 sec and washed with water to remove the excessive stain. Finally samples were visualized under microscope at different magnification

3.10.2 Oxidase test

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colourless reagent becomes an oxidized coloured product. One drop of reagent (N,N,N',N'-tetra-methyl-p-phenylenediamine dihydrochloride) was added onto the bacterial culture on an agar plate. Positive reactions turned the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions were ignored.

3.10.3 Nitrate reduction test

Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. Nitrate broth contained nutrients and potassium nitrate as a source of nitrate. After incubating the nitrate broth, added a 2-3 drops of sulfanilic acid and α-naphthylamine. If the organism has reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. Sulfanilic acid was added; which reacted with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α-naphthylamine to form a red-colored compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it was considered a positive result for nitrate reduction.

3.10.4 Catalase test

The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2$). This reaction is evident by the rapid formation of bubbles. Place a microscope slide inside a petri dish. Collect a small amount of organism from a well-

isolated 18- to 24-hour colony and place it onto the microscope slide. Using a dropper or Pasteur pipette, place 1 drop of 3% H_2O_2 onto the organism on the microscope slide. Do not mix. Immediately cover the petri dish with a lid to limit aerosols and observe for immediate bubble formation (O_2 + water = bubbles). Observing for the formation of bubbles against a dark background enhances readability. Positive reactions are evident by immediate effervescence (bubble formation) microscope to observe weak positive reactions. If using a microscope, place a cover slip over the slide and view under 40x magnification. No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction.

3.10.5 Motility test (Hanging drop method)

Hanging drop preparation is useful for microscopic examination of living organisms, especially bacteria without staining them and to see their motility due to flagella. Clean and flame a hanging drop slide and placed it on the table with the depression uppermost. Spread a little Vaseline or petroleum jelly around the cavity of the slide. Clean a cover slip and apply petroleum jelly on each of the four corner of the cover slip using a matchstick. Place the cover slip on a clean paper with the petroleum jelly slide up. Transfer one loop full of culture in the centre of the cover. Placed the depression slide on the cover slip, with the cavity facing down so that the depression covers the suspension. Press the slide gently to form a cover slip between the cover slip and slide. Lift the preparation and quickly turn the hanging drop coverslip so that the culture drop is suspended. Examine it under low power and then high power microscope. Examine the motility of the organism. It is essential to distinguish between true motility and brownian moment which is an oscillatory movement possessed by all small bodies suspended in a fluid.

3.10.6 Antibiotic profiling of bacterial isolates

Bacterial isolates were grown in nutrient broth until the absorbance reached to 1.0. Absorbance was measured by spectrophotometer. The grown bacterial cells were spread on nutrient agar and antibiotic discs were kept on it. These plates were then incubated at 37°C over night and the inhibition zone was noted. Ready precoated antibiotic discs (Himedia Lab., India) were used to check the sensivity of bacterial isolates. These were vancomycin (Va) 30µg, Ofloxacin (Of) 5 µg , Teicoplanin (Te) 30 µg , Ceftazidime (Ca) 30 µg , Gentamycin (G) 10 µg, Cephotaxime (Cn) 30 µg.

3.11 Molecular characterization

3.11.1 Extraction of genomic DNA from bacteria

Isolation of genomic DNA generally comprises chemical cell disruption by enzymic digestion and detergent lysis; extractions with organic solvents, and selective recovery of the DNA.

Isolation of genomic DNA

A single colony of bacterial isolate was inoculated into 25 ml of nutrient broth in a 250 ml flask and incubated for 14-18 hours at 37°C under shaking condition (120 rpm). Liquid cultures (2.0 ml) were harvested by centrifugation (eppendorf microfuge) at 8,000 rpm for 1 min. The cell pellets were resuspended with 800 µl saline-EDTA, and approximately 50 µl of freshly prepared lysozyme were added. During incubation at 37°C for 30 min, the cell suspension was mixed thoroughly by inverting the Eppendorf tube several times. After addition of 200 µl SDS (10%), the cell suspension was incubated again at 65°C for 15 min. The cell suspension was extracted with organic solvents to remove proteins and cell debris: first, with an equal volume phenol:chloroform:isoamyl alcohol (25:24:1) solution, and centrifuged 10 min at 12,000 rpm. The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). To precipitate extracted nucleic acids, 0.7 volume isopropanol was added to the aqueous phase, followed by 10 min centrifugation at 12,000 rpm. The DNA pellets were washed with 750 µl EtOH (70%) and microfuged another 10 min. Finally, the pellets were resuspended in 40 µl TE buffer/milliQ water and stored at 4°C.

3.11.2 Electrophoresis of DNA on agarose gels

DNA was loaded on agarose gels (0.7 % w/v) prepared in 0.5X TBE, pH 8.0 using a 6X loading dye. Ethidium bromide (0.5µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then electrophoresed at 3 volts/cm for 45-60 minutes and visualized on a U.V. transilluminator.

3.11.3 Quantification of DNA by Nanodrop spectrophotometer

The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement at A_{260}. For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1 cm light path, is equal to a concentration of 50 µg/ml. The quality of the DNA was evaluated by measurement of the A_{260}/A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0 while the A_{230}/A_{260} ratio should be 0.3-

0.9. Ratios (A_{260}/A_{280}) less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

3.11.4 Amplification of 16S rDNA and Purification of PCR products

The polymerase chain reaction (PCR) provides a rapid and highly sensitive method for the primer-mediated enzymatic amplification of specific target sequences in genomic DNA resulting in the exponential increase of target DNA copies. For amplification of 16S rDNA gene following primers were used: Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGGCGGTGTGTTC-3' (Weisberg *et al.*, 1991). The amplification of 16S rDNA from isolates was carried out with a GenAmp thermocycler (Applied Biosystem, USA). Reaction mixture for the PCR contained 10X PCR buffer (Fermentas, USA), each dNTPs at a concentration of 2 mM, 50 mM $MgCl_2$, each primer at a concentration of 0.1 μM and 2.5U of Taq DNA polymerase (Fermentas, USA) in a final volume of 25 μl. PCR conditions were as follows: Preheating at 92°C for 5 min, 35 cycles of 92°C for 15 s, 55°C 30s and 72°C for 30 s and final extension 72°C for 5 min. Successful amplifications were confirmed by agarose gel (0.8% w/v) electrophoresis and ethidium bromide staining. PCR products, as they resulted from amplification of 16s DNA from bacteria samples, were purified by agarose gel electrophoresis prior to cloning. After staining with ethidium bromide, a defined band was visualized under UV irradiation and excised. Besides removing surplus primers, nucleotides, and salts, this method possessed the advantage that incomplete (shorter) amplification fragments are also removed prior to cloning. Subsequently, the DNA was extracted from the gel matrix material, using the SIGMA GenElute gel extraction kit, whereby the DNA is bound to silica gel particles, in the presence of high salt concentrations. Finally purified PCR products were eluted with 30 μl TE buffer (pH 8). In this manner, purified PCR products were applied directly for the cloning in pTZ57R/T vector.

3.11.5 Ligation of 16S rDNA in pTZ57R/T vector

The 16S rDNA amplicon was ligated into pTZ57R/T vector. The reaction mixture was prepared as described below and incubated overnight at 4°C.

Ligation reaction mixture:

Plasmid pTZ57R/T (55ng/μl)	1 μl
Insert (86ng/μl)	0.5 μl

Buffer (10X)	1 µl
T4 Ligase	1 µl
H_2O	6.5 µl

3.11.6 Genetic Transformation using $CaCl_2$

A single colony of *E. coli* DH5α from a freshly grown plate was inoculated into 25 ml of LB broth in a 250 ml flask and incubated the culture for 16-20 hrs at 37°C under shaking condition (120 rpm). Aseptically 200 µl of the above-saturated culture was transferred into 25 ml of fresh LB broth in a 250 ml flask. The culture was further incubated with vigorous shaking at 37°C for 2-3 hrs. To monitor the growth of the culture, the OD_{590} was determined at every one-hour (OD_{590} should be ~ 0.5). The above culture was transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The culture was cooled to 0°C by storing the tubes on ice for 10 min. The cells were harvested by centrifugation at 8,000 rpm for 10 minutes at 4°C. The pellet was resuspend in 10 ml of ice-cold 0.1 M $CaCl_2$ and store on ice for 15 min. Further the cells were recovered by centrifugation at 8,000 rpm for 10 min at 4°C. The cell pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$. The cells in this stage may be stored on ice for 12-24 hours. $CaCl_2$ treatment for 2 hours induces considerably a transient state of "competence" in the *E. coli* cells. One hundred micro liter of the suspension of competent cells was transferred to a sterile and prechilled microfuge tube (1.5 ml capacity). The plasmid DNA sample (~100 ng in a volume of 5 µl or less) was added to each tube. The content of the tubes were mixed gently and stored the tubes on ice for 30 minutes. The tubes were incubated in a circulating water bath that has been preheated to 42°C for exactly 2 minutes without shaking. The tubes were rapidly transferred to an ice bath and chill the cells for 1-2 minutes. One ml of LB broth was added to each tube and incubated the cultures for 45-60 minutes at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. One hundred micro liters of transformed cells was spreaded on each 90 mm Luria agar-Ampicillin-X-Gal-IPTG plates and incubated at 37°C. Transformed colonies should appear in 12-16 hours.

3.11.7 Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5α (Lac Z^-) bacterial host cells were plated on Luria agar medium containing 50 µg/ml ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for colonies containing a recombinant

plasmid. The cloning site in the pTZ57R/T vector is located in the multiple cloning site (mcs) of the plasmid's lacZα gene; if no insert is present, functional β-galactosidase is produced, and the transformed bacterial colony is blue. These few blue colonies occur due to the presence of supercoiled vector molecules, which have escaped linearization. However, if the host cell receives a recombinant plasmid containing a 16S rDNA insert in the lacZα gene, the resulting transformant colony is white (Lac Z^-).

3.11.8 Isolation and purification of plasmid DNA from recombinant bacteria by alkaline lysis method

The plasmid DNA was isolated based on the alkaline lysis method. A single transformed *E. coli* white colony was transferred into 2 ml of Luria broth medium containing appropriate antibiotic (ampicillin, used in a final concentration of 50 μg/ml) in a loosely capped 15-ml tube and incubated the culture overnight at 37°C with vigorous shaking. 1.5-2.0 ml of the above-saturated culture was poured into a microfuge tube and cells were harvested by centrifugation at 8,000 rpm for 1 min. The bacterial pellet was resuspended in 200 μl of ice-cold Solution I by vigorous vortexing to ensure that the bacterial pellet is completely dispersed in this solution. Further 200 μl of freshly prepared Solution II was added and the contents were mixed by gentle inversion of the tubes, five to ten times. Vortexed is avoided here. The tubes were stored on ice for 5 min. Finally 300 μl of ice-cold Solution III was added and mixed by inversion to disperse Solution III through the viscous bacterial lysate. The tubes were stored on ice for 10 min. The tubes were centrifuged at 12,000 rpm for 10 min in a microfuge. The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). To precipitate extracted plasmid DNA, 0.7 volumes isopropanol were added to the aqueous phase, followed by 10 min centrifugation at 12,000 rpm. The DNA pellets were washed with 750 μl EtOH (70%) and microfuged another 10 min. Finally, the pellets were resuspended in 40 μl TE buffer/milliQ water and stored at 4°C for further use.

Composition of Plasmid isolation solutions:-

Solution I

50mM glucose

25mM Tris HCl (pH 8.0)

10mM EDTA (pH 8.0)

Filter sterilized small batches of 100ml and stored at 4°C. Glucose will caramelize if autoclaved.

Solution II (Prepared freshly)

10M NaOH

10% SDS

Solution III

3M potassium acetate (pH 4.5)

3.11.9 Size screening for recombinant plasmids

Clones containing approximately 1.5-kb 16S rDNA inserts were identified by PCR screening, using the rapid protocol for preparation of template DNA from single bacterial colonies and M13-forward/M13-reverse plasmid primers. The amplification products were checked by agarose gel (0.8-1.0% w/v) electrophoresis.

3.11.10 Sequencing

The 16S rDNA inserts were sequenced for both strands using M13 forward and reverse primers, used for pTZ57R/T vectors. The sequence was generated by chain termination method (Sanger *et al.*, 1977) using an Applied Biosystems automatic sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

3.12 Inoculum preparation for degradation studies

All aerobic batch cultivations were carried out in 250 ml Erlenmeyer flasks containing 100ml of liquid culture. Two bacterial strains, C-5 and C-9, which were screened were made to grow on minimal media containing imidacloprid with concentrations of 50, 100, 150 mg/l as sole source of carbon. The control was setup as minimal media with imidacloprid as sole carbon source having same concentrations of 50, 100, 150 mg/l but without the bacterial inoculum. All these experiments were being performed in duplicates and incubated at 37ºC under shaking conditions (130 rpm).

3.13 Growth analysis

Growth of the two bacterial strains, C-5 and C-9 was observed in minimal media containing 50,100,150 mg/l imidacloprid by taking absorbance at 600nm on spectrophotometer on the alternative days for a period of 28 days.

3.14 Determination of pH

pH was checked with the help of pH meter for both the bacterial strains, C-5 and C-9 with different concentrations of imidacloprid in broth culture alternatively for a periods of 28 days.

3.15 Degradation of imidacloprid in broth culture (HPLC analysis)

5 ml of the minimal media in which imidacloprid is being degraded by two isolated bacterial strains C-5 and C-9, was taken after every 7 days for a month to estimate the imidacloprid degradation. Imidacloprid was extracted from this media with the ethyl acetate (media:ethyl acetate = 1:1) and the process was repeated thrice. The extracted solution was transferred to a 100 ml round bottom flask and the extract was evaporated using a rotary evaporator at 45°C. The imidacloprid residue was then redissolved in 1 ml of acetonitrile (ACN) for HPLC analysis. Periodic analysis of imidacloprid concentrations were accomplished using a Waters HPLC (division of Millipore, Milford MA) which has a Reverse Phase C-18 Symmetry Shield column (Waters Millipore) and an ultraviolet (UV) detector. The operating conditions were : 15 min run of a mixture of acetonitrile (55 %) and acidified water pH 3 (45%), injection volume of 20 µl, flow at 1 ml per minute and UV detection at wavelength of 247 nm. Media samples were filtered (0.22µ Millipore filter) prior to analysis. The retention time of imidacloprid under these conditions was 3.348 minute.

Field studies on degradation of imidacloprid contaminated sites

3.16 Soil microcosm experiment

There is a need for studies to be carried out in soil systems which mimic as much as possible those parameters which exist in a natural field system. Growth chamber microcosms which simulated mean field parameters were, in general, better predictors of field behavior. Obviously a compromise has to be reached in the number of variable parameters that can be used in any given study. Too many variables will make the study unmanageable and any results obtained unclear or indecisive. This is one advantage of a microcosm, in that the variables applied can be limited so that real ejects can be measured. However, there can be a danger in over-simplifying a system as dynamic as soil. Consequently, the use of simple microcosms containing only a few grams of soil must go hand in glove with more complex, scaled-up soil microcosms, to validate any results obtained.

Microcosm study involving 6 treatments were carried out in 2 litre capacity glass jars and the experiment was carried out in duplicates (Figure 5.1). Mass culturing of two bacterial isolates were done was done till late exponential phase and centrifuged at 5000 rpm at 4°C for 20 minutes. The supernatant was removed and pellet was washed thrice with double distilled water. Treatment 1 contains sterilized soil and imidacloprid at concentration of 50 mg/kg. Treatment 2 contains sterilized soil, bacterial inoculums (C-5) at concentration of 2×10^6 cells/gram of soil and imidacloprid at a concentration of 50 mg/kg. Treatment 3 contains natural soil, bacterial inoculum (C-5) at concentration of 2×10^6 cells/gram of soil and imidacloprid at a concentration of 50 mg/kg. Treatment 4 contains normal soil and imidacloprid at a concentration of 50 mg/kg. Treatment 5 contains sterile soil, bacterial inoculums (C-9) at a concentration of 2×10^6 cells/gram of soil and imidacloprid at a concentration of 50 mg/kg. Treatment 6 contains normal soil, bacterial inoculums (C-9) at a concentration of 2×10^6 cells/gram of the soil and imidacloprid at concentration of 50 mg/kg. Adequate amount of water was added to maintain soil moisture content. All the components were thoroughly mixed and the glass jars were covered with perforated aluminium foil and placed in light for 30 days. Samples were withdrawn after 7, 14, 21, 28 days incubation to estimate imidacloprid degradation in the soil microcosm.

Table 3.2: Different treatments on the imidacloprid contaminated soil (Soil microcosm)

Treatments	**Soil microcosm**
Treatment 1	Sterile soil + Imidacloprid (SS+I)
Treatment 2	Sterile soil + Imidacloprid + C-5 (SS+I+C-5)
Treatment 3	Normal soil + Imidacloprid + C-5 (NS+I+C-5)
Treatment 4	Normal soil + Imidacloprid + C-5 (NS+I)
Treatment 5	Sterile soil + Imidacloprid + C-9 (SS+I+C-9)
Treatment 6	Normal soil + Imidacloprid + C-9 (NS+I+C-9)

3.17 Extraction of imidacloprid from soil microcosm treatments (HPLC analysis)

The soil materials were thoroughly mixed before extraction of imidacloprid residues. Soil samples (5g) were weighed and extracted with 8 ml acetonitrile + water (80 + 20 by volume) and the process was repeated three times to extract imidacloprid residues in large amount. The suspension was stirred and shaken for 3 hours. The centrifugation was followed at 8000 rpm (4°C) for 20 minutes and the supernatant was filtered through glass wool and the shaking and filteration process was repeated three times. The combined extract solution was transferred to a 100 ml round bottom flask and the extract evaporated using a rotary evaporator using a rotary evaporator at 45°C. The residue was dissolved in 1ml methanol +water (80 +20 by volume) for HPLC analysis.

All samples were analysed on a Waters HPLC and the instrument conditions for the analysis of imidacloprid were as follows : C-18 reversed phase column kept at 25°C using a mobile phase of mixture of acetonitrile (ACN) + acidified water (pH 3) (55 + 45 by volume) at a flow rate of 1ml per minute for 15 minutes. Sample injection volume was 20 µl. Imidacloprid was detected with a photodiode array detector set to monitor absorbance at 247 nm. The retention time of imidacloprid under these conditions were 3.348 minutes. All samples were filtered through 0.2µ Millipore filter before HPLC analysis.

3.18 Analysis of chloride ions (Argentometric method)

In analytical chemistry, argentometry is a type of titration involving the silver (I) ion. Typically, it is used to determine the amount of chloride present in a sample.

The Mohr method uses chromate ions as an indicator in the titration of chloride ions with a silver nitrate standard solution. The sample solution is titrated against a solution of silver

nitrate of known concentration. Chloride ions react with silver (I) ions to give the insoluble silver chloride:

Cl^- (aq) + Ag^+ (aq) → AgCl (s) ($K_{sp} = 1.70 \times 10^{-10}$)

After all the chloride has been precipitated as white silver chloride, the first excess of titrant results in the formation of a silver chromate precipitate, which signals the end point. The reactions are:

$2Ag^+ + CrO_4^{2-} \rightarrow Ag_2Cr_2O_4$ (*s*)

By knowing the stoichiometry and moles consumed at the end point, the amount of chloride in an unknown sample can be determined. This report describes experiments aimed at determining the concentration of chloride in a solid sample

Reagents for analysis of chloride ions (Argentometric method)

1. **0.5 M K_2SO_4 extracting solution—**dissolved 87.1 g of potassium sulphate (K_2SO_4) IN 200 ml distilled water and volume was made upto 1 litre
2. **5% K_2CrO_4 (indicator)** – Dissolve 5 g K_2CrO_4 in about 5 ml distilled water (dH_2O). Add a few drops of silver nitrate until a definite reprecipitate forms. Let stand 12 hours, filter and dilute to 100 ml with dH_2O.
3. **Standard sodium chloride (0.0141 M):** Dissolve 824.0 mg NaCl (dried at 140°C) in dH_2O and dilute to 1000 ml.
4. **Standard AgNO3 solution:** 9.0 g of $AgNO_3$ was weighed out, transferred to 500 ml volumetric flask and made up to volume with distilled water. The resulting solution was approximately 0.1 M. This solution was standardized against 0.0141 M NaCl. In order to adjust the pH of the solutions, small quantities of $NaHCO_3$ were added until effervescence ceased. About 2 ml of K_2CrO_4 was added and the solution was titrated to the first permanent appearance of red $Ag_2Cr_2O_4$.

Procedure

5 g of samples were taken after every 7, 14, 21, 28 days from the microcosm experiment which was set up for the estimation of chloride ions. Chloride ions were extracted from each 5 g of samples using 30 ml of 0.5M K_2SO_4 extracting solution. The samples were shaken on a orbital shaker for 30 minutes. This process was repeated thrice. Collected extract was filtered using Whatman no. 42 filter paper. Release of chloride ions were estimated using argentometric method (Adriano and Doner, 1982). For this, 10 ml of the sample was taken in an Erlenmeyer flask. 3-4 drops of potassium chromate indicator was added and shaken well.

The sample was titrated against standardized $AgNO_3$ solution (0.0141N), till the red colour appeared which disappeared soon. At a point when all chloride ions were precipitated, a stable brick red colour was appeared at the end of the titration. Blanks would be just distilled water and typically require only one or two drops of titrant.

Calculation :

$$\text{Chloride concentration } \frac{\text{mgCl}}{\text{L}} \frac{(A-B)mL \times M \frac{mmol}{mL} \times 35.5 \frac{mgCl}{mmol}}{25ml \times \frac{1L}{1000mL}}$$

Where:

A = ml titrant used for sample

B = ml titrant used for blank (this might be 0 ml)

M= molarity of silver nitrate

3.19 PGPR activites of isolated bacterial strains

Plant growth-promoting rhizobacteria (PGPR**)** was first defined by Kloepper and Schroth to describe soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth.

PGPR have been reported to directly enhance plant growth by a variety of mechanisms: fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones. Direct enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPR has also been reported.

The isolated bacterial strains were checked for their PGPR activities by spreading their grown cultures (OD 0.5) on pikovaskaya (figure 5.2) agar for determination of phosphate solubilisation bacteria, Jensen media (figure 5.3) for nitrogen fixers and rock phosphate (figure 5.4) which degrade rock phosphates by spread plate method. However total nitrogen fixers and phosphate solubilizers were also calculated for the three soil samples.

Table 3.3: Compostion of pikovskaya agar (pikovskaya, 1948)

Ingredients	**Quantity (g/l)**
Glucose	10.0
$Ca_3(PO_4)_2$	25.0*
$(NH_4)_2SO_4$	0.5
NaCl	0.2
$MgSO_4.7H_2O$	0.1
KCl	0.2
Yeast extract	0.5
MnSO4	Trace
$FeSO_4.7H_2O$	Trace
Agar	15.0

Volume was made upto 1000 ml with distilled water

pH adjusted to 7.2 with 5N NaOH and HCl, strelized bt autoclaving at 15 lbs pressure (121ºC) for 15 min.

*Stock suspension of 2.5% $Ca_3(PO_4)_2$ was prepared in distilled water and was autoclaved for preparation of plates or broth, 10 ml of stock suspension was added aseptically to the 90 ml of sterilized medium

Table 3.4: Composition of Jensen media (Jensen, 1932)

Ingredients	**Quantity (g/l)**
Sucrose	20.0
Agar	15.0
$CaCO_3$	2.0
K_2HPO_4	1.0
$MgSO_4.7H_2O$	0.5
NaCl	0.5
$FeSO_4.7H_2O$	0.1
$Na_2MoO_4.2H_2O$	5.0

Volume was made upto 1000 ml with distilled water

pH adjusted to 7.2 with 5N NaOH and HCl, strelized bt autoclaving at 15 lbs pressure (121ºC) for 15 minutes

Table 3.5: Composition of rock phosphate medium

Ingredients	Quantity (mg/l)
Glucose	10.0
Rock phosphate	25.0
(NH4)2SO4	0.5
NaCl	0.2
MgSO4.7H2O	0.1
KCl	0.2
Yeast extract	0.5
MnSO4	Trace
FeSO4.7H2O	Trace
Agar	15.0

Volume was made upto 1000 ml with distilled water

pH adjusted to 7.2 with 5N NaOH and HCl, strelized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes

3.20 Indole acetic activity

Reagents for the estimation of indole acetic acid (IAA) activity

1. **0.5M $FeCl_3$** – dissolved 810 mg $FeCl_3$ in 10 ml distilled water
2. **35% $HClO_4$-**50 ml $HClO_4$ (70%) M was mixed with 50 ml of distilled water
3. **Tryptophan**- 0.1 % w/v
4. **Salper's reagent –** mixed 1 ml of 0.5M $FeCl_3$ with 50ml of 35% v/v $HClO_4$ and the total volume was made upto 51ml. this reagent was freshly prepared.

Procedure for estimation of IAA activity with and without tryptophan (Garden and Paleg, 1957)

1. 1% of the bacterial inoculum was inoculated in 5ml nutrient broth both with and without tryptophan (0.1%) in test tubes for 12 – 14 hrs
2. 2ml of the culture was taken and centrifuged at 10,000 rpm for15 min and supernatant was filtered through whatman filter paper
3. 1 ml of supernatant of each isolate was taken in separate test tubes and 2 ml salper's reagent was added dropwise but rapidly with continous mixing in each tube.
4. The samples were placed in dark for 30 minutes.

5. Development of pink colour was assayed with spectrophotometer at 535 nm

3.21 Nursery plantation experiment

After in-vitro testing of solubilization of Phosphates by two bacterial strains (C-5 and C-9) in shake- flask fermentation conditions. These test cultures were amended in soils and evaluated on maize, in terms of shoot height, root dry weight, shoot fresh and dry weight as per control treatments.

Soil was collected from fields of Thapar University, Patiala was oven dried at 70ºC for 12 hours incubation in between in order to destroy inherent microflora.

Reagents for preparation of slurry

1. **10% sugar solution** – dissolved 3 g of sugar in 30 ml of sterile distilled water
2. **40% gum arabic-** dissolved 12 g of gum arabic in the above solution and mixed well

Methodology for preparation of inoculum

1. Inoculated 1000 µl of bacterial inoculum in 500 ml broth culture
2. Kept in 37°C at 130 rpm for 3-5 days
3. Centrifuged at 8000 rpm for 5 minutes
4. Prepared bacterial inoculums in steri distilled water
5. Repeat the last two steps thrice

Methodology for preparation of slurry

1. Added 10g sugar in 100 ml sterile distilled water and heat the solution
2. After heating , added 40 g of gum arabic in hot solution
3. Mixed well and made a slurry
4. Cool at room temperature
5. And add the inoculum to slurry and mixed well

Seed treatment

Reagents for the seed treatment

1. Maize mature seeds harvested from field-grown plants.
2. 80% Ethanol (~300 ml for ~200 seeds).
3. 50% bleach solution: mix 450 ml of commercial bleach (5.25% hypochlorite) with 450 ml of Millipore water containing 2 drops of the surfactant Tween-20. Use ~900 ml for ~200 seeds.

4. 15% bleach solution: mix 15 ml of commercial bleach (5.25% hypochlorite) with 85 ml of Millipore water containing 1 drop of the surfactant Tween-20. Use ~100 ml for ~200dissected embryos.
5. Millipore water (autoclaved).

Seed treatment protocol

1. Place 50 seeds in a 250 ml beaker along with a stir bar.
2. Add ~ 75 ml of 80% ethanol, cover with the aluminum foil and place the beaker on a stir plate. Stir at medium speed for 3 minutes.
3. Take the beaker to the flow bench and decant the ethanol into a liquid-waste container.
4. Add ~ 75 ml of 50% bleach solution, cover with the aluminum foil and stir for 15 minutes on medium speed.
5. In the flow bench, decant the bleach into the liquid-waste container.
6. Sterilize the seeds a second time by repeating Steps 4 and 5.
7. Rinse the seeds 5 times with sterile Millipore water (~ 75 ml each time).
8. After the last rinse, keep seeds in ~50 ml sterile water (just enough to cover the seeds – do not overfill the container), cover with aluminum foil and leave the beaker inside the flow bench for 24 hours.

Seed inoculation

Seed inoculation of PGPR is done by mixing the PGPR is done by mixing the PGPR culture i.e inoculum with slurry to whish treated seeds are added with the result a uniform coat of PGPR culture around is formed the seed. The inoculated seeds were dried in shade and then sow the seeds in pots.

1. Inoculum was uniformly dispersed in the soil and approximately 50 g of soil was further added.
2. Check the seeds for inoculation density by serial dilution method
3. 4 pots of each colony 5 and colony 9 (added 2.621cells $\times 10^8$ cfu /pot of colony 5 and 2.912 $\times 10^7$ cfu/pot of colony 9) were sown after mixing the inoculum with 5 seeds maize seeds in each pot with the help of slurry.
4. Sowed 5 maize seeds equilaterally. Within a period of a week, three of the plantlets were pulled off
5. Similarly 4 pots of control in which 5 maize seeds in each pot without any bacterial inoculum were sown only with the slurry.

6. Recorded shoot height, fresh weight of shoot, dry weight of shoot and root, after thorough washing, for each treatment after drying at 65° C for 48 hours.
7. Viability of phosphate solubilizing bacteria was checked before the inoculation and after harvesting.

3.22 Available phosphorus in the soil

Procedure for the estimation of available phosphorus (Olsen *et al.*, 1954)

1. Weighed 2.5g soil taken from the pots after 1 month and 50 ml extracting solution was added to it.
2. Kept on a shaker for 30 minutes and was filtered through whattman filter paper no. 42
3. 10ml aliquot of filterate was transferred to a 100ml beaker
4. 1ml of 2.5M H_2SO_4 , 15.5ml distilled water, 8ml reagent B and again followed by 15.5ml of distilled water was added.
5. After 10 minutes, the intensity of the colour was measured at 882 nm against blank
6. Blank was prepared as above without the soil
7. To prepare standard curve, 0, 2, 5, 10, 15 and 20 ml of 50 ppm standard stock solution in 50 ml volumetric flask was measured separately and followed the steps as above.
8. The P concentration of these solutions are 0.04, 0.1, 0.2 ,0.3, 0.4 ppm respectively. After 10 min read the P concentration at 882 nm.

Calculation

Available P in soil (ppm) : P in extract (ppm) × 20 (standard soil to solution ratio)

3.23 Total phosphorus

Sample preparation for elemental analysis

For the release of mineral elements from soil and sediments, di acid (HNO_3-$HClO_4$) oxidation of sample was carried out.

HNO_3/ $HCLO_4$ digestion

1. Weighed 1 g sample of air dried soil, dry shoots ,roots in digestion tube and added 10 ml concentrated HNO_3 digest on electric heater for 1hr at 145°C in acid proof digestion chamber having fume exhaust system
2. Allowed to cool it and 10 ml concentrated HNO_3 and 5 ml $HClO_4$ and heated about 100°C for the first one and then raised the temperature to about 200°C

3. Continued the digestion until the contents become colourless and only white fumes appeared
4. Reduced the acid contents till white matter remains left in the digestion tube
5. After this removed from the heating mental and cooled and added 50% diluted HCl and filtered through whatman filter paper no. 42
6. Gave 2 or 3 washings with 50% diluted HCl and final volume made was 50 ml with diluted 50% HCl

Total phosphorus in soil and plant samples (Kitson and Mellon, 1944)

Ammonium molybdate reacts under acidic conditions to form a heteropoly acid and molybdophosphoric acid. In the presence of vanadium, yellow vanadomolybdate acid is formed. The intensity of colour is propotional to phosphorus concentration.

1. Place 10 ml of acid digests of soil ,shoot and root sample in 50ml volumetric flask, added 10 ml of the vanadate molybdate reagent and dilute to 50 ml
2. Mixed well and read the phosphorus concentration after 10 minutes using spectrophotometer at 420 nm.
3. Prepare the blank by taking 10 ml of distilled water in place of 10 ml of acid digests of soil, shoot and root, sample
4. For standard readings, took 0,1,2,3,4 and 5ml of 100 mg per litre stock phosphorus solution in 50 ml volumetric flask and developed the colour s mentioned above
5. Calibrated the spectrophotometer with known phosphorus concentration and read the concentration of the sample

Calculation

P (mg/kg):

$$\frac{Volume makeup\ after\ acid\ digestion}{Weight\ of\ sample(s)\ to\ develop colour\ (ml)} \times \frac{50}{volume of\ digest\ used} \times P(mg)\ in\ 50ml\ solution$$

3.24 To check the degradation of imidacloprid by test culture in pot experiment (HPLC analysis)

The soil materials were thoroughly mixed in pot and was extracted from different layers of the pot initially and after a month to see the degradation of imidacloprid in pot experiment. Soil samples (5g) were weighed in centrifuge tubes and extracted with 8 ml acetonitrile + water (80 + 20 by volume) and the process was repeated three times to extract imidacloprid residues in large amount. The suspension was stirred and shaken for 3 hours.The

centrifugation was followed at 8000 rpm (4°C) for 20 minutes and the supernatant was filtered through glass wool and the shaking and filteration process was repeated three times. The combined extract solution was transferred to a 100 ml round bottom flask and the extract evaporated using a rotary evaporator using a rotary evaporator at 45°C. The residue was dissolved in 1ml methanol +water (80 +20 by volume) for HPLC analysis.

/All samples were analysed on a Waters HPLC and the instrument conditions for the analysis o/.f imidacloprid were as follows : C-18 reversed phase column kept at 25°C using a mobile phase of mixture of acetonitrile (ACN) + acidified water (pH 3)(55 + 45 by volume) at a flow rate of 1ml per minute for 15 minutes. Sample injection volume was 20 µl. Imidacloprid was detected with a photodiode array detector set to monitor absorbance at 247 nm. The retention time of imidacloprid under these conditions were 3.348 minutes. All samples were filtered through 0.2µ Millipore filter before HPLC analysis.

Chapter 4

Results and Discussions

4.1 Total microbial count in soil samples

Within the last 30 years, the introduction of biotechnology and the modern farming techniques brought about tremendous changes in the conventional farming methods. A virtual revolution has taken place in modern agriculture by the introduction of the hybrid seeds, chemical fertilizers and crop protection chemicals which did lead to increased production. The use of pesticides (particularly herbicides) and synthetic fertilizers has increased dramatically over the past 60 years. However, between 1990 and 2006, the total area treated with pesticides increased by 30% in the UK, and the herbicide-treated area increased by 38%. Green revolution has made our country self sufficient to meet the food needs of our population; but the deleterious effect as a result of modernization of farming, the ecological balance has been altered by the constant application of crop protection chemicals, most of which are deleterious to human and animal health.

The insecticides also affect the microbial population of the soil even though micro-organisms are responsible for most of the degradation of insecticides in the soil. Several investigators (Agnihotri, 1973 and Peeples, 1974) have studied the influence of commonly used pesticides on soil microbes. Thus use of pesticide suppress microbial flora and fauna hence affect soil property (Hussain *et al*., 1986). In farmland habitats, population declines have occurred in about half of plants, a third of microbial species and four-fifths of bird species Marked reduction in fungal species number were recorded in the pesticide treated soils as compared to the normal soil (Robinson and Sutherland, 2002).

Three rhizospheric soil samples: two from pesticide treated area namely Site 1 and Site 2 and one normal soil without imidacloprid treatment were subjected to analysis to determine the effect of chemicals on soil microbial population and their mineralization activities. There has been a marked population decline of many microbial species living in soil (Boatman *et al*., 2007). As reported, there was significant reduction in the microbial flora in the pesticide treated areas. The total microbial count in normal soil was found to be 65.8×10^6 and in pesticide treated soils, Site 1 and Site 2 were 19.2×10^6 and 30.3×10^6 respectively. (Table 4.1). Similarly total fungal count in normal was 52.6×10^3 which was more as compared to Site 1 and Site 2 which was 12.1×10^3 and7×10^3 (Table 4.2) . Almost 3 fold decrease in microbial

population was found in Site 1 and 2 fold decrease in Site 2 respectively with respect to normal. Similarly there was 3 fold decrease in fungal count in Site 1 and 4 fold decrease in Site 2 as compared to normal soil.

The experiment was also aimed at evaluating the changes that occurred in soil nutrient levels as a result of the pesticide treatments. The detoxification capacity of soil depends on its microbial activity. The higher the microbial activity, the greater the capacity of the soil to counteract the effect of a pesticide. Decrease in percentage carbon, nitrogen, phosphorus and pH were recorded in pesticide treated soils.

Table 4.1: Total bacterial count in terms of CFU/g of soil samples

Soil samples	CFU/gm
Normal	65.8×10^6
Site 1	19.2×10^6
Site 2	30.3×10^6

Table 4.2: Total fungal count in terms of CFU/g of soil samples

Soil samples	CFU/gm
Normal	52.6×10^3
Site 1	12.1×10^3
Site 2	7.7×10^3

4.2 Physiochemical analysis:

4.2.1 Determination of pH

Soil chemical analysis results in the pesticide treated soil did not show any significant difference among all treatments, for soil pH which was 8.5, 8.3, 8.2 for the normal, Site 1 and Site 2 soil samples (Table 4.3). This could be due to the fact that the soil of the pesticide treated site had a relatively high buffering capacity based on its high carbonate content (22-28 %) and can fix any change in its pH due to organic matter decomposition.

Table 4.3: Comparison of pH with imidacloprid treated sites and normal site

Soil samples	pH
Normal	8.5±0.02
Site 1	8.3±0.01
Site 2	8.2±0.01

Values sharing a common letter within column are not significant at $P < 0.05$; values are mean ±Standard deviation (n=3)

4.2.2 Determination of soil characteristics

Population of the multifarious organisms like bacteria, fungi etc. is dependent on the nutritive conditions of soil and certain other factors such as soil texture, pH, water contents, aeration and so on. The physiochemical nature of the soil is important for persistence, metabolism and binding of pesticides in soil (Husaain *et al*., 1986 and Tayaputch *et al*., 2001).

Besides deleterious effects on microbial life, insecticides also affect the population and activity of beneficial organisms in soil (Bhuyan *et al.*, 1992; Hussain *et al*., 2001). Thus physical, chemical and climatic factors affect the growth and survival of the soil fauna and soil microorganisms.

Soil chemical properties may also be altered by accumulation of residual pesticides and their metabolites. These processes may disrupt the ecological balance in the soil micro environment, first by simplifying the microbial population, and possibly by reducing soil fertility and its ability to support life. Accumulation of pesticides in resistant or tolerant species may provoke episodes of toxicity to organisms higher in the food chain.

The main process that occurs in the soil is the organic matter decomposition. The heterotrophic population needs organic carbon to grow. The organic matter in normal soil was found to be 0.54% as compared to Site 1 and Site 2 with 0.24 and 0.27% (Table 4.4). 0.89% organic matter was observed as compared to in 0.41% Site 1 and 0.46% in Site 2. There was 53% and 48% decrease in organic matter pesticide treated soil namely Site 1 and Site 2 as compared to normal soil (Table 4.4)

Nitrogen supply and demand is essential to improve the efficiency of nitrogen used in agriculture systems due to both economic and environment concerns. It is much affected by the pesticide treatment. Moreover, pesticides have been shown to adversely affect N_2 fixation, either directly by affecting the rhizobia (Anderson *et al.*, 2004), disrupting the signaling between legume-derived phytochemicals (luteolin, apigenin) and Rhizobium Nod receptors

(Fox *et al.,* 2007). Similarly there was significant decrease of 61% in pesticide treated soils as compared to the normal soil. The total nitrogen was found to be 0.84% in normal soil and 0.33% in both Site 1 and Site 2 soil samples (Table 4.4).

Phosphorus is second only to nitrogen as a mineral nutrient required by both plants and microorganisms. However, some microbiological populations can solubilize the fixed phosphorus, turning it available for plants. These bacteria can be seen as a bio-fertilizer, as the plant themselves develop some mechanisms to invite these bacteria to grow near their roots. Again there was significant decrease in the available and total phosphorus values in pesticide treated soils. The available and total P in normal soil was found to be 16.6 and 216 mg/kg whereas in Site 1 it was 5.84, 72mg/kg and for Site 2 it was found to be 6.62 and 93.2 mg/kg (Table 4.4). Nearly 64% and 60% decrease in available phosphorus and 67% and 57% decrease in total phosphorus was found in Site 1 and Site 2 respectively as compared to normal soil. Thus phosphorus was found to be most sensitive to imidacloprid as it showed significant decrease in their values.

Thus presence of imidacloprid affects soil physiochemical properties which disturbs the soil ecosystem thus soil fertility and microbial flora and fauna.

Table 4.4: Comparison of physiochemical properties in normal and imidacloprid treated soil

Soil samples	**Available P (mg/kg)**	**Total P (mg/kg)**	**Organic carbon (%)**	**Organic matter (%)**	**Total nitrogen (%)**
Normal	16.6±0.06a	216±6a	0.52±0.01a	0.89±0.01a	0.84±0.02a
Site 1	5.84±0.13b	72±10b	0.24±0.00b	0.41±0.00b	0.33±0.01b
Site 2	6.62±0.22b	93.2±4b	0.27±0.01b	0.46±0.01b	0.33±0.001b

Values sharing a common letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

4.3 Enzyme activities in soil

Despite the beneficial impacts of insecticides in improving and stabilizing agricultural productivity by control of obnoxious weeds, fungi and insects, these allocthonous organic chemicals are known to contaminate soil ecosystem and pose threat to a balance equilibrium among various groups of microorganisms in soil, which play an important role in recycling plant nutrients. Needless to mention the process of ammonification, nitrification, nitrogen fixation, mycorrhizal association and solubilization of phosphates, enzyme activity of

microorganisms contributes to the soil fertility. These organisms in association with other soil microorganisms exist in harmony creating a biological equilibrium in soil. This equilibrium is quite often temporarily affected when pesticides either directly or indirectly reach the soil.

Soil has been a home for living biomass; conceding innumerable microbial activities leading to mineralization, immobilization of nutrients, which have been often the source of nutrients for the plant species, besides the microorganisms.

Cycon *et al*. (2005) investigated the effects of insecticide, herbicide and fungicide on the enzymatic activities in sandy soil for 28 days under laboratory conditions. The results showed that the influence of tested pesticides on dehydrogenase, acid and alkaline phosphatase and urease was diversified. Furthermore, these studies confirmed that enzyme activities are a useful tool for the detection of pesticide side effects in soil and they can be used as indicators of soil pollution

Soil dehydrogenase activity is often used as a measure of the metabolic activity of microorganisms in the soil. Any toxicant from the external environment added to the soil may alter the microorganisms and thus dehydrogenase enzymes. Several variations in dehydrogenase activity have been observed in soils with different insecticides and nutrients treatments. Scheunert *et al*. (2001) found that under laboratory conditions dehydrogenase activity was depressed significantly with the pesticide treatment in soil. On the contrary, Amrit and Amarjeet (2005) observed that dehydrogenase activity is not impaired owing to imidaclorprid application in treated mung field. The results came out in contrast to Amrit and Amarjit, there was decrease in the dehydrogenase activity in pesticide treated areas. Dehyrogenase activity was more in normal soil with 10.92 μM as compared to 7.2 μM in Site 1 and Site 2 (Table 4.5). The results came out as percent inhibitory values for pesticide treatments on dehydrogenase activity was found to be 28% decrease in both pesticide treated soils as compared to normal soil. The decrease in enzyme activity with the increase in concentration and spray schedule is due to the lethal action of applied insecticides on microorganisms, which in turn affect the enzymatic process. Similarly, various results have been reported in which decrease of dehydrogenase acivity was observed with different pesticides. Chandrayan and Sethunathan (1980), Purushothaman *et al*., (1974) observed inhibition of dehydrogenase activity by an insecticide, cytrolane. Similarly, inhibition of dehydrogenase was observed by the application of bromophos pesticide to the soil (Srimathi *et al*., 1986). Nelson (1985) also found significant decrease in dehydrogenase activity with

pesticide treatment and Mayanglambam *et al.* (2005) also observed 35.5 % inhibition of dehydrogenase activity by quinalphos application.

The acid and alkaline phosphtase activity was found to be 484 and 412 µM/g/hr in normal soil whereas 233 and 285 µM/g/hr in Site 1 and in 243and 342 µM/g/hr in Site 2 (Table 4.5). The percent inhibitory values for pesticide treatments on acid and alkaline phosphatase activity was found to be 18% , 31% decrease in Site 1 and 14% ,17% in Site 2 soil samples respectively as compared to normal due to affected microbial flora with the pesticide treatment. The inhibitory effect of pesticides on phosphatase may be attributed to the lethal actions of insecticides on P- solubilizers population which alter the membrane permeability of the microorganisms releasing phosphatase enzymes. Similarly reduction in phophatase activity was observed in many pesticide treated soils. This statement is supported by Voets *et al*. (1974) in their study on the effect of atrazine on phosphatase activity in a forest soil who concluded that the inhibition of the phosphatase activity was up to 61.8%. Similar results were also noticed by Krishnmurthy (1989) by employing fenvelerate and Kennedy and Arathan (2004) with carbofuran; Madhuri and Rangaswamy (2002) with dichlorvos, phorate and methomyl; Kalam *et al*. (2004) with propiconazol and Kennedy and Arathan (2004) with carbofuran. Kalam *et al*. (2004) observed maximum inhibition of soil phosphatase activity (46.6%) in presence of propiconazole, a fungicide (100 mg kg-1) after 120 days. On the contrary, Madhuri and Rangaswamy (2002) observed that soil samples receiving 2.5 kg ha-1 of the insecticides dichlorvos, phorate and methomyl and also in soil samples receiving 5.0 kg ha-1 of the insecticides, chloropyrifos and methyl parathion [parathion-methyl], the activity of phosphatase was significantly more at 20 days period of incubation and decreased progressively with increasing period of incubation.

The most substantial index of biological activity in soil is its enzymatic activity and that it can give an idea of the biochemical process in the soil. The enzyme acts as indicator of the soil fertility. He *et al. (*2003) reported that pesticides application significantly inhibited soil urease activity and the characteristic parameters of soil urease, such as its activity, maximum reaction velocity V and velocity constant k excluding K, were decreased with increasing concentrations. Urease activity was found to be 13 µM/g/hr as compared to 10 µM/g/hr in Site 1 and Site 2 (Table 4.5). The per cent inhibitory values of effect of pesticides treatments on urease activity calculated was 22% and 20% decrease in Site 1 and Site 2 as compared to normal soils. This decreased in urease activity may be attributed to decrease in microbial population by addition of pesticides as enzyme activity is directly related to the microbial

count in soil as reported by Dinesh *et al*. (2000). The per cent inhibitory values of effect of pesticides treatments on urease activity calculated was 22% and 20% decrease in Site 1 and Site 2 as compared to normal soils. This decreased in urease activity may be attributed to decrease in microbial population by addition of pesticides as enzyme activity is directly related to the microbial count in soil as reported by Dinesh *et al*. (2000). Similarly, inhibitory effect on urease activity upon insecticide application was observed by Lethbridge and Burns (1976) with organophosphorus. Basavaraj (1984) recorded inhibition of urease activity by 2,4-D, carbofuran and quintozene applied in combination to soil, Srimati *et al*. (1986) with bromophos, Elliot (1989) concluded that fungicides application will affect the urea hydrolysis in soil in the initial stages. Basavaraj (1984) with quinalphos at 100 *ppm*, Kennedy *et al.* (2004) with carbofuran at 1.5 kg/ha and Laksmikantha (2000) with fenvalerate, quinalphos and endosulfan at double recommended concentration (RC) and 4RC.

Table 4.5: Comparison of enzyme activities in normal and pesticide treated soil

Soil samples	Acid phosphatase (µM/g/hr)	Alkaline Phosphatase (µM/g/hr)	Soil Dehyrogenase (ppm)	Urease activity (µM/g/hr)
Normal	284.85±1a	412.20±4a	10.20±0.7a	13.6±0.6a
Site 1	233.67±3c	285.13±2c	7.29±0.2b	10.6±0.3a
Site 2	243.84±6b	342.64±5b	7.28±0..6b	10.9±0.5a

Values sharing a common letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

4.4 Determination of AM fungal percent colonization

There was significant decrease in percent AM colonization in pesticide treated soil as normal soil showed maximum colonization of 33.3% (Figure 4.1) followed by 8.6% in Site 1 and nil in Site 2 (Table 4.6) .This shows that indiscriminate use of pesticides and fungicides leads to a reduction in microbial flora and diversity and effects soil ecosystem. These results are in agreement with the data obtained by Cabello (1995, 1997) who found in field experiments that pesticide contamination affects the AMF population associated with plants. Thus, the determination of AMF development in roots could be a sensitive indicator of changes in soil

pollutant toxicity. As mycorrhizal fungi are important in agriculture and forestry as bidirectional nutrient transfer between host and fungal endophyte (i.e., drain of host carbon and uptake of soil mineral nutrients) drive many nutrient cycling processes in soil. The advantages of a diverse and healthy mycorrhizal community include better survival and nutrition of plants in stressed environments. Thus the determination of AMF development in roots could be a sensitive indicator of changes in soil pollutant toxicity (insecticides, pesticides)

There may be three reasons for the decrease in AMF colonization. Firstly by the reduction in the transfer of host photosynthate to the fungus. Walton *et al.* (1994) suggested that when a chemical stress is present in soil, a plant may respond either by increasing or changing exudation (carbon allocation) to the rhizosphere. Plants in the field responded to the presence of imidacloprid in the soil by expending part of their photosynthate to produce root exudates, thereby diminishing the supply of carbon to mycorrhizal fungi. The fungus could be directly affected by the action of imidacloprid.

Secondly, such mycorrhizal fungus behavior might be because of the production of inhibitory metabolites by the microbes when they degrades imidacloprid in the soil

Thirdly that microorganisms can take imidacloprid from the soil and accumulate it. However, when taken up by the hyphae, the toxin is not cleaved by any enzyme and is retained without modification. This demonstrates that, the fungus is able to remove the pollutant (pesticide residues) from the substrate which affects the soil properties and consequently microbial diversity and nature equilibrium. Such removal with bioimmobilization of the pollutant in may diminish the concentration of the free toxicant around roots in the soil. This could be of interest for the bioremediation of soils. Thus accumulation of imidacloprid may decrease AMF colonization.

Table 4.6: Comparison of the AM mycorrhizal fungal colonization in potato plants grown in pesticide treated areas and normal sites

Soil samples	**Percent colonization (%)**
Normal	33.3
Site 1	8.6
Site 2	Nil

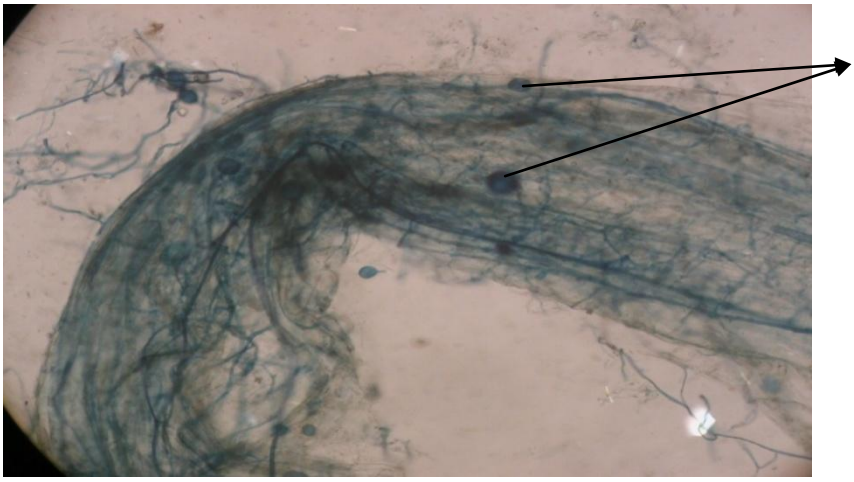


Figure 4.1: AM fungal colonizaion in normal soil

4.5 **Growth kinetics in minimal media**

The bacterial strains isolated from the soil samples were tested for the utilization of insecticide imidacloprid by each bacterial strain as a carbon source in minimal media. The bacterial growth and utilization of imidacloprid was estimated. The growth was accessed by taking absorbance at 600 nm of the bacterial suspension observed for C-5, C-9 in minimal media containing imidacloprid at three different concentrations (50, 100, 150 mg/l) for a period of 28 days. Both isolates were able to grow and utilize imidacloprid as their carbon source with with a lower O.D. Additionally, insecticides not only damage structural proteins essential for growth of the organism but also responsible for geno-toxicity (Pham *et al*., 2004) and eventually leads to the decreased functioning and survival of organisms exposed to high concentration of insecticide (Kumar *et al*., 2001). 0.4 O.D of the culture with bacterial inoculation was reported as maximum in the presence of imidacloprid at a concentration of 10 ppm (Ahemad *et al*., 2001). The growth of isolated bacterial strains was less due to the toxicity of the insecttcide. The growth profile showed maximum growth at 50 mg/l (Figure 4.2) (Table 4.7) followed by 100 mg/l (Figure 4.3) (Table 4.8) and 150 mg/l (Figure 4.4) (Table 4.9). Both the isolates showed normal growth curve with a short lag phase followed by the log phase and then comes near to the stationary phase in a month. 0.32 O.D was observed in the experiment as maximum in the presence of imidacloprid at a concentration of 50 ppm (Figure 4.2) (Table 4.7). Both the isolates took time of about 5-7 days to adapt and the growth increases. Both isolates took less time to adapt when imidacloprid concentration was less which means lag phase is small as compared to high concentrations. Growth was faster in

microorganisms haiving less imidacloprid concentration. Thus more the concentration of imidacloprid less is the growth which shows imidacloprid at higher concentration either inhibit the growth of microorganisms or hinder their activity or either kills most of the microorganisms. Thus imidacloprid at higher concentration proves toxic to microorganisms. Thus due to the deficiency of carbon source their growth is less in comparison to that of lower concentration. Maximum growth takes place after 10 days in all bacterial isolates. C-9 showed more growth in comparison to C-5 which means C-9 is having better ability to degrade imidacloprid and to use it as a carbon source. However both isolates showed almost same growth pattern and showed more growth in less concentration.

Table 4.7: Effect of imidacloprid (50 mg/l) on the growth rate of C-5 and C-9

Time (Days)	**Control**	**C-5**	**C-9**
1	0.028±0.03g	0.005±0.006g	0.002±0.01g
3	0.061±0.07fg	0.016±0.01g	0.018±0.01f
5	0.125±0.002ef	0.141±0.01f	0.018±0.01ef
7	0.123±0.003ef	0.018±0.01ef	0.221±0.01de
9	0.146±0.01def	0.220±0.01de	0.247±0.01d
11	0.169±0.01cde	0.240±0.01cd	0.259±0.01c
13	0.187±0.01cde	0.269±0.01bc	0.298±0.01bc
15	0.192±0.007bcde	0.285±0.01abc	0.322±0.01abc
17	0.211±0.007abcde	0.309±0.01ab	0.331±0.01ab
19	0.228±0.007abcd	0.314±0.005ab	0.354±0.01ab
21	0.239±0.007abc	0.313±0.01ab	0.362±0.02ab
23	0.254±0.01abc	0.318±0.02ab	0.361±0.02a
25	0.277±0.01ab	0.317±0.01ab	0.356±0.01a
27	0.285±0.01a	0.327±0.01a	0.355±0.02a
29	0.296±0.004a	0.329±0.008a	0.279±0.01a

Values sharing a common letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

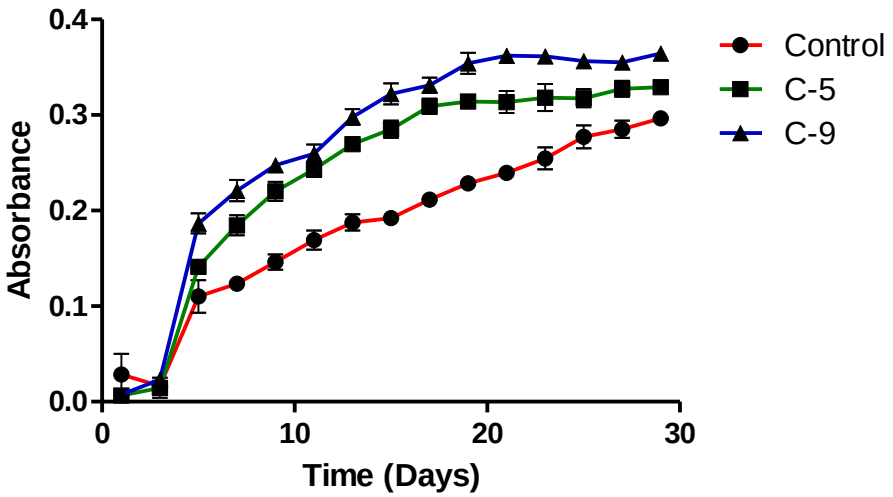


Figure 4.2: Effect of imidacloprid (50 mg/l) on the growth rate of C-5 and C-9

Table 4.8: Effect of imidacloprid (100 mg/l) on the growth rate of C-5 and C-9

Time (Days)	**Control**	**C-5**	**C-9**
1	0.002±0.03f	0.005±0.002g	0.002±0.006e
3	0.056±0.06ef	0.111±0.01f	0.120±0.02de
5	0.125±0.002de	0.133±0.01ef	0.160±0.05cd
7	0.123±0.002cde	0.150±0.01def	0.180±0.07bcd
9	0.146±0.01cd	0.117±0.02def	0.184±0.02bcd
11	0.169±0.01bcd	0.195±0.01cde	0.186±0.01bcd
13	0.187±0.01abcd	0.215±0.01bcd	0.234±0.02abcd
17	0.210±0.01abc	0.247±0.04abc	0.247±0.04abc
19	0.220±0.01ab	0.249±0.04abc	0.253±0.01abc
21	0.230±0.01ab	0.282±0.01ab	0.253±0.00abc
23	0.254±0.02a	0.289±0.01a	0.295±0.01ab
25	0.267±0.01a	0.303±0.01a	0.305±0.01a
27	0.265±0.007a	0.311±0.01a	0.337±0.01a
29	0.267±0.01a	0.321±0.01a	0.339±0.01a

Values sharing a common letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

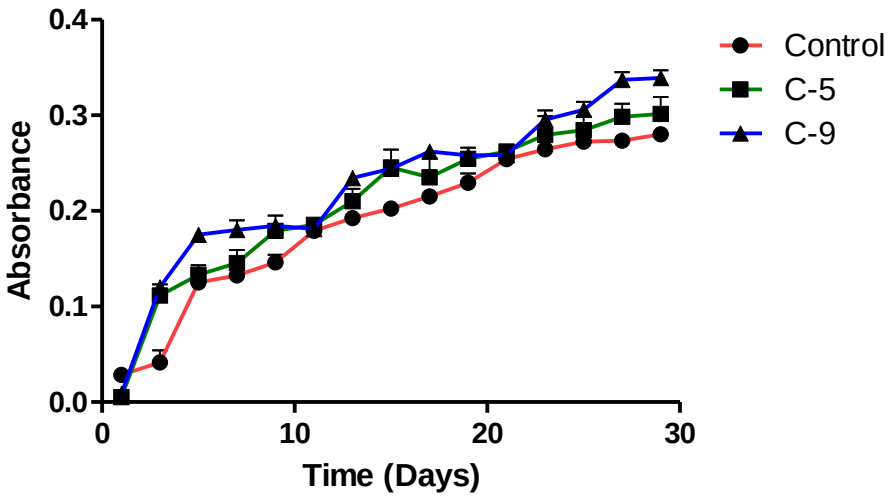


Figure 4.3: Effect of imidacloprid (100 mg/l) on the growth rate of C-5 and C-9

Table 4.9: Effect of imidacloprid (150 mg/l) on the growth rate of C-5 and C-9

Time (Days)	**Control**	**C-5**	**C-9**
1	0.004±0.001e	0.005±0.01c	0.004±0.005d
3	0.007±0.002e	0.009±0.01bc	0.005±0.007d
5	0.107±0.007d	0.011±0.01abc	0.007±0.009d
7	0.117±0.01d	0.017±0.09abc	0.010±0.009cd
9	0.136±0.02cd	0.074±0.01abc	0.012±0.08bcd
11	0.154±0.03bcd	0.133±0.01abc	0.069±0.03abc
13	0.182±0.02abc	0.147±0.01abc	0.148±0.03ab
15	0.187±0.01ab	0.150±0.02abc	0.170±0.03a
17	0.210±0.02a	0.167±0.02abc	0.226±0.03a
19	0.217±0.01a	0.174±0.007abc	0.235±0.03a
21	0.221±0.004a	0.191±0.01abc	0.238±0.02a
23	0.221±0.003a	0.210±0.007abc	0.243±0.01a
25	0.223±0.002a	0.219±0.007ab	0.252±0.01a
27	0.229±0.003a	0.225±0.02a	0.261±0.01a
29	0.229±0.007a	0.244±0.03a	0.269±0.01a

Values sharing a common letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

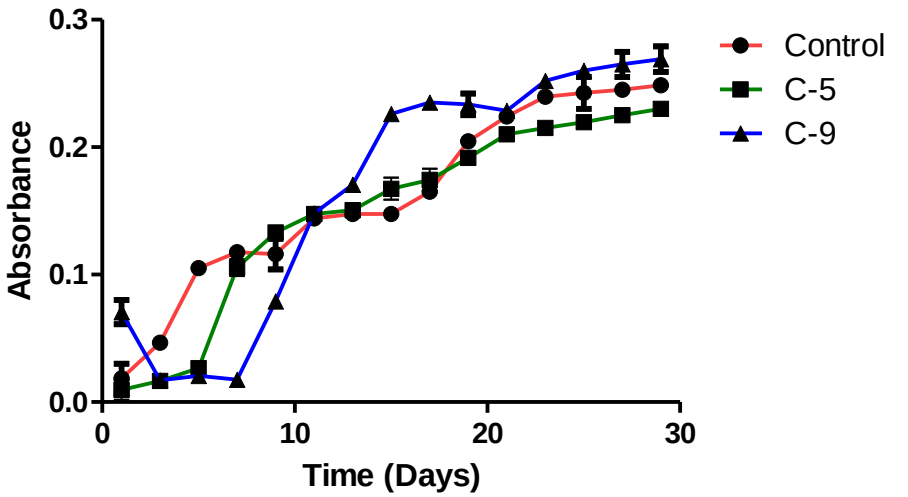


Figure 4.4: Effect of imidacloprid (150 mg/l) on the growth rate of C-5 and C-9

4.6 Effect on pH by degradation studies

The effect on pH by imidacloprid concentration was studied for bacterial isolates C-5 and C-9 (Figure 4.5, 4.6, 4.7). The pH of the broth culture was initially set at 7.2. As the optical density of the culture increases pH of the culture decreases. The decrease in pH was not much significant though. But the decrease in pH correlates with the increase in absorbance. The pH decrease was seen more in C-9 as compared to C-5 (Table 4.10, 4.11, 4.12). However no results of the effect of imidacloprid on pH has been reported in literature. At lower concentrations pH decrease was more as compared to higher concentration. The more the optical density of the culture, more organic acids or carbon dioxide wii be released by microorganisms, more will be the pH decrease. As some microorganisms produce acid as they grow which results in decrease of the pH. Thus pH decrease was more in culture with imidacloprid at the concentration range of 50 mg/l as compared to that of 150mg/l.

Table 4.10: Effect of imidacloprid (50 mg/l) on the pH of the media

Time (Days)	Control	C-5	C-9
1	7.225±0.02bc	7.220±0.01ab	7.215±0.3a
3	7.205±0.04a	7.205±0.06a	7.180±0.04ab
5	7.200±0.01ab	7.180±0.01abc	7.175±0.003ab
7	7.190±0.01ab	7.101±0.01bc	7.135±0.02ab
9	7.180±0.01ab	7.115±0.02bc	7.105±0.02b
11	7.170±0.01ab	7.090±0.01c	7.095±0.02b
13	7.170±0.01ab	7.070±0.08cd	7.045±0.02b
15	7.180±0.01ab	6.960±0.01de	6.930±0.01c
17	7.165±0.04ab	6.910±0.01e	6.795±0.02d
19	7.165±0.02ab	6.890±0.01e	6.745±0.01de
21	7.110±0.01ab	6.850±0.01ef	6.670±0.01e
23	7.065±0.01a	6.845±0.02ef	6.625±0.04ef
25	7.045±0.06a	6.755±0.02f	6.540±0.04fg
27	7.035±0.02	6.635±0.02g	6.475±0.03g
29	6.960±0.01	6.565±0.02g	4.475±0.02g

Values sharing a common letter are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

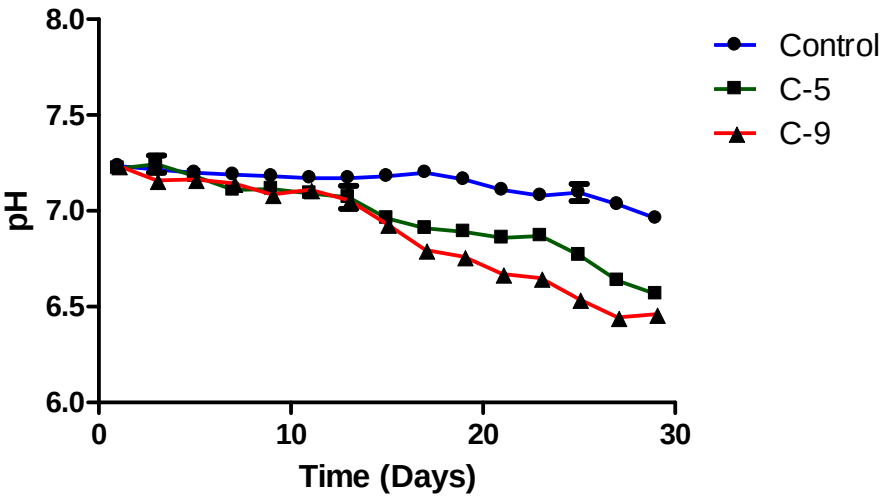


Figure 4.5: Effect of imidacloprid (50 mg/l) on the pH of the media

Table 4.11: Effect of imidacloprid (100 mg/l) on the pH of the media

Time (Days)	Control	C-5	C-9
1	7.240±0.02a	7.225±0.03a	7.230±0.02a
3	7.230±0.02ab	7.190±0.01a	7.200±0.01a
5	7.140±0.03abcd	7.175±0.02a	7.215±0.02a
7	7.210±0.02abc	7.150-±0.02ab	7.200±0.01a
9	7.200±0.01abc	7.130±0.01abc	7.180±0.01a
11	7.170±0.02abc	7.110±0.01abcd	7.110±0.01b
13	7.165±0.02abc	7.075±0.1abcd	7.070±0.01b
15	7.170±0.01abc	7.080±0.02abcd	7.055±0.02b
17	7.155±0.02abcd	6.990±0.02bcde	6.960±0.01cd
19	7.155±0.02abcd	6.960±0.01cdef	6.935±0.02c
21	7.135±0.02bcd	6.955±0.02cdef	6.930±0.02cd
23	7.115±0.02cd	6.940±0.05def	6.900±0.01cd
25	7.060±0.04de	6.910±0.04def	6.880±0.01de
27	7.010±0.01e	6.835±0.02ef	6.805±0.007ef
29	6.960±0.01e	6.800±0.04f	6.710±0.03f

Values sharing a common letter are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

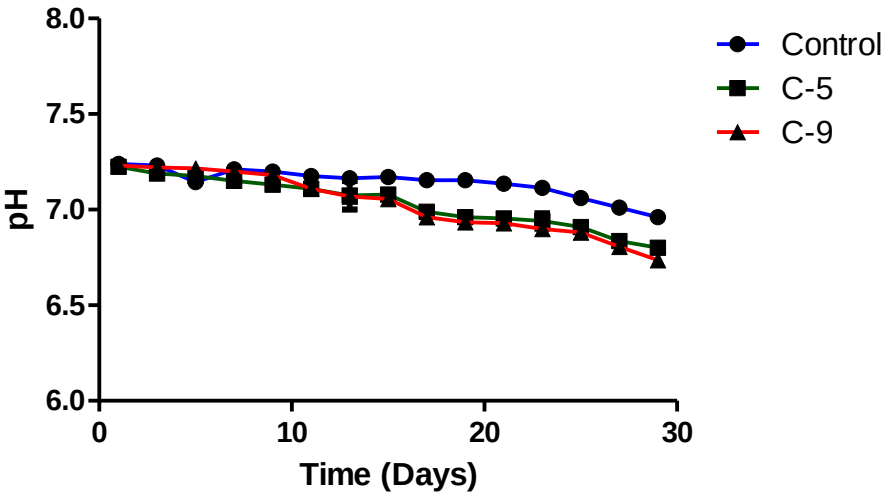


Figure 4.6: Effect of imidacloprid (100 mg/l) on the pH of the media

Table 4.12: Effect of imidacloprid (150 mg/l) on the pH of the media

Time (Days)	Control	C-5	C-9
1	7.200±0.01abc	7.220±0.01a	7.220±0.01a
3	7.230±0.02a	7.215±0.02a	7.210±0.01a
5	7.220±0.01a	7.205±0.02a	7.190±0.01ab
7	7.220±0.02a	7.210±0.02a	7.200±0.01a
9	7.215±0.02a	7.175±0.007ab	7.170±0.01abc
11	7.200±0.01abc	7.130±0.02abc	7.100±0.01bcd
13	7.210±0.02ab	7.100±0.02bcd	7.09±0.05cd
15	7.170±0.01abcd	7.075±0.20cde	7.075±0.1d
17	7.160±0.01abcd	6.035±0.02def	7.015±0.1d
19	7.160±0.01abcd	6.990±0.01efg	6.920±0.04e
21	7.175±0.03abcd	6.970±0.01fgh	6.900±0.01e
23	7.115±0.02bcde	6.935±0.02ghi	6.875±0.01e
25	7.110±0.01cde	6.905±0.03ghi	6.845±0.02e
27	7.100±0.01de	6.988±0.02hi	6.850±0.01e
29	7.035±0.04e	6.870±0.02i	6.825±0.02e

Values sharing a common letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

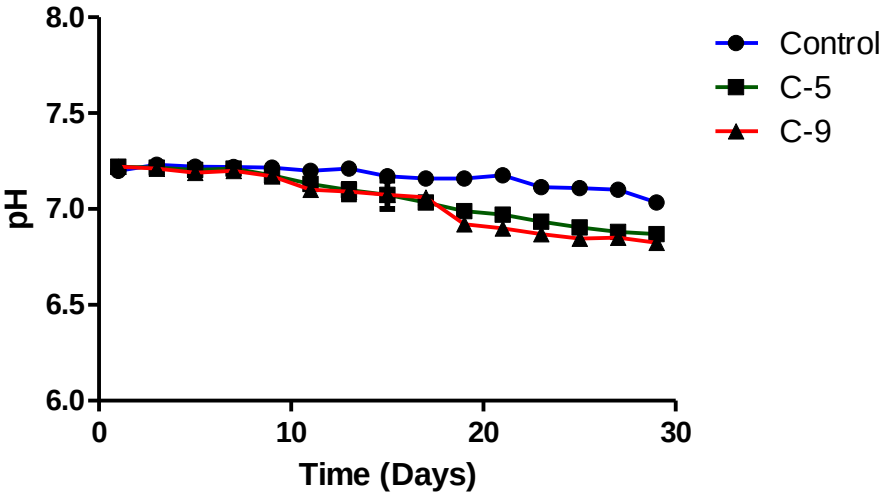


Figure 4.7: Effect of imidacloprid (150 mg/l) on the pH of the media

4.7 Degradation of imidacloprid (HPLC analysis)

Before any samples were analyzed via HPLC, a serial dilution of known concentration imidacloprid from 1mg/l to 0.001mg/l were prepared and analyzed to calibrate the instrument. The mean retention time for the imidacloprid dilutions across all concentrations and sampling periods ranged from 3.348 minutes in all standards and samples. Anhalt *et al*., 2007 reported 64% of the degradation of imidacloprid at the concentration of 43 mg/l by an isolated soil organism *Leifsonia*. The degradation of imidacloprid by *Leifsonia* strain PC-21 was the first report of imidacloprid degradation by an isolated microorganism in 2007. On the better side, our studies resulted in almost 85% degradation of imidacloprid at the concentration of 50 mg/l (Table 4.13) (Figure 4.7). However, not much imidacloprid degrading strains has been isolated in literature. HPLC analysis revealed that colony C-5 and C-9 showed high potential of degrading imidacloprid and utilize it as a energy source mainly carbon source. Slight decrease in degradation was noted at higher concentrations which means microbes capability of degrading imidacloprid decreased as the concentration of imidacloprid in broth rises (Table 4.15, 4.17) (Figure 4.10, 4.12). Moreover C-9 colony showed better degradation results as compared to C-5. Maximum degradation was noticed in culture having imidacloprid at low concentration which was 50 mg/l. C-9 showed maximum degradation of 86% and C-5 showed 70% at this concentration after a month. As the concentration increases to 100mg/l, C-9 showed total degradation of 79% and C-5 showed 65%. The degradation was reduced to 75% and 62% for C-9 and C-5 with the increasing concentration of 150mg/l. Increase in concentration of imidacloprid decreased the growth of these isolates that is at higher concentrations microorganisms degrade less imidacloprid because of its toxicity to microorganism. However C-5 and C-9 showed similar growth pattern. The degradation pattern of imidacloprid were shown below by their peaks in which retention time was 3.348. The degradation of imidacloprid was maximum at 50 mg/l (Figure 4.9), followed by 100 mg/l (Figure 4.11) and least degradation at 150 mg/l (Figure 4.13).

Table 4.13: Degradation of imidacloprid (50 mg/l) in minimal media

Time (Days)	Control	C-5	C-9
7	2.77±0.3c	20.15±1d	26.3±1a
14	160±1b	34.45±1c	42.75±1b
21	32.8±1a	42.1±0.5b	67.35±1c
28	32.45±1a	70.75±1a	86.35±1d

Values sharing the common letter within the column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

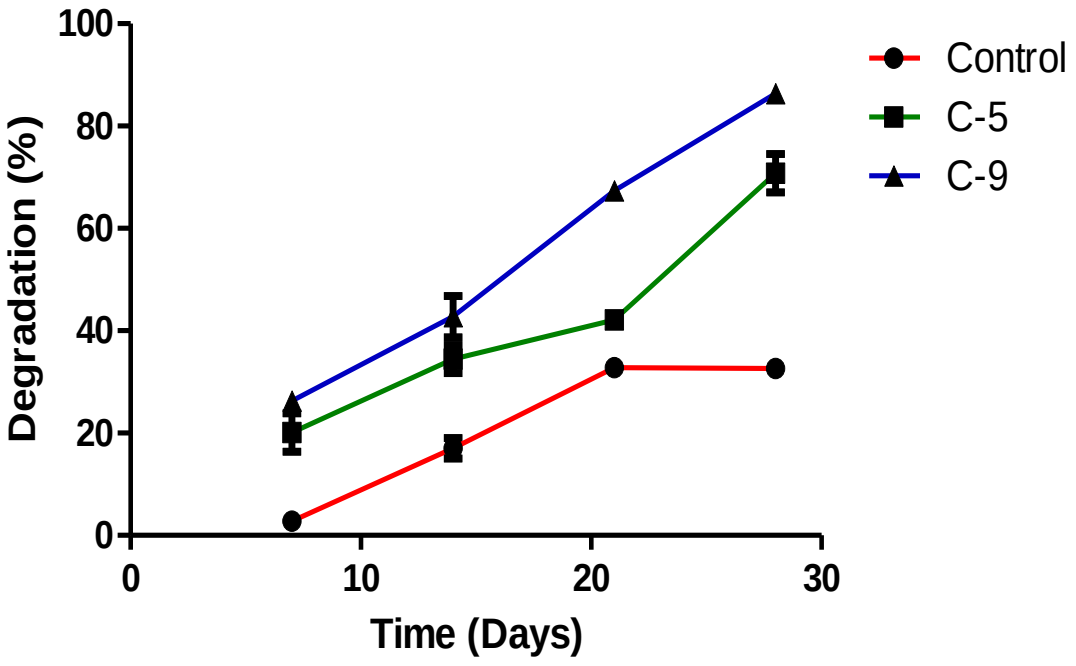


Figure 4.8: Percent degradation of imidacloprid (50 mg/l) in minimal media

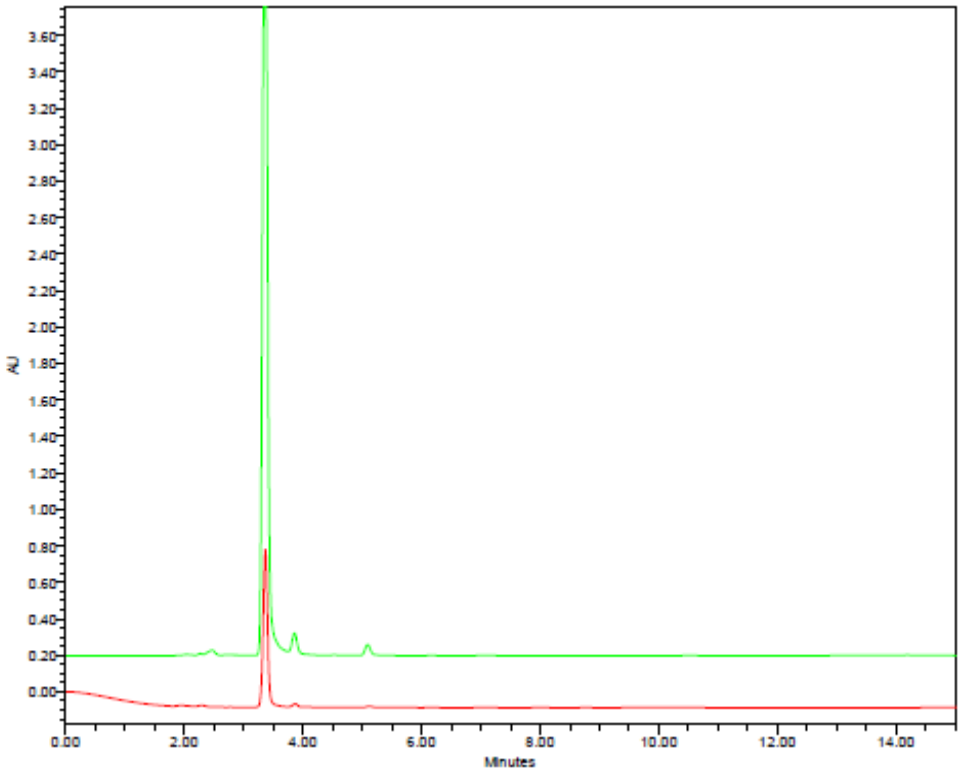


Figure 4.9: Control

Degradation of imidacloprid (50mg/l)

Table 4.14: Two way ANOVA table showing the interaction between three samples and number of days on degradation of imidacloprid (50 mg/l)

Source of Variation	Df	SS	MS	F
Interaction	6	823.6	137.3	103.4***
Degradation (%)	2	4877	2439	1837***
Time (Days)	3	7368	2456	1850***
Residual	12	15.93	1.328	

Table 4.15: Degradation of imidacloprid (100 mg/l) in minimal media

Time (Days)	Control	C-5	C-9
7	8.85±3c	16.75±3c	26.5±2d
14	13.9±1bc	34.25±2b	44.0±5c
21	20.1±1ab	43.50±3b	52.9±2b
28	27.2±2a	64.25±4a	73.35±1a

Values sharing a common letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

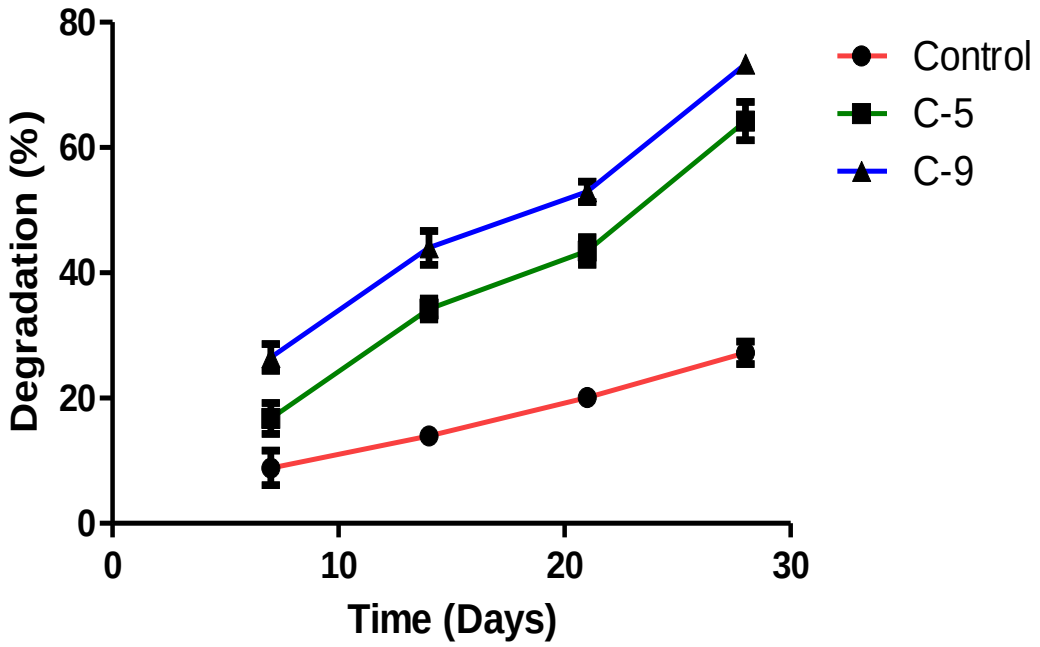


Figure 4.10: Percent degradation of imidacloprid (100 mg/l) in minimal media

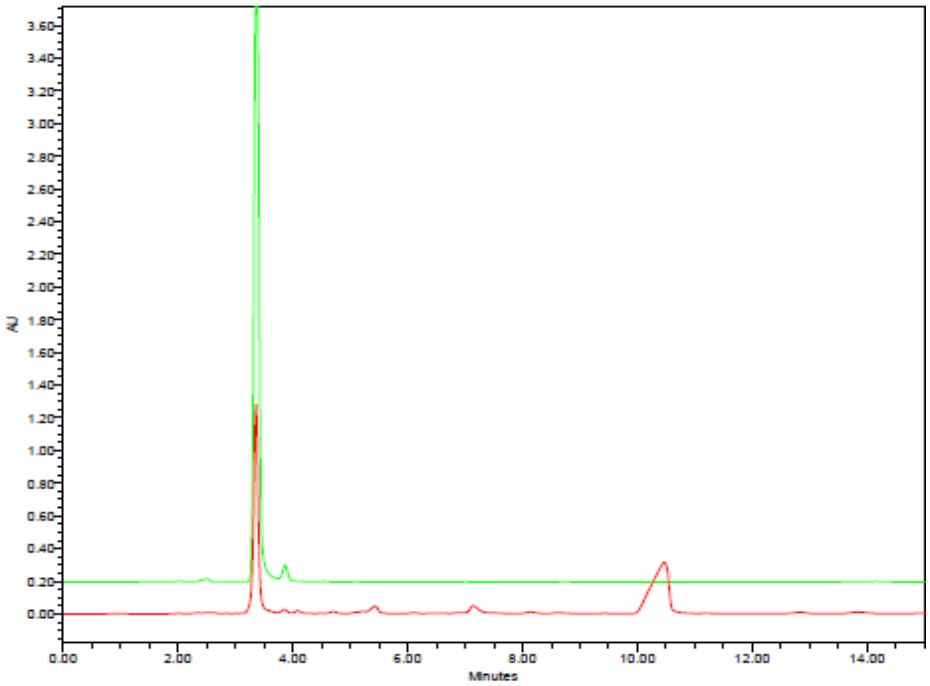


Figure 4.11: Control without bacterial inoculums

Degradation of imidacloprid (50mg/l)

Table 4.16: Two way ANOVA table showing the interaction between three samples and number of days on degradation of imidacloprid (100 mg/l)

Source of Variation	Df	SS	MS	F
Interaction	6	560.4	93.40	9.647***
Degradation (%)	2	4227	2113	218.3***
Time (Days)	3	4442	1481	153.0***
Residual	12	116.2	9.681	

Table 4.17: Degradation of imidacloprid in broth culture at the concentration of 150 mg/l

Time (Days)	Control	C-5	C-9
7	8.60±1c	17.05±1c	23.75±3d
14	16.15±1b	29.85±2bc	43.7±3c
21	18.8±1b	37.75±2b	55.5±2b
28	25.7±3a	61.7±4a	66.9±3a

Values sharing a common letter within column are not significant at P < 0.05; values are mean ± Standard deviation (n=3)

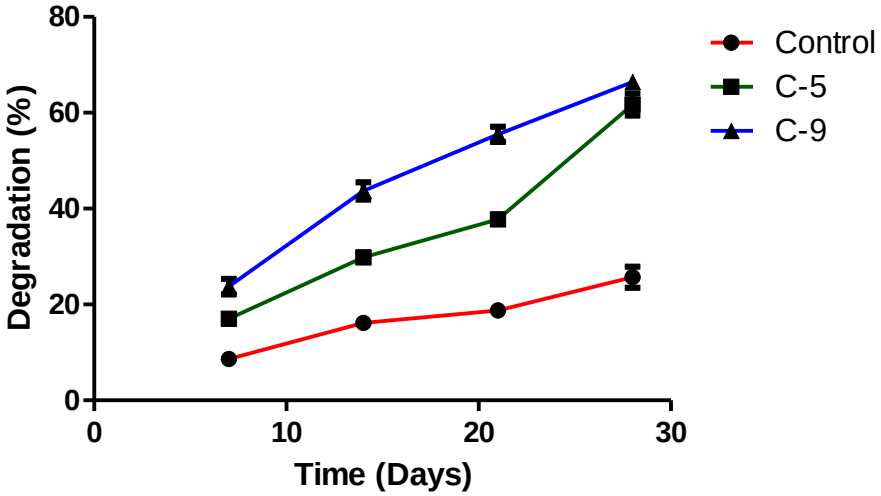


Figure 4.12:Percent degradation of imidacloprid (150 mg/l) in minimal media

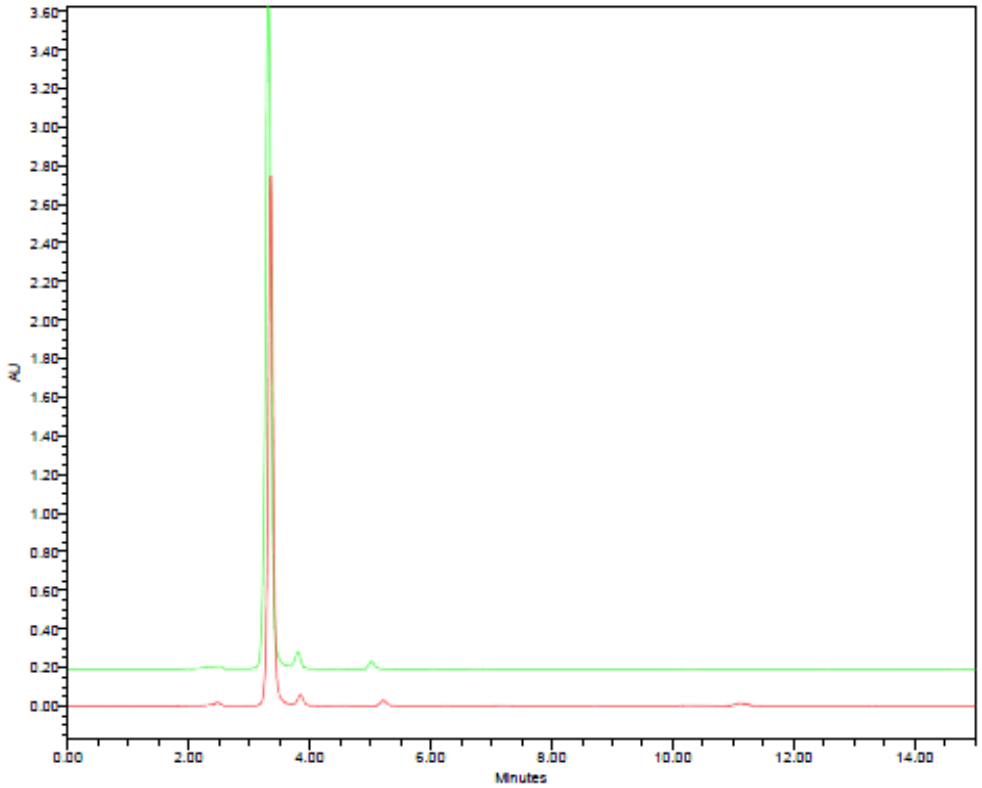


Figure 4.13: Control without bacterial inoculums

Degradation of imidacloprid (150 mg/l)

Table 4.18: Two way ANOVA table showing the interaction between three samples and number of days on degradation of imidacloprid (50 mg/l)

Source of Variation	Df	SS	MS	F
Interaction	6	580.9	96.82	13.22***
Degradation (%)	2	3873	1936	264.4***
Time (Days)	3	3919	1306	178.4***
Residual	12	87.87	7.322	

4.8 Physiological characterization of bacterial colonies

All the bacterial isolates were found to be rod shaped, Gram positive and negative and thus form blue and pinkish colonies. Both the bacterial isolates showed positive motility test. The ability to reduce nitrate was also found in both C-5 and C-9. However C9 showed positive oxidase test as compared to C5 which showed negative test. Both bacterial isolates showed negative catalase test (Table 4.19).

Table 4.19: Biochemical characterization of bacterial isolates

Isolates	Gram stain	Shape	Motility test	Nitrate reduction test	Oxidase test	Catalase test
C-5	+	Rod	+	+	-	-
C-9	-	Rod	+	+	+	-

4.8.1 Antibiotic sensitivity of isolated bacterial colonies

Total of 6 different antibiotics were tested on both bacterial isolates C-5 and C-9.
Both of the bacterial isolates were found to be sensitive and showed zone of inhibtion to the following antibiotics - vancomycin (Va) 30µg , Ofloxacin (Of) 5 µg , Teicoplanin (Te) 30 µg, Ceftazidime (Ca) 30 µg, Gentamycin (G) 10 µg , Cephotaxime (Cn) 30 µg. (Table 4.20).
Both bacterial isolates were found more sensitive to antibiotic Ofloxacin .C-5 was found to be least sensitive to Cephotaxime whereas C-9 was found to be least sensitive to Ceftazidime and Cephotaxime (Figure 4.14, 4.15).

Table 4.20: Different zones of inhibtion (cm) in response to different antibiotics

Antibiotic	**C-5 (cm)**	**C-9 (cm)**
Teicoplanin (Te)	2	2.1
Ofloxacin (Of)	3.2	3
Vancomycin (Va)	1.8	2.0
Ceftazidime (Ca)	2	1.1
Gentamycin (G)	2.5	2.2
Cephotaxime (Cn)	0.7	1.1

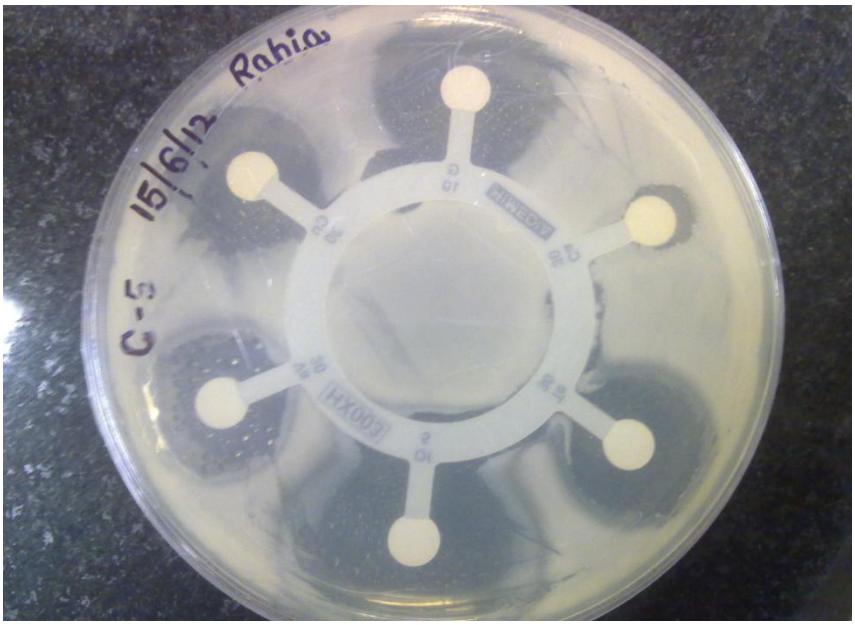


Figure 4.14 : Different zones of inhibtion by C-5 due to antibiotic sensitivity

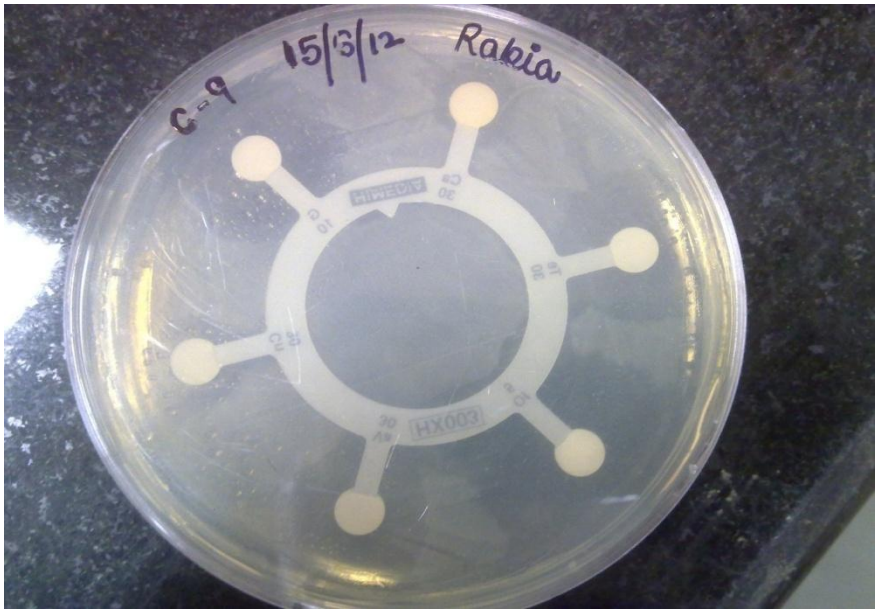


Figure 4.15: Different zones of inhibtion by C-9 due to antibiotic sensitivity

4.9 Characterization of bacterial samples

Polymerase chain reaction (PCR) can produce products to the more highly conserved 5S, 16S, and 23S ribosomal subunits which can potentially differentiate species and also show intra specific differences (Wakabayashi *et al.,* 1999). The different bacterial isolate selected were identified on the basis of molecular properties (Figure 4.16).

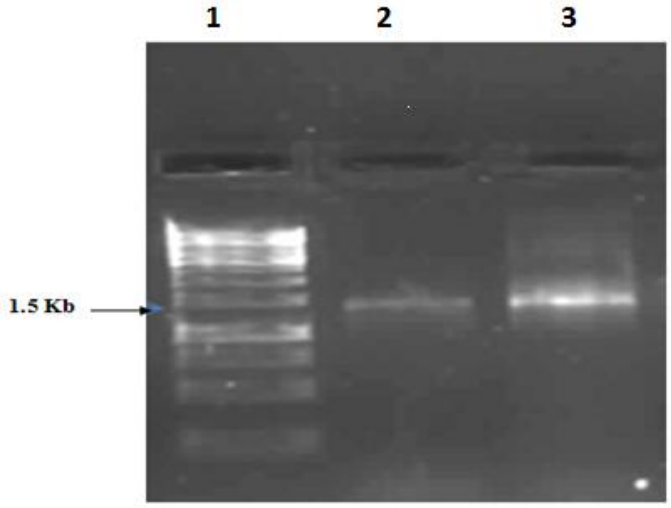


Figure 4.16: 16S amplification in lane 1, 2 and 3. Lane 2 contains 16 S product of C-5, lane 3 of C-9

4.10 Soil microcosm (HPLC)

The biodegradation of imidacloprid in soil has been reported (Sarkar *et al*., 2001; Rouchaud *et al*., 1994). The isolated bacteria *S. maltophilia* NJ1 strain and *S. maltophilia* CGMCC 1.1788 used in this degradation of imidacloprid , Gram (−) rod, frequently occurring in water and soil (Claude *et al*., 1995) degrading 60% imidacloprid. The microcosm experiment set up resulted in more degradation as compared to that reported by Claude *et al*. (1995). As a result of biodegradation after 30 days in HPLC analysis, it was observed that 17.4% disappeared from treatment 1 which was sterile soil and imidacloprid (50 mg/l). Treatment 2, which contained sterilized soil amended with bacterial inoculum C-5 and imidacloprid (50mg/l) showed degradation of 71%, while treatment 3, contained natural soil augmented with bacterial inoculums C-5 and imidacloprid (50 mg/l) showed degradation of 54.6% (Figure 6.17) (Table 4.21). In treatment 4, natural soil and imidacloprid (50mg/l) showed degradation of 37%. Whereas for the C-9, treatment 5 which contained sterilized soil amended with bacterial inoculum C-9 and imidacloprid (50mg/l) showed maximum degradation of 82%. In treatment 6, which contained natural soil augmented with bacterial inoculum C-9 and imidacloprid (50 mg/l) showed degradation of 57.6% (Figure 4.19) (Table 4.23). Thus this shows in soil microcosm study bacterial isolates C-5 and C-9 degraded imidacloprid most efficiently in treatment 2 and treatment 5, utilizing imidacloprid as a sole source of carbon and energy because here no other energy source was present other than imidacloprid. Two way ANOVA has been done to show the interaction between different soil treatments and number of days on imidacloprid degradation (50 mg/l) by C-5 and C-9 (Table 4.22, 4.24).

However C-9 showed better degradation of 11% as compared to C-5 (Figure 4.18, 4.20). While less degradation was observed in control, after 30 days of incubation, which might be due to chemical reactions at the set conditions which was only sterile soil and imidacloprid. The degradation of imidacloprid was decreased in treatment 3 and 6 when it was augmented in natural soil where indigenous micro floras were present, it might be due to incompatible growth conditions for bacterial strain C-5 and C-9 in presence of natural microflora. Similarly, degradation of imidacloprid in treatment 4 also decreased in absence of C-5 and C-9, which are efficient degrader of imidacloprid.

Table 4.21: Imidacloprid degradation by C-5 for different soil treatments

Time (Days)	SS+I	SS+I+C-5	NS+I+C-5	NS+I
7	5.65±0.9b	18.95±1c	16.15±1c	8.1±1d
14	7.45±1b	61.5±2b	46.1±5b	23.6±1c
21	16.4±1a	63.8±2b	46.6±1b	26.1±2b
28	17.65±1a	71.7±1a	56.25±2a	37.7±1a

Values sharing a common letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

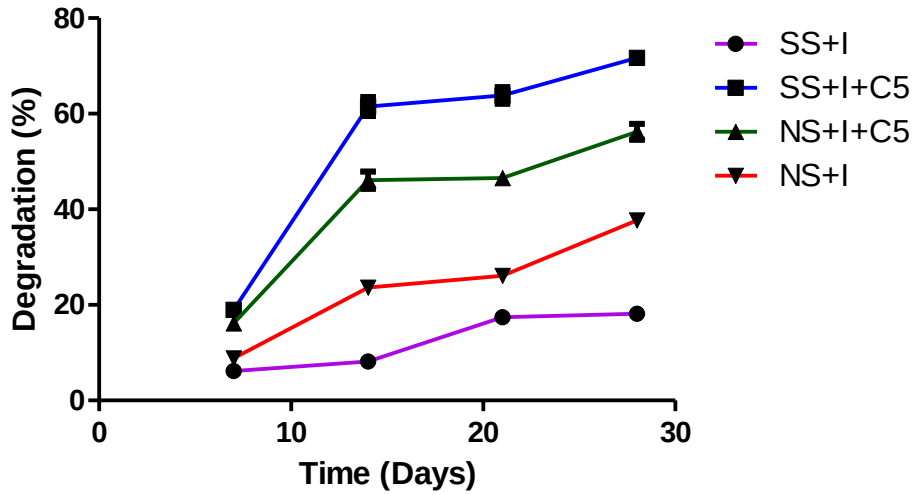


Figure 4.17: Percent degradation of imidacloprid (50 mg/l) by C-5 for different soil treatments

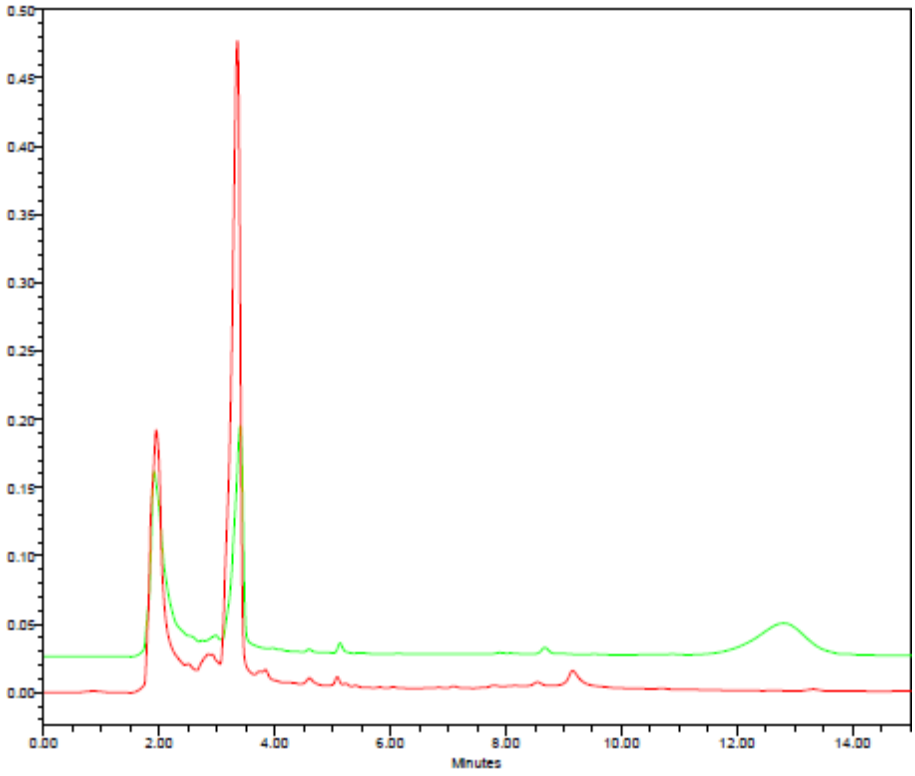


Figure 4.18: Control without bacterial inoculums

Imidacloprid degradation by C-5

Table 4.22: Two way ANOVA table showing the interaction different soil treatments and number of days on imidacloprid degradation (50 mg/l) by C-5

Source of Variation	Df	SS	MS	F
Interaction	9	1314	146.0	33.13***
% Degradation	3	8091	2697	611.9***
Time (Days)	3	4967	1656	375.7***
Residual	16	70.52	4.407	

Table 4.23: Chloride ion release by C-9 for different soil treatments

Time (Days)	SS+I	SS+I+C-9	NS+I+C-9	NS+I
7	6.15±1b	49.35±2d	16.65±2c	9.15±1d
14	7.55±1b	67.15±3c	41.5±1b	25.05±2c
21	17.95±2a	76.35±4b	47.1±1b	27.6±1b
28	17.6±1a	83.45±1a	56.6±1a	37.65±1a

Values sharing a common lowercase letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

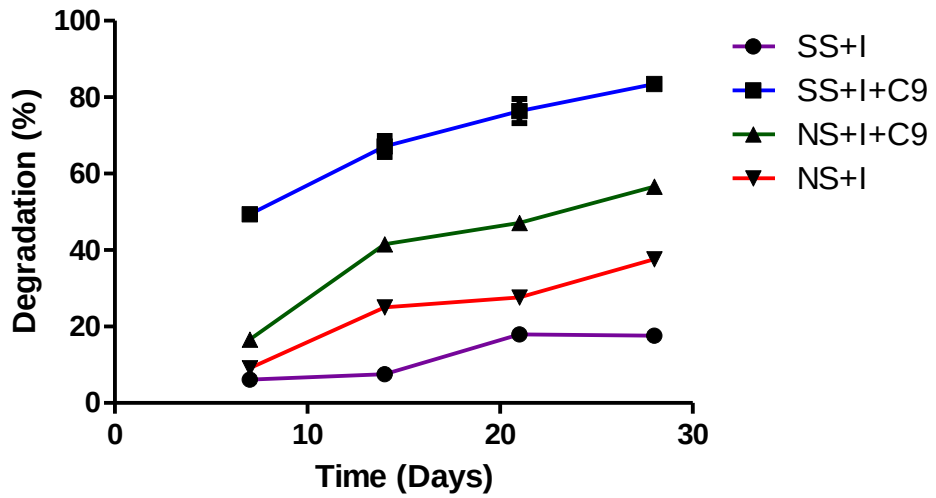


Figure 4.19: Percent degradation of imidacloprid (50 mg/l) by C-9 for different soil treatments

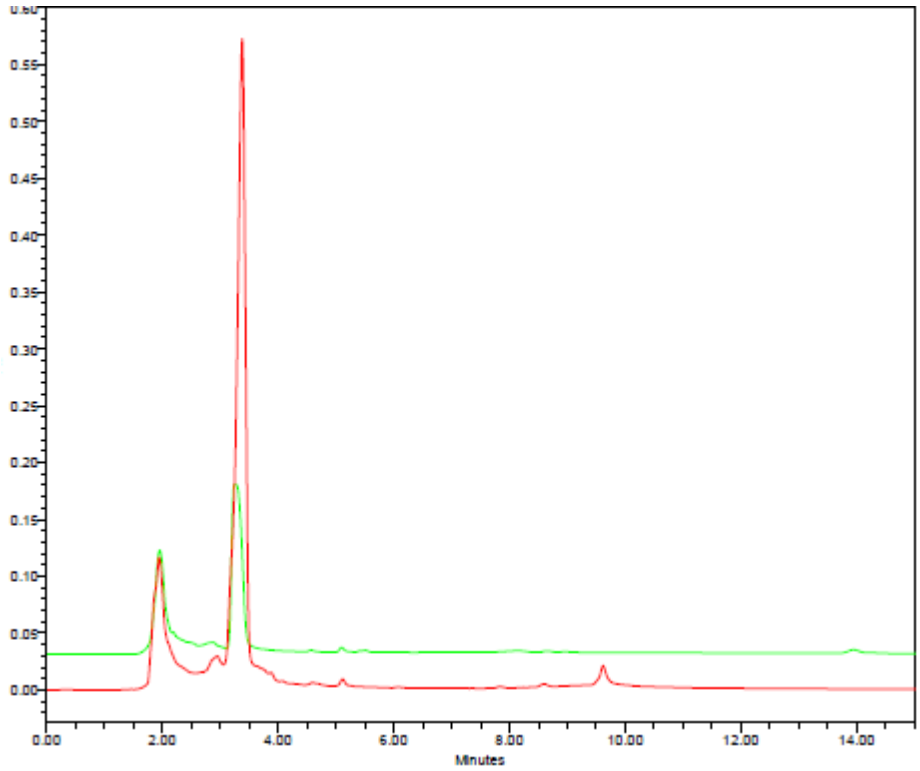


Figure 4.20: Control without bacterial inoculum

Imidacloprid degradation by C-9

Table 4.24: Two way ANOVA table showing the interaction different soil treatments and number of days on imidacloprid degradation (50 mg/l) by C-9

Source of Variation	Df	SS	MS	F
Interaction	9	543.5	60.39	13.28****
Degradation (%)	3	14377	4792	1054****
Time (Days)	3	3583	1194	262.7****
Residual	16	72.76	4.547	

4.11 Chloride release assay

During the course of soil microcosm experiment it was observed that as imidacloprid was degraded and concentration of inorganic chloride ion in soil jars increases. Chloride ion concentration gradually increased with the passage of time. The two are interrelated i.e., concentration of chloride ion is directly linked with the depletion of imidacloprid in soil

microcosm experiment. All the six treatments were analyzed by argentometric method to see the chloride ion release in the microcosm setup (Table 4.25, 4.27). During imidacloprid degradation, at the end of the month maximum chloride ion release of 29.98 and 27.76 mg/l was observed in C-9 and C-5 having sterile soil with bacterial inoculums and imidacloprid (50 mg/l) which was directly propotional to the degradation studies through HPLC which showed maximum degradation (Figure 4.21, 4.22).Thus the minimum chloride ion increased was seen in treatment 1 containing sterile soil and imidacloprid (50 mg/l). Two way ANOVA has been done to show the interaction between different soil treatments and number of days on chloride ion release by C-5 and C-9. However no results have been reported so far for the chloride ion release by imdacloprid degradation.

Table 4.25: Chloride ion release by C-5 for different soil treatments

Time (Days)	**SS+I**	**SS+C-5**	**NS+C-5**	**NS+I**
7	2.90±0.4b	12.08±0.3c	3.76±0.1d	2.70±0.1d
14	5.46±0.2ab	19.72±0.3b	10.07±0.1c	6.56±0.1c
21	4.70±1ab	24.69±1.1a	11.58±0.1b	11.16±0.7b
28	11.2±0.4a	27.98±0.1a	15.09±0.4a	14.08±0.3a

Values sharing a common letter are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

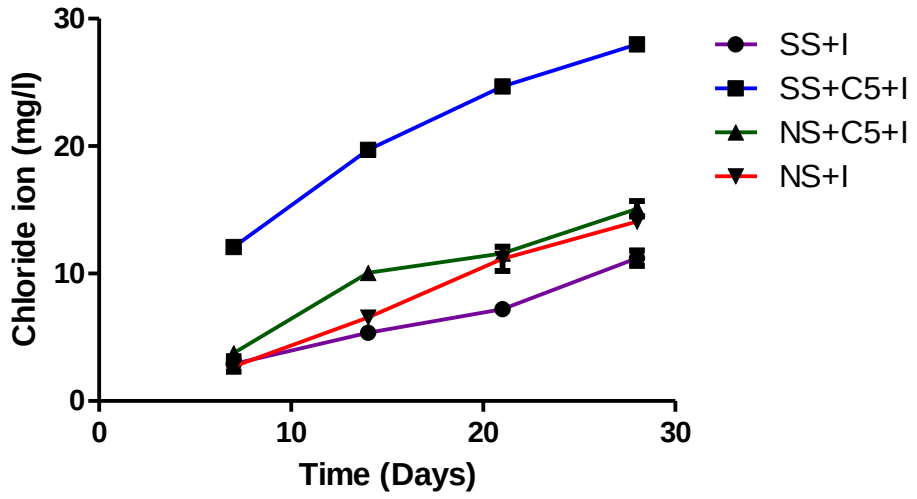


Figure 6.21: Percent chloride release by C-5 for different soil treatments

Table 4.26: Two way ANOVA table showing the interaction of different soil treatments and number of days for chloride ion release by C-5

Source of Variation	Df	SS	MS	F
Interaction	9	48.77	5.418	6.167***
Chloride ion release	3	1006	335.2	381.5***
Time (Days)	3	596.8	198.9	226.4***
Residual	16	14.06	0.8786	

Table 4.27: Chloride ion release by C-9 for different soil treatments

Time (Days)	SS+I	SS+I+C-9	NS+I+C-9	NS+I
7	2.90±0.4d	12.24±0.7d	4.53±0.8c	3.20±0.2d
14	6.06±0.1c	18.57±0.4c	10.42±0.6b	6.70±0.2c
21	8.73±0.1b	23.40±0.2b	12.70±0.08b	10.5±0.2b
28	11.05±0.3a	30.04±0.4a	26.10±0.6a	13.9±0.2a

Values sharing a common letter are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

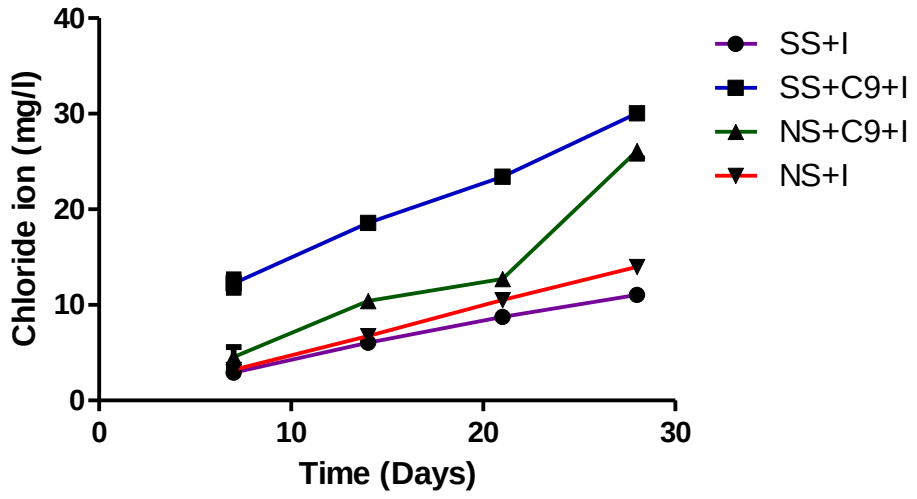


Figure 4.22: Percent chloride release by C-9 for different soil treatments

Table 4.28: Two way ANOVA table showing the interaction of different soil treatments and number of days for chloride ion release by C-9

Source of Variation	Df	SS	MS	F
Interaction	9	141.9	15.77	26.92***
Chloride ion release	3	940.2	313.4	535.0***
Time (Days)	3	901.7	300.6	513.1***
Residual	16	9.373	0.5858	

4.12 Phosphate solubilizers and nitrogen fixers

All the three type of soils were tested for their microbial count of phosphate solubilizers and nitrogen fixers by serially diluting them on pikovskaya and jensen media followed by spread plate method and thus incubated for 48 hrs. It was found that in normal soil nitrogen fixers were more as compared to other soils collected from Site 1 and Site 2 and phosphate solu bilizers were less as compared to Site 1 and Site 2. In normal soil phosphate solubilizers were almost two fold less then pesticide treated soil Site 2 i.e., a much significant reduction in their count (Table 4.29). It may be said that most of phosphate solubilizers got resistant to pesticides and thus multiply in large amount as compare to other microflora. Thats why C-5 and C-9 both are phosphate solubilizers as they got adapted to pesticide treated soil. Therefore phosphate solubilizers increased instead of decreasing in pesticide treated soil as compared to that of nitrogen fixers.

Table 4.29: Comparison of microbial count of nitrogen fixers and phosphate solubilizers in normal and pesticide treated soil

Soil	Nitrogen fixers	Phosphate solubilizers
Normal	24×10^6	20.2×10^6
Site 1	20.6×10^6	25.1×10^6
Site 2	14.2×10^6	43×10^6

4.13 PGPR activities of isolated bacterial strains

Two isolates of soil bacteria (C-5 and C-9) were tested for their plant growth promoting activities *in vitro*. Plant growing rhizobacteria (PGPRs are known to influence plant growth by various direct or indirect mechanisms. Parameters assessed were phosphate solubilization, nitrogen fixation and IAA activity (with and without tryptophan). Phosphate solubilization and nitrogen fixation production were tested qualitatively by plating the bacterial isolates in pikovskaya, rock phosphate and jensen media, respectively. IAA production was tested colorimetrically using ferric chloride perchloric acid reagent. Both bacterial isolates C-5 and C-9 showed clear zone on pikovskaya plates which was a positive indication that both bacterial isolates were capable of using phosphate i.e., both were phosphate solubilizing bacteria. However C-5 and C-9 showed negative result for both jensen and rock phosphate medium. C-9 showed moreclear zone than C-5 which means that C-9 is better phosphate solubilizing bacteria then C-5 (Figure 4.23). The microorganisms showed IAA production acivity of growth regulators by phosphate solubilizing bacteria has been studied by Ponmurugan and Gopi (2006). IAA activity of two bacterial isolates were observed and it was noted that there was more than 3 fold increase in the activity of C-9 and 2 fold in C-5 in the presence of tryptophan (Figure 4.24) (Table 4.30). Moreover the IAA activity of these colonies explains the more plant growth in inoculated colonies in pot experiment. There is increasing evidence that phosphobacteria improve plant growth due to biosynthesis of plant growth substances rather than their action in releasing available phosphorus. Further evaluation of the isolates exhibiting multiple plant growth promoting traits on soil plant system is need to uncover their efficacy as effective PGPR.

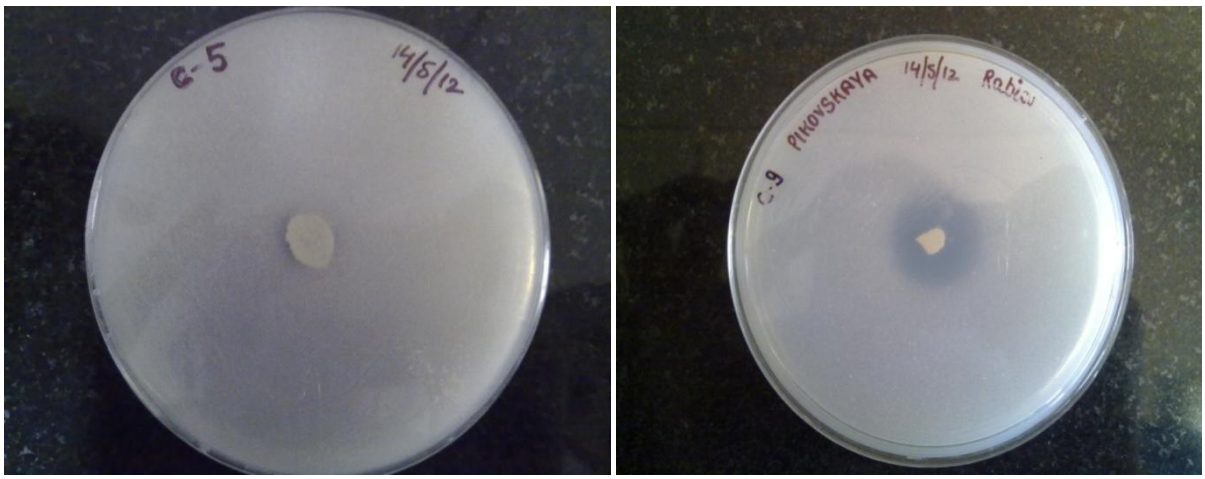


Figure 4.23: Clear zone of phosphate solubilisation in both colonies C-5 and C-9.

Table 4.30: comparison of IAA activity in both colonies C-5 and C-9 without tryptophan and in supplementation of tryptophan

Bacterial isolates	Without Tryptophan	With Tryptophan
C-5	5.85±0.2a	8.81±1.5a
C-9	12.4±0.7b	27.6±1.7b

Values sharing a letter within column are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

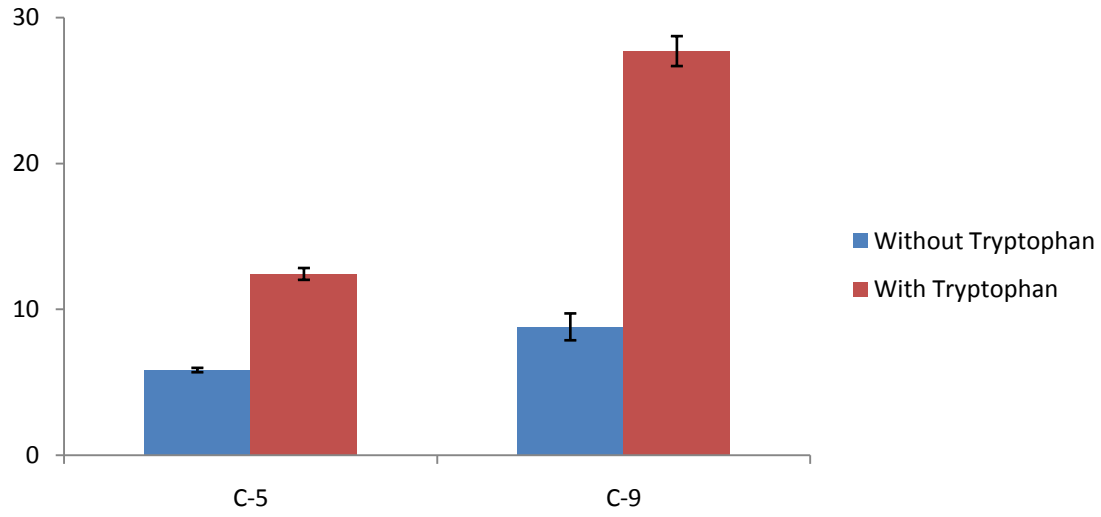


Figure 4.24: comparison of IAA activity in both colonies C-5 and C-9 without tryptophan and in supplementation of tryptophan

4.14 Nursery plantation experiment

Many studies in relation to crop improvement by P solubilisation bacteria were carried out either in pot cultures or field conditions (Whitelaw *et al*., 1997; Saber *et al*., 2009 and Omar, 1989). Microbial populations are key components of soil plant entity where they are associated in a framework of interactions affecting plant development (Vassilev *et al*., 2006). In the present study significant increase in plant growth was recorded with the inoculation of P solubilizing bacterial isolates C-5 and C-9. The microorganisms involved in P solubilization can enhance plant growth by increasing the availability of plant growth promoting substances and other trace elements. There was significant increase in the height of C5 as compared to control where more increase was found in C-9 i.e., almost 2 fold

increase in the shoot length was found as compared to control (Figure 4.25). Similarly increase in shoot fresh and dry weight was observed in inoculated colonies C-5 and C-9 in comparison to that of control. PSMs also known as PGPB (Plant growth promoting bacteria) may effectively increase the surface area of roots (Bashan *et al*., 2004) and the root weight (Bertrand *et al*., 2001). Similar findings were reported by Puente *et al*. (2004). Similarly, root weight was found to be more in C-9 as compared to control (Table 4.31). This shows higher nutrient uptake by inoculated roots significantly improved seedling growth Kucey *et al*., (1989) reported that besides providing P, phosphate solubilization microorganisms produces considerable amounts of N and plant growth promoting substances in the rhizosphere. Kundu and Gaur (1984) also reported, increased P uptake and plant growth in various crops inoculated with PSMs. PSB are also reported to produce metabolites such as phytoharmones which aid in plant growth (Kloepper *et al*., 1989). Two way ANOVA has been done for the interaction between different treatments and soil types on the growth parameters (Table 4.32). Thus the inoculation of these bacterial isolates considerably increased P uptake and enhanced plant growth as compared to uninoculated soil. These results could be attributed to the ability of these microorganisms to solubilize phosphorus already present in the soil. The growth parameters of maize plant were higher in treatment having phosphate solubilizing bacteria.

Table 4.31: Comparison of various growth parameters between inoculated (C-5 and C-9) and uninoculated strains in the pot experiment

Treatments	**Shoot length (cm)**	**Shoot fresh weight (g)**	**Shoot dry weight (g)**	**Root dry weight (g)**
Control	37.05±1.4d	14.95±0.9c	9.295±1b	0.61±0.14a
C-5	46.78±2.4c	17.55±0.3b	13.4±1.5b	0.87±0.06a
C-9	51.75±1.3d	18.7±0.5	15.85±0.9b	0.955±0.4a

Values sharing a common letter are not significant at $P < 0.05$; values are mean ±Standard deviation (n=3)

Table 4.32: Two way ANOVA table showing the variation between soil types and treatments on growth parameters

Source of Variation	Df	SS	MS	F
Interaction	6	115.5	19.25	14.15***
Treatments	2	146.8	73.40	53.98***
Soil type	3	6157	2052	1509***
Residual	12	16.32	1.360	

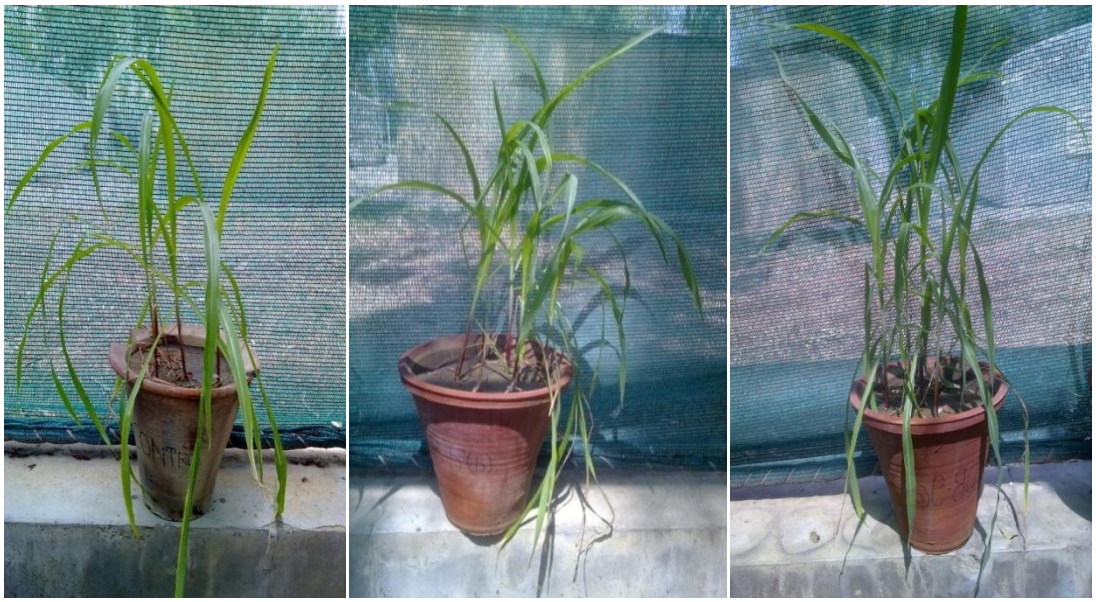


Figure 4.25: Comparison of shoot length in both control (uninoculated) left; and with inoculated bacterial strains C-5 (middle) and C-9 (right)

Slight reduction in pH was noticed after final harvest. Son *et al*., (2003) reported that, phosphate solubilisation was mainly due to the acidification of the culture by bacteria; however high level of phosphate solubilisation may not be achievable in soil because most soils have a great pH buffering capacity. Moreover the final pH did not reduce to strongly acidic levels. It is known that the production of organic acids by soil microorganisms and commensurate pH decrease is the major mechanism of phosphate solubilisation (Whitelaw *et al*., 1999). Since the final pH values in the present study were not as low as those in the study of Son *et al*. (2003). Thus there was reduction of 0.2 units in pH of the soil from 8.3 to 8.1 after harvesting.

The available P increased in the soil having bacterial inoculums as compared to control (Table 4.33). There was almost 3 fold increase in the available P in C-9 as compared to control (Figure 4.26). C-5 also showed significant increase in the available P content as compared to the control. P solubilising microorganisms can solubilise and mineralize P from organic and inorganic pools of total soil P and may be used as inoculants to increase P availability to plants (Kucey *et al*., 1989; Richardson, 1994, 2001; Illmer *et al*., 1995; Whitelaw *et al*., 1999). Phosphate solubilising bacteria are slowly emerging as important organisms used to improve soil health as *in vitro* studies have demonstrated that they reduce P deficiency in soil (Yosef *et al.*, 1999). Thus the P content was more in pots inoculated with PSMs. Previous studies involving plants inoculated with PSMs showed growth enhancements and increased P contents but large variations were found (Kucey *et al*., 1989).

Table 4.33: Comparison of available P after harvesting in both control and bacterial strains

Soil samples	**Available P (mg/kg)**
Control	6.135±0.02
C-5	11.2±0.14
C-9	16.88±0.16

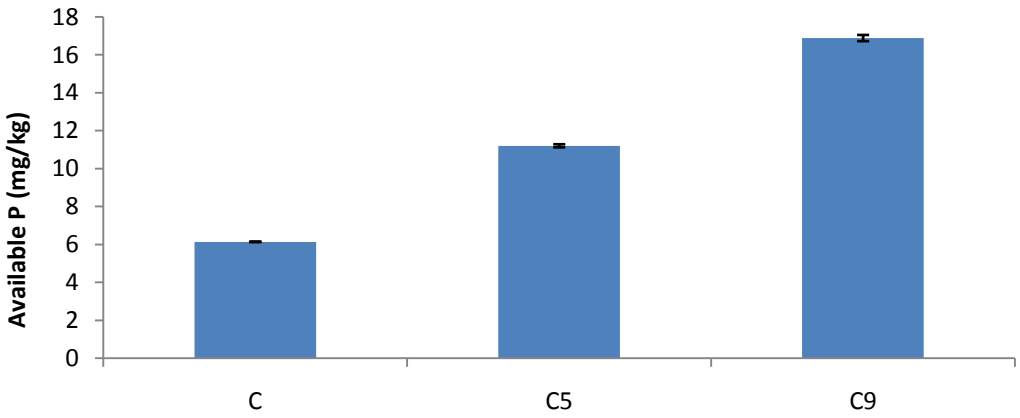


Figure 4.26: Available P (mg/kg) in inoculated and uninoculated soils after harvesting

The total P content decreased after harvesting maize in the soil therefore addition of PSMs increased the available P of the soil significantly. The total P content was more than 2 fold in soils of inoculated colonies as compared to control i.e., sifgnificant increase was observed

(Table 4.34) Thus 3 fold increase in total P of C-9 and two fold of C-5 was recorded in stem of three plants (Figure 4.27). But the total P in roots was found to be more in C-5 as compared to C-9 in contrast to above results where total P content was more in C-9. Thus total P content was less in C-9 almost comparable to control. Thus this shows PSMs increased the P content and thus their uptake from the soil. Two way ANOVA analysis has been done as shown below (Table 4.35)

Table 4.34: Comparison of the Total P (mg/kg) in different tissues of maize plant in various treatments

Treatment	**Soil**	**Stem**	**Root**
Control	93.65±1c	70.5±1a	90.615±0.8a
C-5	195±4b	109.5±1a	100.5±4a
C9	210.5±2a	139±22a	96±3a

Values sharing a common letter are not significant at $P < 0.05$; values are mean ± Standard deviation (n=3)

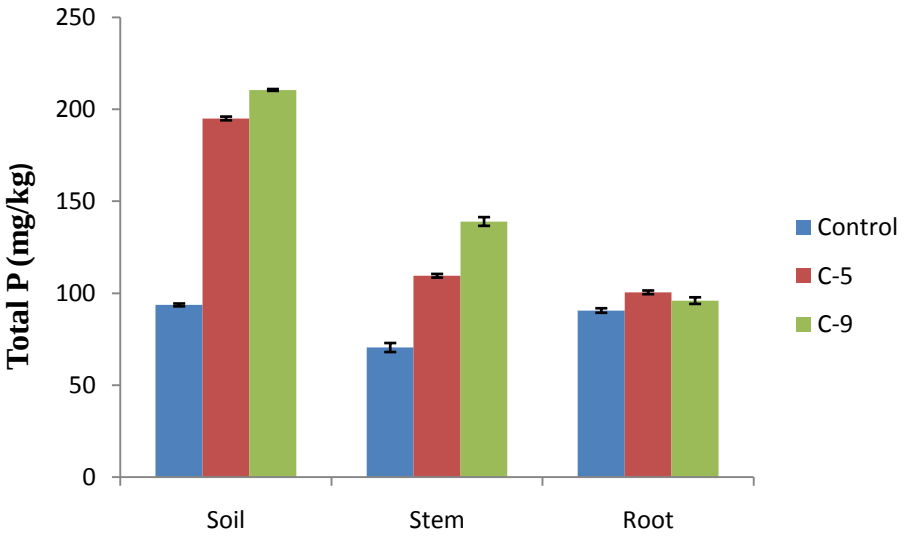


Figure 4.27: Total P (mg/kg) in different tissues of maize plants in inoculated and uninoculated samples after harvesting.

Table 4.35: Two way ANOVA table showing the variation between soil types and treatments on Total P content

Source of Variation	Df	SS	MS	F
Interaction	4	7466	1867	7.937**
Treatments	2	17429	8714	37.06***
Soil Type	2	13465	6732	28.63**
Residual	9	2117	235.2	

After 4 weeks the viability of inoculated P solubilizing bacteria were tested after 4 weeks and it was found that there was significant increase in the viability of bacteria which was 24.9×10^6 and 37.4×10^6 before inoculation in pot inoculated with C-5 and C-9. After 4 weeks the viability was found to be 2.1×10^8 and 33.5×10^8. In control soils where inoculums was not added, it was found no halozones were present around the grown colonies which suggested that phosphate solubilizing bacteria were absent in the control soils.

The samples were also tested for imidacloprid degradation with the HPLC analysis and it was found that the degradation of imidacloprid was 63% in control and 84 and 93 % in C-5 and C-9 repectively (Figure 4.28). C-9 showed a maximum degradation of 93% (Figure 4.29). Thus the reason for more degradation may be because the imidacloprid is photodegradable. It degrades at high rate. Secondly however plants produce some chemicals which helps in the degradation of imidacloprid at a faster rate. Phytoremediation has been a proposed approach for remediating pesticide-contaminated soils. Plants that are able to survive high concentrations of pesticide mixtures could contribute to pesticide waste degradation in soil as a result of intense microbial activity in the root zone or rhizosphere (Anderson *et al*., 1994). The rhizosphere effect is important because of the potential reduction of pesticide wastes due to coincidental metabolism by microbial populations (cometabolism) or by catabolism of chemicals for use as a carbon or nitrogen source (Coats, 1993). Plants can also contribute to the removal of pesticide wastes through uptake into the plant tissue (Cunningham *et al.*, 1997). Little is known about the fate of pesticide mixtures in soil at point-source contamination levels. The current study was conducted to investigate whether interactions among herbicides in mixtures influence the degradation of individual herbicides. The effects of aging herbicide mixtures on soil respiration and plant germination and survival were also

measured. Additionally, studies were conducted to determine the influence of native prairie grasses on the removal of pesticide wastes in soil

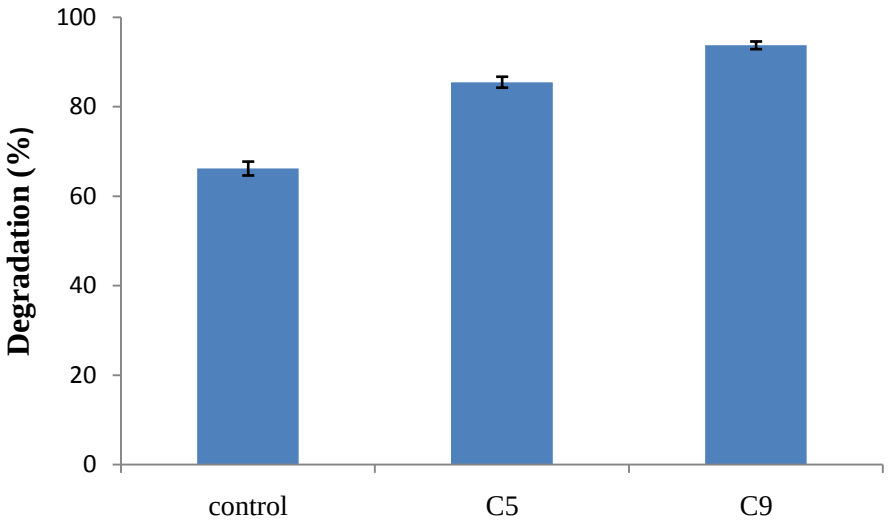


Figure 4.28: Imidacloprid degradation after a period of 28 days in the pot experiment in both inoculated and uninoculated strains.

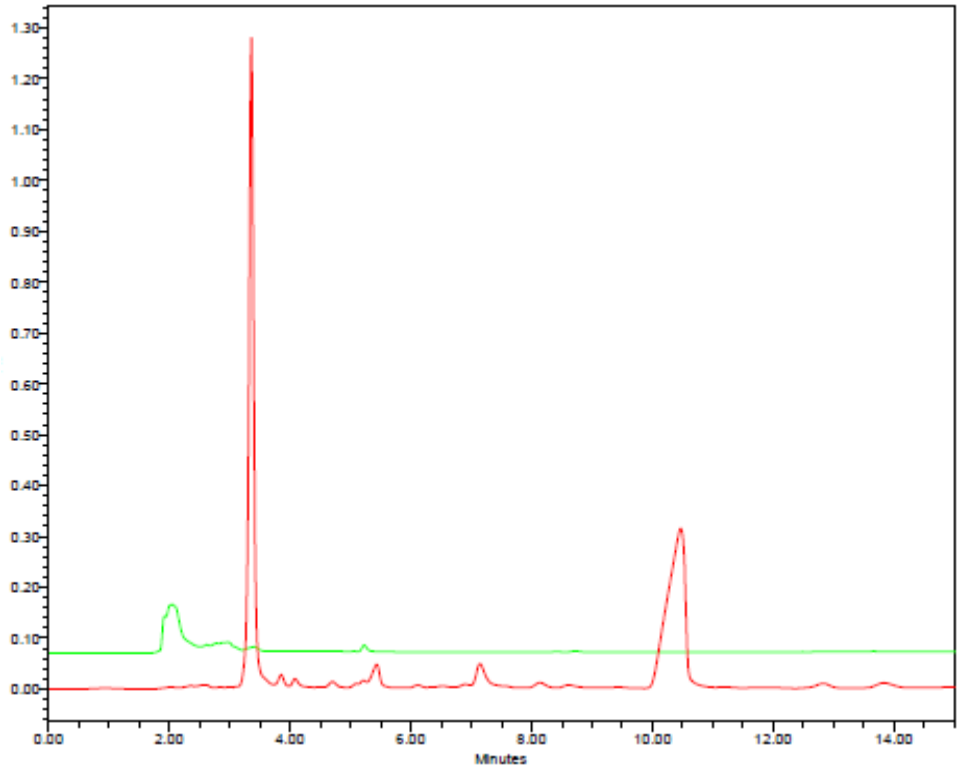


Figure 4.29: Imidacloprid present initially before nursery plantation experiment
Degradation of imidacloprid after nursery plantation experiment (Retention time 3.348)

Thus the increase in plant growth, available and total P in soil inoculates with PSMs may assist in solving problems encountered for crop production economy and could contribute to reversing the trend of soil degradation and actually encourage soil conservation during cultivation of the land. Thus, the applications of PSMs is recommended as a sustainable way of increasing crop yield and also improving the physio chemical properties of the soil.

Salient findings

- Reduction in physiochemical characteristics and enzymatic activities of the pesticide treated soil Site 1 and Site 2 as compared to the normal soil. Reduction in the available ant total Phosphorus, nitrogen, organic carbon content of the pesticide treated soil. Reduction in the enzymatic activities like acid and alkaline phosphatise, urease and soil dehydrogenase to almost 2 fold as compared to normal soil.
- Significant reduction in the total microbial count in the pesticide treated soil as compared to the normal soil.
- Two efficient imidacloprid degrading strain to degrade this pesticide wastes by bioremediation to get rid of polluted soils which effect soil fertility, crop yield disturbing the crop production economy and thus disturbes the nature equilibrium.
- Two efficient imidacloprid degrading bacteria C-5 and C-9, degrading imidacloprid as a sole carbon source when inoculated in minimal media with concentration of 50, 100 and 150ppm.
- After the morphological and biochemical analysis of efficient bacterial isolates (C-5 and C-9) molecular characterization was carried out on the basis of 16S rRNA .
- Two bacterial isolates C-5 and C-9 degrades imidacloprid (50mg/l) in natural environmental conditions in the soil microcosm as the various interactions of the microbial activities in the soil. C-9 came out to better imidacloprid degrading strain as compared to C-5.
- The two bacterial isolates C-5 and C-9 were efficient phosphate solubilizers with the appearance of a halozone on pikovskaya agar plates.
- Significant increase in growth parameters of maize plant when P solubilizing bacteria inoculated in the pot experiment with more IAA activity improved soil properties as there was 2 fold increase in available P and total P of the samples inoculated with PSMs as compared to the control.
- 10% more degradation in the imidacloprid content in the experimental setup of pot cultures in comparison to the soil microcosm as plants that are able to survive high concentration of pesticide mixtures could contribute to pesticide waste degradation as a result of intense microbial activity in the root zone or rhizosphere. Thus microbial activities in the rhizospheric soil leads to more degradation of imidacloprid.

Summary

The rapidly growing industrialization along with an increasing population has resulted in the accumulation of a wide variety of chemicals. Pesticides are used in sizeable areas and applied to soil surfaces and accumulate beneath the ground surface, reaching rivers and seas thus contaminating ground water which is harmful for the entire ecosystem. Insecticides are proven to be toxic when inhaled in more than required quantity.. Thus, the frequency and widespread use of man-made "xenobiotic" chemicals has led to a remarkable effort to implement new technologies to reduce or eliminate these contaminants from the environment. Commonly used pollution treatment methods (e.g. land-filling, recycling, pyrolysis and incineration) for the remediation of contaminated sites have also had adverse effects on the environment, which can lead to the formation of toxic intermediates. As the natural microbiota is continuously exposed to pesticides therefore, it is no surprise that these microorganisms, that inhabit in polluted environments, are armed with resistance by catabolic processes to remove the toxic compounds. Biological degradation by organisms (fungi, bacteria, viruses, protozoa) can efficiently remove pesticides from the environment, especially organochlorines, organophosphates and carbamates used in agriculture and this process of degradation is said to be Bioremediation

Moreover use of pesticides leads to soil infertility in long run affecting soil physiochemical properties and enzymatic activities which consequently results in decreased crop yield.

Thus in order to prevent the crop economy, laboratory studies were undertaken first to check the physiochemical properties and enzymatic activities in pesticide treated soils leading to soil infertility. For this study, soil samples were collected from the potato (*Solanum tuberosum*) grown fields of variety ATL and FC-1 where pesticides were applied for the last ten years. Thus in order to see the effect of pesticides on contaminated soils, third soil samples was collected from the area without prior pesticide treatment. Thus the samples collected from the field of potato varieties of ATL and FC-1 were designated as Site 1and Site 2 the third sample was designated as normal soil without prior pesticide treatment. However the soil microbial flora and physiochemical properties were significantly affected because of the pesticide treatment which disturbs the nature equilibrium. Reduction in enzyme activities can be used as a tool for the detection of pesticide side effects in soil and thus indicators of soil pollution. So environmental friendly, non hazardous and cost effective technique needs to be applied in order to degrade these pesticides from the atmosphere. Thus

for remediation of one of the insecticide imidacloprid, biological agent i.e., microorganisms were used. The effect of imidacloprid on nervous system urges a need to remove this pollutant for safety issues. Moreover studies suggested that imidacloprid was leaching through soil and reaching ground water. Thus in order to prevent ground water from the contamination of imidacloprid, this insecticide residuals needs to be removed by the process of bioremediation. Bioremediation is considered as an efficient and cheap biotechnological approach to clean up the polluted environment with the help of microbes. The survival of microorganism under insecticidal stress can provide efficient, cheaper and environmental friendly solution for the bioremediation of xenobiotic polluted soil (Arnett *et al.*,2000; Shakoori *et al*., 2001). Thus for the degradation of the imidacloprid two efficient bacterial strains C-5 and C-9 were isolated from the Site 2, which were capable of degrading imidacloprid in the minimal media where these isolates used imidacloprid as a sole carbon and energy source. The effect of concentration of imidacloprid on the degradation kinetics was studied by inoculating the two bacterial strains C-5 and C-9 in minimal media with imidacloprid concentration of 50,100, and 150 mg/l. the effect of ph on imidacloprid degradation was observed. More the drop in pH, more was the microbial activity of isolated bacterial strains C-5 and C-9 which results in more imidacloprid degradation. Degradation of imidacloprid was reported more in lower concentration of 50 mg/l as the higher concentration of imidacloprid was reported to be toxic for some microorganisms. Thus the inability of the microorganisms to use imidacloprid as a sole carbon source was effected due to change in the metabolism of microbes. C-9 was found out to be more efficient in degrading imidacloprid. Similarly the degradation of imidacloprid was also observed in soil microcosm experiment. Treatment 2 with sterile soil, imidacloprid and bacterial strain C-5 results in more degradation as compared to the normal soil with imidacloprid and bacterial isolate C-5. This shows the efficacy of bacterial strain C-5 to degrade more imidacloprid as compared to the normal soil in which indigenous microbes were present and leads to the incompatibility of the bacterial strain C-5 to grow at the same rate. Similar results were observed with the C-9 bacterial strain. However C-9 was more effective in degrading imidacloprid in soil microcosm. The release of chloride ions was found to be in stochiometry with the degradation process. More the degradation of imidacloprid more would be the chloride ion release. C-5 and C-9 were found to be efficient phosphate solubilizers with the appearance of clear zone on pikovskaya agar plates. The two bacterial isolates were then checked for their PGPRs activities. IAA activity production of growth regulators was more by phosphate solubilizing bacteria.Thus high IAA activity and phosphate solubilizing properties of the isolates lead to increase in plant growth parameters thus

increase in crop yield improving soil fertility. Phosphate solubilising bacteria are slowly emerging as important organisms used to improve soil health as *in vitro* studies have demonstrated that they reduce P deficiency in soil. Thus degradation of imidacloprid was also checked in nursery plantation experiment after harvesting and it was found that the degradation was more (90%) in pot experiment with PSMs (C-5 and C-9) as plants contribute to pesticide waste degradation in soil as a result of intense microbial activity in the root zone or rhizosphere by coincidental metabolism which degrades imidacloprid.

Thus the increase in plant growth, available and total P in soil inoculates with PSMs may assist in solving problems encountered for crop production economy and could contribute to reversing the trend of soil degradation and actually encourage soil conservation during cultivation of the land. Thus, the applications of PSMs is recommended as a sustainable way of increasing crop yield and also improving the physio chemical properties of the soil in order to maintain the nature ecosystem.

References

Abbink, J, The biochemistry of imidacloprid. Pflanzenschutz-Nachrichten, 1991; 44: 183-195.

Agilent Technologies, Agilent 1200 Series diode array and multiple wavelength detectors, 2006; Manual, Waldbronn, Germany.

Agnihotri SP, Pandey SY, Jain HK, Dewar HS, Rapid single step extraction and clean up method for the estimation of residues of insecticides in soil. The insecticide act and pesticide residue control, 1981, CFTRI Mysore.

Aguera, A, Almansa E, Malato S, Maldonado MI, Fernandez-Alba AR, Evaluation of photocatalytic degradation of imidacloprid in industrial water by GC-MS and LC-MS. Analusis 1998; 26: 245-251.

Ahemad M, Mohammad S, Ecotoxicological assessment of pesticides towards the plant growth promoting activities of Lentil (Lens esculentus)-specific Rhizobiumsp. strain MRL3. Springer Science+Business, 1998; Media, LLC 201.

Amamm RI, Ludwig W, Schleifer KH, Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbial Review, 1995; 59: 143-169.

Amrit K, Amarjeet K, Impact of imidacloprid on soil fertility and nodulation in mung bean (*Vigna radiata)*. Asian Journal of Water Environmental Pollution, 2005; 2: 63-67.

Anderson C, Hu XP, Song D, Effect of Imidacloprid granules on subterranean termite foraging activity in ground touching non structural wood. Journal of Sociology, 2007; 50: 861-866.

Anhalt JC, Moorman TB, Koskinen WC, Biodegradation of imidacloprid by an isolates soil organism. Journal of Environmental Science and Health, part B, 2007; 42: 509-514.

Bacey J, Environmental Fate of Imidacloprid. Technical Report, Environmental Monitoring Department of Pesticide Regulation, 2005 Sacramento.

Bai D, Lummis S, Leicht W, Breer H, Sattelle D, Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neurone. Pesticide Science, 1991; 33: 197-204.

Basavaraj B, Effect of pesticides on the activity of urease, phosphatase and dehydrogenase in black and red soils of Karnataka. Agriculture Science, 1984; 13: 467- 542 .

Bashan Y, Holguin G, de-Bashan LE, Azospirillum-plant relationships: physiological, molecular, agricultural, and environmental advances, Canadian Journal of Microbiology, 2004; 50: 521–577.

Baskaran S, Kookana RS, Naidu R, Determination of the insecticide imidacloprid in water and soil using high-performance liquid chromatography. Journal of Chromatography, Part A, 1997; 787: 271-275.

Bertrand H, Nalin R, Bally R, Cleyet-Marel JC, Isolation and identification of the most efficient plant growth-promoting bacteria associated with canola (Brassica napus). Biology and Fertility of Soils, 2001; 33:152–156.

Bhuyan S, Sahu SK, Adhya TK, Sethunathan N, Accelerated aerobics degradation of hexaclorocyclohexane in suspension of flooded and non flooded soils pretreated with hexachlorocyclohexane, Journal of Boilogy and Fertility of Soils, 1992; 12: 279-288.

Boatman ND, Impacts of agricultural change on farmland biodiversity in the UK, Biodiversity under threat, 2007; 1-32.

Bowers LM, Toxicity of NTN 33893 2F to the blue-green alga *Anabaena flos-aquae*. Bayer Corporation, 1996a, Kansas City, Missouri; Report No. 107549. 31:222-226.

Buckingham SD, Lapied B, Le Corronc H, Grolleau F and Sattelle DB, Imidacloprid actions on insect neuronal acetylcholine receptors. Journal of Experimental Biology, 1997; 200: 2685-2692.

Byrtus G, Anderson A, Saffran K, Bruns G and Checknita L, Determination of new pesticides in Alberta's surface waters. The Water Research User Group, Alberta Environment, 2002.

Cabello MN, Efecto de la contaminación con hidrocarburos sobre hongos formadores de micorrizas vesiculo-arbusculares (MVA). Bol Micol (Chile), 1995; 10:77–83.

Chandrayan K, Sethunathan N, effect of HCH, carbaryl, benomyl and atrazine on dehydrogenase activity in flooded soil, Bulletin of Environmental Contamination and Toxicology, 1980; 24: 379-382.

Chaudhry GR, Chapalamadugu S, Biodegradation of Halogenated Organic Compounds Microbiological Reviews. Microbiology and Molecular Biology Reviews, 1991; 55: 59-79.

Claude B, Davin-Regli A, De Micco P, A simple method for selective isolation of Stenotrophomonas maltophilia from environmental samples, Journal of Applied Environmental Microbiology, 1995; 61: 1653–1654.

Coats JR, Cink JH, Effect of concentration, temperature, and soil moisture on the degradation of chlorpyrifos in an Urban Iowa soil. Bulletin of Environmental Contamination and Toxicology, 1993; 5: 62-70.

Cohen SZ, Creeger SM, Carsel RF, Enfield CG, Potential pesticide contamination of groundwater from agricultural uses. In: Treatment and disposal of pesticide wastes. American Chemical Society Symposium 1984; 297 – 325.

Cox C, Dicamba. Journal of Pesticide Reform, 1994; 14: 30-35.

Cox L, Changes in sorption of imidacloprid with incubation time. Bayer Corporation, Kansa City, Missouri, 1997; pp 28.

Cox L, Koskinen WC, Yen PY, Influence of soil properties on sorption-desorption of imidacloprid. Journal of Environment Science and Health, Part B, 1998; 33: 123-134.

Cox L, Koskinen WC and Yen PY, Sorption-desorption of imidacloprid and its metabolites in soils. Journal of Agricultural and Food Chemistry, 1997; 45: 1468-1472.

Cox C, Insecticide Fact Sheet: Imidacloprid. Journal of Pesticide Reform, 2001; 21: 15-21.

Cycon M, Kaczynska A, Piotrowska SZ, Soil enzyme activities as indicator of soil pollution by pesticides. Pesticides, 2005; 2: 35-45.

Cunningham SD, Shann JR, David E, Anderson A. Phytoremediation of Contaminated Water and Soil. ACS Symposium Series,1992; 664: 2–17

Debarati P, Gunjan P, Janmejay P, Rakesh VJK, Accessing Microbial Diversity for Bioremediation and Environmental Restoration. Trends in Biotechnology, 2005; 23: 135-142.

Diaz E, Bacterial Degradation of Aromatic Pollutants: A Paradigm of Metabolic Versatility. International Microbiology, 2004; 7: 1139-6709.

Dinesh R, Dubey RP, Ganeshamurthy AN, Prasad GS, Organic manuring in rice-based cropping system: effects on soil microbial biomass and selected enzyme activities. Journal of Current Sciences, 2000; 79: 1716-1720.

Dua M, Singh A, Sethunathan N, Johri AK, Biotechnology and bioremediation: Success and limitations. Applied Microbial Biotechnology, 2002; 59: 143-152

Eiben R and Rinke M, Subchronic toxicity study on Wistar-rats (administration in the feed for 96 days). INCHEM Toxicological Evaluations: Imidacloprid; International Programme on Chemical Safety, 1989, World Health Organization: Geneva, Switzerland.

Elbert A, Becker B, Hartwig J and Erdelen C, Imidacloprid - a new systemic insecticide. Pflanzenschutz-Nachrichten Bayer, 1991; 44: 113-136.

Elliot GC, Effects of inhibitors of microbial activity on urea hydrolysis and nitrification in a soilless potting medium, Acta horticulturae, 1989; 238: 173-180.

Erenchun N, Balugera G, Goicolea MA, Barrio RJ, Imidacloprid metabolites. Analytical Chemistry Journal, 1997; 349: 199.

Fernández-Alba AR, Valverde A, Agüera M, Contreras S, Chiron J, Chromatogr A, Introduction about imidaclropid, 1996; 72: 93.

Fernandez-Perez ME, Gonzalez-Prada MD, Controlled release of imiacloprid from a lignin matrix: water Release kinetics and soil mobility study. Journal of Agricultural and Food Chemistry, 1980; 46: 3828-3834.

Finley SD, Broadbelt LJ, Hatzimanikatis V, In Silico Feasibility of Novel Biodegradation Pathways for 1,2,4-Trichlorobenzene, BMC Systems Biology, 2010; 4: 1752-0509.

Fossen M, Environmental Fate of Imidiacloprid; Department of Pesticide Regulation. Environmental Monitoring: Sacramento, CA, 2006; 16: 256-258.

Fox JE, Gulledge J, Engelhaupt E, Burow ME, McLachlan JA, Pesticides reduce symbiotic efficiency of nitrogen-fixing rhizobia and host plants. Preeding of National Academy of Science 2007; 104:10282–10287.

Gahloff JE, Koehler PG, Penetration of the Eastern subterranean termite into soil treated at various thicknesses and concentrations of dursban TC and Premise 75. Journal of Economic Entomology 2001; 94: 486-491.

Gavrilescu M, Fate of Pesticides in the Environment and its Bioremediation. Engineer in Life Science, 2005; 5: 497-526.

Gonias ED, Oosterhuis DM, Bibi AC, Brown RS, Yield, growth and physiology of Trimax TM treated cotton.In D. M. Oosterhuis (Ed.), Summaries of Arkansas Cotton Research, AAES Research Series, 2003; 521: 139-144.

Guay I, Recommendation of criteria for water quality for imidacloprid for protection of aquatic life. Directorate of monitoring the state of the environment, service advice and expertise, the Ministry of Environment of Quebec, 1998, Quebec.

He WX, Jiang X, Yu GF, Bian YR, Effect of dimehypo on soil urease activity. Acta Pedologica Sinica, 2003; 40: 750-755.

Hemmingson BB, Use of exogenously grown bacteria for the clean up of contaminated soil and water, Human impact in self recruiting populations (ed. G. Sundness), 1993; 3: 67-77.

Hocker B, Redlich D, Parlour H, Monitoring of insecticide in low land river. Fresenius Environmental Bulletin, 1995; 7(9A-10A): 699-703.

Hovda LR, Hooser SB, Toxicology of newer pesticides for use in dogs and cats. Veterinary Clinic Small Animals, 2002; 32: 455-467.

Hussain A, Asi MR, Equable Z, Dissipation and degradation of 14C-DDT in tando Jam (sindh) Soil Under Field Conditions, Pakistan Journal of Analytical Chemistry, 2001; 2: 14-18.

Hussain A, Azam F, Malik KA, Bound residues of 14 C Carbofuran in soil. International Atomic Energy Agency Technical Document, 1986; 306: 13-21.

Hussain S, Siddique T, Arshad M, Saleem M, Bioremediation and Phytoremediation of Pesticides. Critical Review in Environmental Science and Technology, 2009; 39: 843-907.

Illmer P and Schinner F, Solubilization of inorganic phosphates by microorganisms isolated from forest soil. Journal of Soil Biology and Biochemistry, 1995; 24: 389–395.

Jain AK, West T, Xang S, Post W, Assesing the impact of changes in climate and carbon dioxide on potential carbon sequesteration in agriculture soil. Geophysical researcher letter, 2002; 32: 239-252.

Kalam A, Tah J, Mukherjee AK, Pesticide effects on microbial population and soil enzyme activities during vermicomposting of agricultural waste, Journal of Environmental Biology, 2004; 25: 201-208.

Karpouzas DG, Singh BK, Microbial degradation of organophosphorus xenobiotics: metabolic pathways and molecular Basis. Journal of Advanced Microbiology and Physiology, 2006; 51:119–185.

Kato I, Koenig KL, Shore RE, Frizzera G, Use of pesticides in food. Journal of Advanced Microbiology and Physiology, 2000; 13: 965-974.

Kennedy ZJ, Arathan SS, Influence of carbofuran on the activity of soil enzymes in submerged rice soil ecosystem, Journal of Biotechnology of Microbes and Sustainable Utilization, 2004; 4: 322-326.

Kitson RE, Mellon MG, Colorimetric determination of phosphorus as molybdivanadophosphoric acid. Industrial and Engineering Chemistry - Analytical Edition, 1944; 16: 379–383.

Kloepper JW, Lifshitz R and Zablotowiz RN, Free living bacterial inoculate for enhancing crop productivity. Journal of Trends in Biotechnology, 1989; 7: 39–43.

Koester J, Metabolites of Imidacloprid. Brighten Crop Protection Conference, 1999; 2: 901-906.

Koterba MT, Banks WSL, Shedlock RJ, Pesticide in shallow ground water in the Delmarwa Peninsula. Journal of. Environmental Quality, 1993; 22: 500-518.

Kreutzweiser DK, Good D, Chartrand T, Scarr R, Thompson D, Non-target effects on aquatic decomposer organisms of imidacloprid as a systemic insecticide to control emerald ash borer in riparian trees. Ecotoxicology and Environmental Safety, 2007.

Krishnamurthy KO, Effect of pesticides on phosphate solubilizer microorganisms. University of Agricultural Sciences, Dharwad, 1989.

Krohn J, Hellpointner E, Environmental fate of imidacloprid, Bayer plant protection news 2002; 55: 12-14.

Kucey RMN and Leggett ME, Increased yields and phosphorus uptake by Westar Canola *(Brassica napus L)* inoculated with a phosphate-solubilizing isolate of *Penicillium bilaji*. Canadian Journal of Soil Science, 1989; 69: 425–432.

Kucey RMN, Janzen HH, Leggett ME, Microbially mediated increases in plant-available phosphorus, Journal of Advances in Agronomy, 1989; 42: 199–228.

Kumar N, Anubhuti Bora JI, Amb MK, Chronic toxicity of the triazole fungicide tebuconazole on a heterocystous, nitrogenfixing rice paddy field cyanobacterium, Westiellopsis prolific Janet. Journal of Microbiology Biotechnology, 2004; 20: 1134–1139.

Kundu BS and Gaur AC, Rice response to inoculation with N2-fixing and P-solubilizing microorganisms. Journal of Plant and Soil, 1984; 79: 227–234.

Laksmikantha HC, Effect of foliar insecticides on soil microorganisms and their biochemical processes in soils of Gulbarga. University of Agricultural Sciences, Dharwad, 2000; 65: 45-48.

Leicht W, Imidacloprid - A chloronicotinyl insecticide. Pesticide Outlook, 1993; 4: 17-21.

Lethbridge G and Burns RG, Inhibition of soil urease by organophosphorus Insecticides. Journal of Soil Biology Biochemistry, 1976; 8: 99-102.

Madhuri RJ and Rangaswamy V, Influence of selected insecticides on phosphatase activity in groundnut (*Arachis hypogeae* L.) soils. Journal of Environmental Biology, 2002; 23: 393-397.

Mandic AI, Lazic SD, Okresz S, Gaal FF, Journal of Analytical Chemistry , 2004; 60: 1134-1138.

Matsuda K, Sattelle DB, Mechanism of Selective Actions of Neonicotinoids on Insect Acetylcholine Receptors, New Discoveries in Agrochemicals: American Chemical Society Symposium Series, 2005; 172-183.

Matsuda K, Buckingham SD, Freeman JC, Squire MD, Baylis HA, Sattelle DB, Effects of the alpha subunit on imidacloprid sensitivity of recombinant nicotinic acetylcholine receptors. British Journal of Pharmacology, 1998; 123: 518-524

Matsuda K, Sattelle DB, Mechanism of Selective Actions of Neonicotinoids on Insect Acetylcholine Receptors. New Discoveries in Agrochemicals: American Chemical Society Symposium Series, 2005; 172-183.

Mayanglambam T, Vig K, Singh DK, Quinalphos persistence and leachingunder field conditions and effects of residues on dehydrogenase and alkaline phosphomonoesterases activities in soil. Bulletin of Environmental Contamination and Toxicology, 2005; 75: 1067-1076.

Miles Inc., Imidacloprid: pesticide leaching potential model. Report No. 105008, Miles Inc., Hamilton, NY, 1993; 3: 687-691

Mishra V, Lal R, Srinivasan S, Enzymes and Operons Mediating Xenobiotic Degradation in Bacteria, Critical Reviews in Microbiology, 2005; 27: 133-166.

Moza PN, Hustert K, Feicht E, Kettrup A, Photolysis of imidacloprid in aqueous solution. Chemosphere, Critical Reviews in Microbiology, 1998; 36: 497-502.

Mullins JW, Imidacloprid: a new nitroguanidine insecticide. American Chemical Society Symposium, Madison, 1993; 54: 184-198.

Mulye HS, Environmental evaluation of imidacloprid insecticide and the end-use product Admire 240F. Pesticides Division, Commercial Chemicals Evaluation Branch, Environmental Protection Service, Environment Canada, 1995; 94:1705-1706.

Mulye HS, Supplementary review of imidacloprid technical and the end-use product, Environmental Assessment Division, Pest Management Regulatory Agency, Health Canada, 1996.

Mulye HS. Supplementary review of imidacloprid technical and the end-use product, Admire 240F insecticide. Environmental Assessment Division, Pest Management Regulatory Agency, Health Canada, 1997, Submission Numbers: 95-1229 and 96-2186.

Nelson EE and Li CY, Persistence of benomyl and captan and their effects on microbial activity in field soils, Bulletin of Environmental Contamination and Toxicology, 1985; 34: 533-540.

Nemeth LG, Füleky G, Morovjan and Csokan P, Sorption behaviour of acetochlor, atrazine, carbendazim, diazinon, imidacloprid and isoproturon on Hungarian agricultural soil. Chemosphere, 2002; 48: 545-552.

Omar SA, The role of rock-phosphate-solubilizing fungi and vesicular - arbuscular - mycorrhiza VAM) in growth of wheat plants fertilized with rock phosphate, World Journal of Microbiology and Biotechnology, 1998; 14: 211–218.

Orme S, Kegley S, PAN Pesticide Database, Pesticide Action Network. Imidacloprid. www.pesticideinfo.org. Accessed: January 2004.

Parsek MR, McFall SM and Chakrabarty AM, Microbial degradation of toxic environmental pollutanats, Ecological and Evolutionary considerations. Journal of Biosorption and Bioremediation, 1995; 35: 175-188.

Pham CH, Min J, Gu MB, Pesticide induced toxicity and stress response in bacterial cells, Bulletin Environmental Contamination and Toxicology, 2004; 72: 380–386.

Pikovskaya RI, Mobililazation of phosphorus in soil in connection with vital activities of some microbial species. Microbiologiya, 1948; 17: 362–370

Poelarands GJ, Zandstra M, Bosma T, Kulakov LA, Larkin MJ., Marchesi, JR, Weightman AJ, Janssen, DB, Haloalkane-utilizing *Rhodococcus*strains isolated from geographically distinct locations possess a highly conservedgene cluster encoding haloalkane catabolism. Journal of bacteriolog.,2000; 182: 2725-2731.

Ponmurugan P, Gopi C, Distribution pattern and screening of phosphate solubilizing bacteria isolated from different food and forage crops. Journal of Agronomy 5, 2006; 600–604

Plumlee, KH, Wismer T, Novel Insecticides. Clinical Veterinary Toxicology; Ed.; Mosby Mosby: St. Louis, MO, 2004; 184-185.

Puente ME, Li CY, Bashan Y, Microbial populations and activities in the rhizoplane of rock-weathering desert plants. II. Growth promotion of cactus seedlings. Journal of Plant Biology, 2004; 6: 643–650.

Purushothaman D, Marimuthu T, Keravan, R, Respiration and dehydrogenase activity of soil as influenced by application of cytrolane. Indian Journal of Experimental Biology,1974; 12: 580- 581.

Richardson AE, Hadobas PA, Hayes JE, Extracellular secretion of *Aspergillus phytase* from Arabidopsis roots enables plants to obtain phosphorus from phytate. Plant Journal, 2001; 25: 641–649.

Robinson RA, Sutherland WJ, Post-war changes in arable farming and biodiversity in Great Britain, Journal of Applied Ecology, 2002; 39: 157-176.

Rouchaud J, Gustin F, Wauters A, Soil Biodegradation and Leaf Transfer of Insecticide Imidacloprid Applied in Seed Dressing in Sugar Beet Crops, Bulletin of Environmental Contamination and Toxicology, 1994; 53: 344-350.

Rouchaud J, Gustin F, Wauters A, Imidacloprid insecticide metabolism in sugar beet field crops, Bulletin of Environmental Contamination and Toxicology, 1996; 56: 29–36.

Saber WLA, Ghanem KM, El-Hersh MS, Rock phosphate solubilization by two isolates of *Aspergillius niger* and *Penicillium sp*. and their promotion to mung bean plants, Research Journal of Microbiology, 2009; 4: 235–250.

Sarkar MA, Biswas PK, Roy S, Kole RK, Chowdhury A, Effect of pH and type of formulation on the persistence of imidacloprid in water, Bulletin Environmental and Contamination Toxicology, 1999; 63: 604-609.

Sarkar MA, Sankhajit R, Kole R, Chowdhury A, Persistence and metabolism of imidacloprid in different soils of West Bengal, Journal of Pest Management and Sciences, 2001; 57: 598–602.

Scheurnert I, Gunthner A, Rosenbrock P, Long term fate and effects of the herbicide Bromoxynil in soil cropped with maize. International Atomic Energy Agency Technical Document 2001; 1248; 89-98.

Schoefs O, Perrier M and Samson R, Estimation of Contaminant Depletion in Unsaturated Soils Using a Reduced-order Biodegradation Model and Carbon Dioxide Measurement, Applied Microbiology and Biotechnology, 2004; 64: 53-61.

Scholz K, Spiteller M, Influence of groundcover on the degradation of 14C-imidacloprid in soil. Brighton Crop Protection Conference, Journal of Pests and Diseases, 1992; 883-888.

Schroeder ME, Flattum RF, The Mode of Action and Neurotoxic Properties of the Nitroethylene Heterocycle Insecticides. Pesticide Biochemistry and Physiology, 1984; 22: 148-160.

Schroll R, Brahushi R, Dorfler U, Kuhn S, Fekete J, Munch JC, Biomineralisation of 1,2,4-Trichlorobenzene in Soils by an Adapted Microbial, Environmental Pollution, 2004; 127: 395-401.

Shadnia S, Fatal intoxication with imidacloprid insecticide. American Journal of Emergency Medicine, 2007; 26: 634-634.

Shakoori AR, Chaudhary A, Ali SS, Carbosulphan and quinaphols degrading bacteria isolated from insecticide contaminated soil and industry effluents have potential to clean up the environment. Proceeding of Pakistan Congress of Zoology, 2000; 20: 199-222.

Sheets LP, Imidacloprid: A Neonicotinid Insecticide. Handbook of Pesticide Toxicology, 2nd ed.; Krieger RI. Academic Press: San Diego, CA, 2001; 2: 1123-1130.

Siddiqui MKJ, Srivastava S, Srivastava SP, Mehrotra PK, Mathur N, Tandon I, Persistent chlorinated pesticides and intra-uterine foetal growth retardation: a possible association. Int Arch Occup Environ Health, 2003; 76: 75-80.

Skujins JJ, Braal L, McLaren AD, Characterization of phosphatase in a terrestrial soil sterilized with an electron beam. Enzymology, 1962; 25: 125–133

Somich CJ, Muldoon MT, Kearney P, On site treatment of pesticide waste and rinsate using ozone and biologically active soil, Journal of Environmental Science Technology, 1990; 24: 745-749.

Son TTN, Diep CN, Giang TTM, Effect of bradyrhizobia and phosphatensolubilizing bacteria application on Soybean in rotational system in the Mekong delta. Journal of Soil Biology and Ecology, 2006; 14: 48–57.

Srimathi MS, Karnath NGK, Majumdar SK, Influence of bromophosphate on some biological activities of soil, Journal of Soil Biology and Ecology, 1986; 6: 9-15.

Stephan CE, Mount DI, Hansen DJ, Gentile JH, Chapman GA, Brungs WA, Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses. U.S. Environmental Protection Agency, Washington, DC. 1985; 23: 562-567

Struger JT, Fletcher P, Martos B, Gris G, Pesticide concentrations in the Don and Humber River Watersheds Environment Canada, Ontario Ministry of the Environment, and City of Toronto, 2002; 22-26

Suenaga HM, Mitsuoka T, Ura, Watanabe T, Furukawa K, Directed evolution of biphenyl dioxygenase: Emergence of enhanced degradation capacity for benzene, toluene and alkylbenzenes. Journal of Bacteriology, 2002; 183: 5441-5444

Sutherland WJ, Robinson RA, Post-war changes in arable farming and biodiversity in Great Britain. Journal of Applied Ecology, 2002; 39: 157-176.

Sur R, Stork A, Uptake, translocation and metabolism of imidacloprid in plants. Bulletin of Insectology, 2006; 56: 35-40.

Tabatabai MA, Bremner JM. Use of p-Nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology and Biochemistry, 1969; 1: 301–307.

Tayaputch N, Pampan P, Phaikaew Y, Shukiatwatana L, Dumrags B, Impact on long term usage on soil microbial activities and 14 C-Monotrophos degradation. Second FAO/IAEA Research coordination meeting Bangkok, 1997; 22-26.

Thielert W, A unique product: The story of the imidacloprid stress shield. Pflanzenschutz-Nachrichten Bayer, 2006; 59: 73-86.

Thomson A, Parsek A, Pesticide synthesis. Chemical Petrochem and Process, 1996; 62: 334-382

Thyssen J and Machemer L, Imidacloprid: Toxicology and Metabolism. Journal of Clinical Toxicology, 2001; 39 (6): 617-621.

Tomizawa M and Yamamoto I, Binding of nicotinoids and the related compounds to the insect nicotinic acetylcholine receptor. Pesticide Science, 1992; 17: 231-236.

Tomizawa M, Casida JE, Minor structural changes in nicotinic insecticides confer differential subtype selectivity for mammalian nicotinic acetylcholine receptors. British Journal of Pharmacology, 1999; 127: 115-122.

Tomizawa M, Casida JE, Neonicotinoid Insecticide Toxicology, Mechanisms of Selective Action, Annual Review of Pharmacology and Toxicology, 2005;45:247-268.

Tomlin C, The Pesticide Manual. Tenth Edition, The British Crop Protection Council and the Royal Society of Chemistry, Bath Press, Bath; 2000.

Tomlin CDS, The Pesticide Manual, A World Compendium, 14th ed.; British Crop Protection Council, Surry, England, 2006; 813-814.

Vaccari DA, Strom PF, Alleman JE, Environmental Biology for Engineers andScientists. John Wiley&Sons, 2006, DOI: 10.1002/0471741795, ISBN-10: 0471722391.

Vassilev N, Franco I, Vassileva M, Azcon R, Improved plant growth with rock phosphate solubilized by Aspergillus niger grown on sugar-beet waste. Bioresource Technology, 1996; 55: 237–241.

Vilchez, JL, El-Khattabi J, Fernandez A, Navalon A, Determination of imidacloprid in water and soil samples by gas chromatography-mass spectrometry. Journal of Chromatography A, 1996; 746: 289-294.

Vilchez JL, El-Khattabi R, Navalon A, Photochemical-fluorimetric method for the determination of the insecticide imidacloprid in water samples. Analytica Chimica Acta, 1998; 371: 247-253.

Voets JP, Meerschman P and Verestrate W, Soil microbiological and biochemical effects of longterm atrazine application. Journal of Soil Biology and Biochemistry, 1974; 8: 149-152.

Walkley AJ, Black IA, Estimation of organic carbon by chromic acid titration method. Soil Science 1934; 37: 29–38.

Walton BT, Hoylman AM, Perez MM, Anderson TA, Johnson TR, Guthrie EA and Christman RF, Rhizosphere microbial communities as a plant defense against toxic substances in soils, ACS symposium, American Chemical Society, 1994; 82–92.

Wamhoff H, Schneider V, Photodegradation of imidacloprid. Journal of Agriculture Food Chemistry,1999; Vol 47: 1730-1734.

Watanbe K, Kodama Y, Kaku N, Diversity and abundance of bacteria in an underground soil storage cavity. BMC Microbiology, 2002; 2:23

Ware, GW, Whitacre, DM, The Pesticide Book. Meister Pro Information Resources, 2004; 70-71.

Whitelaw MA, Growth promotion of plants inoculated with phosphate-solubilizing fungi. Journal of Advances in Agronomy, 2000; 69: 100–151.

Whitelaw MA, Harden TJ and Bender GL, Plant growth promotion of wheat inoculated with *Penicillium radicum sp*. Australian Journal of Soil Research, 1997; 35: 291–300.

Whitelaw MA, Harden TJ and Helyar KR, Phosphate solubilisation in solution culture by the soil fungus *Penicillium radicum.* Soil Biology and Biochemistry, 1999; 31; 655–665.

Wilmes R, Range-finding tests for abiotic degradation. Miles Report No. 94366. Bayer AG, Monheim, Germany,1988; 11-12.

Wismer T, Novel Insecticides. Clinical Veterinary Toxicology; 2004; 184-185.

Woese CR, Kandler O, Wheelis ML, Towarda a natural system of organisms: Proposal for the domains Archae,Bacteria, and Eucarya. Proceeding natural Academy Science,1990; 87: 4576-4579.

Wohlers K, Dissociation constant of NTN 33893. Miles Report No. 99863. Bayer AG, Leverkusen, Germany,1988; 4: 242 -256.

Wu I, Lin, J, Cheng E, Acute Poisoning with the Neonicotinoid Insecticide Imidacloprid in N-methyl Pyrrolidone. Clinical Toxicology, 2001; 39: 617-621.

Xu G, Zheng W, Li Y, Wang S, Zhang J and Yan Y , FRP. Int Biodetermination Biodegradation, 2008; 62: 51–56.

Yamamoto I, Nicotine to nicotinoide. In: Yamamoto, I.,Casida,J.E. (Eds.),Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor. Springer,Tokyo, 1999; 3-27.

Yosef BB, Rogers RD, Wolfram JH, Richman EJ, *Pseudomonas cepacia* mediated rock phosphate solubilization in kaolinite and montmorillonite suspensions. Soil Science Society of America, 1999; 63:1703–1708.

Zheng W, Liu W, Kinetics and mechanism of the hydrolysis of imidacloprid. Pesticide Science, 1999; 55: 482-485.